Molecular analysis of bacterial diversity in kerosene-based drilling fluid from the deep ice borehole at Vostok, East Antarctica

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Abstract

Decontamination of ice cores is a critical issue in phylogenetic studies of glacial ice and subglacial lakes. At the Vostok drill site, a total of 3650 m of ice core have now been obtained from the East Antarctic ice sheet. The ice core surface is coated with a hard-to-remove film of impure drilling fluid comprising a mixture of aliphatic and aromatic hydrocarbons and foranes. In the present study we used 16S rRNA gene sequencing to analyze the bacterial content of the Vostok drilling fluid sampled from four depths in the borehole. Six phylotypes were identified in three of four samples studied. The two dominant phylotypes recovered from the deepest (3400 and 3600 m) and comparatively warm ($-10\,^{\circ}$C and $-6\,^{\circ}$C, respectively) borehole horizons were from within the genus *Sphingomonas*, a well-known degrader of polyaromatic hydrocarbons. The remaining phylotypes encountered in all samples proved to be human- or soil-associated bacteria and were presumed to be drilling fluid contaminants of rare occurrence. The results obtained indicate the persistence of bacteria in extremely cold, hydrocarbon-rich environments. They show the potential for contamination of ice and subglacial water samples during lake exploration, and the need to develop a microbiological database of drilling fluid findings.

Introduction

There is considerable interest in biology in investigating the microbial forms that thrive in extreme environments, especially under extreme hot or cold conditions. It is now known that some Archaea can survive the standard sterilization procedure of autoclaving at 121 °C (Kashefi & Lovley, 2003), and some bacteria can live in super cooled clouds, sea ice and surface snow of the South Pole at temperatures up to $-20\,^{\circ}$C (e.g. Carpenter *et al.*, 2000; Sattler *et al.*, 2001; Breezee *et al.*, 2004; Junge *et al.*, 2006).

However, a major problem in the search for life in extreme environments [including astrobiological studies beyond Earth (Morrison, 2001)] is 'forward contamination', the accidental contamination of samples and the environment during exploration and sample collection (Rummel, 2001). In such environments, microbial biomass and activity are likely to be low and susceptible to the effects of contamination, as has been noted for accretion ice of the Vostok core (Bulat *et al.*, 2004) and some surface soil sites in the Atacama Desert (Navarro-Gonzalez *et al.*, 2003).

The search for microbial signatures in the extreme polar environments of Earth continues to present many challenges (Priscu *et al.*, 2005). One important question in the analysis of polar ice cores is the extent to which they may have been contaminated during sampling. For example, at Vostok, the number of microbial cells in deep ice has been estimated as of the order of 100–1000 cells mL^{-1} of melted water (Abyzov, 1993; Karl *et al.*, 1999; Priscu *et al.*, 1999; Abyzov *et al.*, 2001; Christner *et al.*, 2001; Christner, 2002). Other results, however, indicate that both accretion ice (subglacial lake water frozen onto the base of the glacial ice sheet) and deep glacial ice are exceptionally clean, i.e. contain essentially no indigenous microorganisms (or their DNA suitable for analysis) (Bulat *et al.*, 2004). Such disparities may reflect differences among samples, or may be the result of the sampling volume of the ice, analytical methodologies and last, but not least, decontamination protocols. To address the potential contamination issue there is a need for improved understanding of the diversity and biomass of microbiota that may contaminate the ice samples during drilling and core retrieval.

One approach to the question of microbial contaminants is to analyze the drilling fluids used during the ice core retrieval process at each site. This information is also of interest in identifying the microbial diversity of communities that can survive or grow in hydrocarbon-rich environments at frigid temperatures. In the present study we focused on the bacterial content of drilling fluid (DF) obtained from the deep 5G-1 borehole at Vostok station (78°27′ S, 106°52′ E) on the East Antarctic ice sheet. Organic chemical analyses of this fluid show that it is a complex mixture of mostly saturated hydrocarbons (alkanes) with nine to 16 carbon atoms typically present in various types of aviation kerosene (e.g. TS-1, JP-8) and foranes (e.g. Forane 141B), which act as densifiers. Alkanes and foranes tend to be present in a rough proportion of 5 : 1 (Russian Antarctic Program, 2003). Aviation kerosene can also contain branched and aromatic hydrocarbons (e.g. alkylbenzenes and naphthalenes), and forane represents dichlorofluoroethane. This fluid readily coats the ice surface as a cohesive layer (Fig. 1) and may penetrate into the ice interior through micro-fissures or cracks. The Vostok drilling fluid was transported to the site in common use tanks and was not sterilized during use. Therefore, contamination of the ice was possible during both drilling and core retrieval.

Given the long-term and complex 'evolution' of the DF inside the borehole (during the 1990–2001 time interval) and its markedly varying temperature profile (from ultra-low temperatures near the surface ($-57\,^{\circ}C$) to close to $-6\,^{\circ}C$ in the accretion ice horizon at the bottom of the drill hole), such a mixture cannot be realistically simulated in laboratory conditions. We therefore present here data on the microbial content and molecular diversity of Vostok DF sampled directly from the borehole in 2001 following drilling operations during 1990–1998 and after a 3-year time interval when no drilling operations or measurements were performed. During this 'conservation' period the borehole was sealed with a cap at the top of the drill casing (Russian Antarctic Program, 2003).

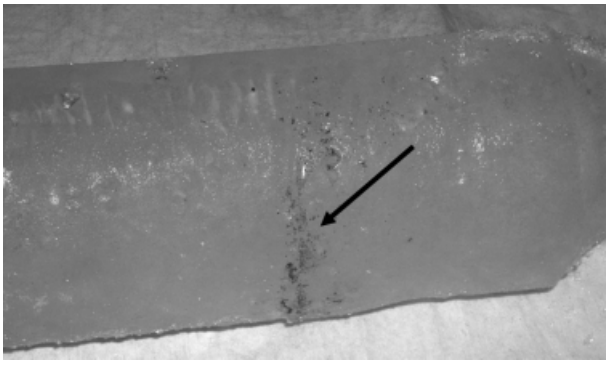

Fig. 1. Traces of Vostok DF (indicated by arrow) on the surface of an ice core sample retrieved from 2054 m depth.

The long-term persistence of the DF in a borehole is of especial concern when dealing with estimates of microbial content because although human-made kerosenes and foranes are originally clean and sterilized during the refining process, they may develop DF-degrading microbial communities during subsequent storage and handling.

The objectives of the present work were to estimate the microbial content of DF as a source for potential forward-contamination of ice cores and to evaluate the DF as a possible extreme econiche for hydrocarbon-degrading bacteria.

Materials and methods

Sample collection

Several samples of Vostok DF were collected in 2001 by the 46th Russian Antarctic Expedition (2000/2001) directly from the 5G (5G-1) borehole in the depth interval 110–3600 m following the 3-year conservation period (Russian Antarctic Program, 2003). A remotely tripped steel sampler featuring a pump and isolating valves was employed to obtain discrete samples. Before each run the sampler was rinsed and filled with pure kerosene released into the borehole just before reaching the sample-collecting depth. Upon returning the DF sample to the surface, the DF aliquot was transferred into plastic bottles prerinsed with pure kerosene.

Originally, samples were collected for density measurements and were kept in plastic bottles for 1 year at Vostok station in ice core storage at an average local temperature of $-55\,^{\circ}C$. Then they were resampled using sterile 50-mL Falcon centrifuge tubes and finally delivered (at $-20\,^{\circ}C$) to a laboratory for DNA analysis. The samples selected for the present study are characterized in Table 1. Though the same samples and preliminary analytical results have already been reported (Russian Antarctic Program, 2003), the cell/DNA extraction procedures and further analyses reported in the present study were significantly different and much more precise.

It should be noted that the formation of DF mixture in a borehole is rather complicated. The forane is injected, as a densifier, at specified depth horizons down to 2755 m to equalize hydrostatic pressure while pure kerosene is added to the borehole from above to compensate for the volume of cored ice removed. In addition, the DF retrieved from the borehole with a cable and a coring device over a period of 2–3 h was replaced by pure kerosene from above. This led to kerosene enrichment in the upper part of the drill hole as there was forane loss through evaporation during the ice core sampling period.

Additional samples were collected in 2005 for cell enumeration by the 50th Russian Antarctic Expedition (2004/2005) from similar borehole horizons. They were transferred

Table 1. Characterization of Vostok DF samples and distribution of bacterial phylotypes recovered (refer to Table 3)

	Drilling fluid sample number			
	110	2750	3400	3600
Sample depth (m)	110	2750	3400	3600
Ice origin	Glacier	Glacier	Glacier	Lake
DF composition (ratio)	Mostly kerosene*	Kerosene/forane (5 : 1)	Kerosene/forane (5 : 1)	Kerosene/forane (5 : 1)
On-site temperature, °C	−57	−23	−10	−6
DF exposure at 'physiological' temperature†, years	–	–	∼3	∼4
DF residence time in borehole, years	> 3	∼6	∼10	∼11
No. of clones‡	5	0	15	8
No. of phylotypes	2	0	2	2
Closest phylotype (no. of clones)	*Gammaproteobacteria Haemophilus influenzae* dfB4-15 (1)	–	*Alphaproteobacteria Sphingomonas natatoria* dfB6-5 (13)	*Alphaproteobacteria Sphingomonas* sp. dfB7-5 (3)
	Unidentified dfB4-6 (4)	–	Unidentified dfB6-21 (2)	*Bacteroidetes Haloanella gallinarum* dfB7-7 (5)

*A mixture of newly added kerosene and the original DF sampled from the bottom of the borehole and exposed to the atmosphere for few hours at the drilling site leading to forane loss by evaporation.

†'Physiological' temperatures are defined here as the lowest temperature conditions (not below −20 °C) at which bacterial activity has been experimentally proved [Breezee *et al*. (2004), Junge *et al*. (2006), The MEPAG (2006)].

‡Bacteria which were not found in the recognized contaminant databases established for Vostok DF.

into sterile 100-mL Schott bottles that were maintained at −20 °C until analysis some months later in our laboratory.

Bacterial cell enumeration

Bacterial cell concentrations were determined by flow cytometry using water phase subsamples (refer to the Molecular biology section). Samples were fixed with 0.2 μm filtered glutaraldehyde at a final concentration of 0.25%, incubated for 2 h before being frozen in liquid nitrogen and then stored at −80 °C until analysis. Samples were thawed at room temperature and incubated for 15 min in the presence of SYBR-Green I (1/10 000 final concentration, Marie *et al*., 1999). Analysis was performed using a FACSARIA flow cytometer equipped with a 488-nm wavelength excitation and the standard filter setup for SYBR-Green I.

Molecular biology

The initial approach for cell/dissolved DNA recovery from DF was by filtration, a protocol that has worked well with ice meltwater (Bulat *et al*., 2004). However, the DF contained invisible impurities that formed a cohesive film over the membranes and blocked the pores. There was therefore the need to develop an alternative protocol.

The common TE (Tris-EDTA) buffer made with pure molecular biology grade water (MoBio) and concentrated 'ready-to-use' TE buffer (Sigma) was added to DF samples, and the mixture was subjected to mid-rate vortexing for 30 min at room temperature followed by subsequent separation of water phase and its treatment under vacuum for 20 min to remove kerosene-forane traces. A control extraction procedure (with no DF added) was performed in a parallel.

The genomic DNA was successfully extracted using the QIAamp DNA Mini kit, Qiagen (protocol D for bacteria) and aliquots up to 1.0 μL were tested in PCR runs using 20 μL total volume. A semi-nested broad-range bacterial 16S rRNA gene 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 (Bulat *et al*., 2004).

PCR products embracing the V3 region of 16S rRNA genes were cloned into the TOPO TA (Invitrogen) cloning vector and the corresponding rRNA gene libraries were constructed. After plating, positive transformants were screened for inserts by PCR using flanking vector primers. The PCR products were cleaned with a QIAquick PCR Purification Kit (Qiagen) and sequenced. The 405–431 base pair (bp) sized amplicons from sample libraries were compared against the GenBank with the BLAST program (Altschul *et al*., 1997) to identify the sequence. At the same time they were tested against on-site established contaminant databases (containing the sequences recovered from

different relevant sources of contamination) with the STARBLAST software (DNAstar) to evaluate the sequences for contaminant status. Those sequences, disregarding their identification, which showed no match to the contaminant database entries at the level of bacterial species (more than 97% sequence similarity for rRNA gene sequences (Stackebrandt & Goebel, 1994), were considered to originate from the Vostok DF.

The contaminant databases were produced in a similar way to DF samples and were particularly established to address negative PCR results (an amplification control to check the purity of the PCR reagents with no DNA added), mock cell and DNA extraction (an extraction control to check the purity of the cell and DNA extraction reagents with no sample added) and laboratory environment effects (a control to check the overall purity of laboratory surfaces by testing dust collected).

To clarify further the status of findings in terms of physical-chemical features of the environment (Vostok DF in the borehole) special contaminant indexing criteria were developed which among other clauses included the above-mentioned contaminant databases along with contaminants recorded in other analyses (Table 2). These criteria allowed us to rank sequences according to high or low probability of being a contaminant (e.g. human pathogens vs. water contaminants in other studies, respectively) (Table 2). Those sequences which failed to meet contaminant indexing criteria were considered to have developed *in situ* and likely to be DF-degrading microorganisms.

No chimeric sequences were detected either by visual inspection of corresponding alignments or by the Ribosomal Database Project II Chimera Check online tool (http://rdp.cme.msu.edu/cgis/chimera.cgi?su=SSU).

The sequences reported in this paper have been deposited in the GenBank database under accession numbers DQ422864–DQ422869.

Results

The DF samples selected for the present study represented four major points of the borehole depth-temperature-age profile (Table 1). The main differences between the samples were the DF chemical composition, the local temperature in the borehole, the exposure time at physiological temperatures in the borehole, and the overall residence time in the borehole. The physiological temperature in our definition means the lowest temperature conditions (not below $-20\,^{\circ}C$) at which bacterial activity has been proved experimentally (Breezee *et al.*, 2004; Junge *et al.*, 2006). The same temperature threshold decisive for microbial reproduction is now proposed for use in looking for extraterrestrial life (The MEPAG, 2006).

Sample #110 was the youngest (residence time in the borehole), representing the upper cold level (inside the coring casing), containing mostly kerosene and subject to the most human impact. Samples #3400 and #3600 were the oldest (in terms of residence time), originating from the deepest levels and exposed to 'physiological' temperatures for 3–4 years. The last sample, #2750, was intermediate by age and depth horizon with *in situ* temperatures cold enough to stop microbial metabolism. These last three samples contained DF enriched by forane. In addition, sample #3600 originated from the accretion (lake water-derived) ice horizon, and samples #2750 and #3400 were from the deep glacial ice horizon.

The subsequent cloning and sequencing of these samples revealed 19 bacterial phylotypes of 90 clones. Of them, only six phylotypes (28 clones) successfully passed our contaminant databases and were therefore considered to originate from the Vostok DF (Tables 1 and 3).

All six phylotypes were recovered in the uppermost and deepest DF samples, whereas none was detected in the intermediate sample #2750 (Table 1). This is in agreement with flow cytometry data on cell enumeration performed for new additionally collected DF samples of similar deep horizons. The 2500 m and 2700 m depths revealed no cells, whereas the deepest horizons (3300 and 3564 m) were observed to contain about 10^2 cells mL^{-1} (data not shown).

Representatives of the extensive genus *Sphingomonas* (*Alphaproteobacteria*) (*Sphingomonas* sp. and *Sphingomonas natatoria*-relatives) were dominated by clone numbers (16 clones of 28 in total) and were found only in the deepest samples, #3400 and #3600, where physiological temperatures prevailed for 3–4 years (Table 1). These two species, as shown by descriptions of their closest neighbours in world-wide databases, have the capability to degrade mono- and

Table 2. Key contaminant indexing criteria for taxa developed *in situ* in Vostok borehole

Score	Regulation
+3	Pathogens, commensals and saprophytes of humans, animals and plants
+3	Autotrophs (photoautotrophs, chemoautotrophs)
+3	Thermophiles
+2	Taxa recorded in on-site established contaminant databases (negative PCR, mock cell and DNA extraction, laboratory environment)
+2	Alone clone, single sequence
+1	Taxa shown to be contaminants in other analyses e.g. Tanner *et al.* (1998), Cisar *et al.* (2000), Kulakov *et al.* (2002)

Table 3. Bacterial phylotypes in Vostok DF identified by 16S rRNA gene sequencing

Phylotype Accession number*	Sequence similarity (%) (size, bp)	Division Nearest taxon/clone in GenBank	Features/source (references)	Conclusion
dfB7-5 DQ422868*	100-99.5 (405)	SPH429240 *Sphingomonas aerolata*	Airborne, UK (Busse *et al.*, 2003)	*Alphaproteobacteria* *Sphingomonas* sp. DF-degrading
		SPH429239 *Sphingomonas faenia*	Airborne, Finland (Busse *et al.*, 2003)	
		SPH429238 *Sphingomonas aurantiaca*	Airborne, Finland (Busse *et al.*, 2003)	
		AY439276 *Sphingomonas* sp. GICT1	Greenland GISP2 ice core 3042 m (Miteva *et al.*, 2004)	
		AF548567 *Sphingomonas* sp. eh2	Endolithic sample, Antarctica (Hughes & Lawley, 2003)	
		AF395031 *Sphingomonas* sp. M3C203B-B	Taylor Dome ice core 203 m, Antarctica (Christner, 2002)	
		AF324199 *Sphingomonas* sp. V1	Lake Vostok accretion ice core 3593 m, Antarctica (Christner *et al.*, 2001)	
		AF184221 *Sphingomonas* sp. Ant20	PAH-contaminated soil near Scott Base, Antarctica, PAH-degrading (Aislabie *et al.*, 2000)	
		AY571827 *Sphingomonas* sp. clone 437D	AH-contaminated soil near Scott Base, Antarctica, PAH-degrading (Saul *et al.*, 2005)	
		DQ234505 Arctic soil bacterium R5TW3	Oligotrophic forest soil, Finland (Mannisto & Haggblom, 2006)	
		AY526679 *Sphingomonas* sp. Muzt-J11	Muztag Ata ice core 21.4 m from the Pamirs Platea (Xiang *et al.*, 2005)	
dfB6-5 DQ422869	100-99.0 (405)	U20772 *Blastobacter* sp. SMCC B0477	Deep terrestrial subsurface, aromatic hydrocarbon degrading [(Fredrickson *et al.*, 1995), (Balkwill *et al.*, 1997)]	*Alphaproteobacteria* *Sphingomonas natatoria*-relative DF-degrading
		AY328639 Uncultured bacterium DSSF16	Drinking water distribution system (Williams *et al.*, 2004)	
		AY898067 Uncultured clone M8907C08	Water of a hospital therapy pool (Angenent *et al.*, 2005)	
		AF532054 Uncultured clone 3607bR-48	Lake Vostok accretion ice core 3607 m, Antarctica, identified as *Sphingomonas natatoria* and assigned to contaminants (Bulat *et al.*, 2004)	
		AF479381 Glacial ice bacterium M3C1.8K-TD9	Taylor Dome glacial ice core, Antarctica (Christner, 2002)	
		AB024289 *Erythromonas ursincola*	No data on PAH degradation ability (Hiraishi *et al.*, 2000), easily forms biofilms owing to high aggregation capability (Rickard *et al.*, 2002)	
		AB024288 *Sphingomonas natatoria*		
dfB4-15 DQ422864	98.4 (430)		Periodontal microbial communities (DQ003635)	

Table 3. Continued.

Phylotype Accession number*	Sequence similarity (%) (size, bp)	Division Nearest taxon/clone in GenBank	Features/source (references)	Conclusion
		DQ003635 *Haemophilus* genomosp. P3 oral clone MB3_C38		*Gammaproteobacteria* *Haemophilus influenzae* Human-associated Contaminant
	97.9 (430)	X87976 *Haemophilus influenzae*	Human pathogen (Quentin *et al.*, 1996)	
dfB7-7 DQ422865	97.2 (425)	AB035150 *Haloanella gallinarum*	Human pathogen (Pham *et al*., 1999)	Bacteroidetes *Haloanella gallinarum*-relative Human-associated Contaminant
	96.7 (425)	AY244776 *Haloanella gallinarum*	Pathogens isolated from human tissues (AY244776)	
dfB4-6 DQ422866	100 (346† of 431 bp)	AF544866 Uncultured clone D1C-74	Healthy tissues of diseased Caribbean coral (Pantos *et al*., 2003)	Unidentified Human/soil-associated Contaminant
	100 (250† of 431 bp)	AJ874226 Uncultured clone Rum_12d	*Rumex acetosa* rhizosphere (Dohrmann & Tebbe, 2005)	
dfB6-21 DQ422867	100-99.8 (408)	AJ295559 Uncultured bacterium wr0198	Rape rhizosphere, Germany (Kaiser *et al*., 2001)	Unidentified Soil-associated Contaminant
		AY093470 Uncultured bacterium MB-B2-105	Marine deep subsurface sediments (Reed *et al*., 2002)	
		AY375144 Uncultured bacterium D5	Deep sea sediments (Zeng *et al.*, 2005)	

*Here and further GenBank accession number.
†Overlapped region in sequences.

polyaromatic hydrocarbons (Table 3, references within). Therefore, the two *Sphingomonas* phylotypes were ultimately assigned as DF-degraders that can likely utilize aromatic hydrocarbons *in situ*.

Three more phylotypes (assigned with different divisions and detected in samples #110 and #3600) showed a substantial similarity to sequences of human/animal pathogens or saprophytes. According to the Vostok DF contaminant indexing criteria (Table 2, highest score) these phylotypes were therefore contaminants (Table 3). The remaining phylotype from sample #3400 showed substantial sequence similarity to uncultured bacterial DNA clones isolated both from agricultural soil/plant rhizosphere and from deep marine sediments. According to the contaminant indexing criteria (Table 2, highest score) this phylotype was also ultimately considered a contaminant (Table 3).

The six phylotypes recovered were put into a specialized Vostok DF microbial database which will serve as an extra source of contamination checks in future Vostok ice core microbiological studies.

Discussion

Review on microbiology of hydrocarbon-contaminated sites

In the laboratory, many microorganisms (mainly bacteria) are able to degrade or metabolize saturated and aromatic hydrocarbons present in oil and its refining process products as well as the sulphur compounds associated with oil. Hydrocarbon-degrading bacteria have also been isolated from many different environmental sources including oil and its refinery products, natural deep subsurface oil field reservoirs, oil-contaminated ground and sea waters, sediments and soils. There have been several recent detailed reviews on microbial hydrocarbon metabolism which have considered the complex mechanisms of transformation of hydrocarbons (Kanaly & Harayama, 2000; Van Hamme *et al.*, 2003; Head *et al.*, 2006).

Among hydrocarbon-degrading bacteria recovered directly from oil and its refinery products the representatives of sulphate-reducing bacteria of the *Desulfobacteriaceae* family (*Deltaproteobacteria*) (Rabus *et al.*, 1996), sulphur-reducers, of which the *Actinobacteria* and *Bacillus/Clostridium* group (*Firmicutes)* (Rees *et al.*, 1997; Folsom *et al.*, 1999) and *Thermotogaceae* (*Thermotogae*) (L'Haridon *et al.*, 1995) are the most investigated. Recently, representatives of *Sphingomonas* were found to be one of commonest microbial contaminants to be extracted live from US Air Force aviation fuel (kerosene-based JP-8 fuel) (Rauch *et al.*, 2006).

Numerous living bacteria have also been recovered from natural (usually deep-subsurface) high-temperature oil field reservoirs (e.g. L'Haridon *et al.*, 1995). Recent detailed studies (Orphan *et al.*, 2000; Bonch-Osmolovskaya *et al.*, 2003) strongly suggest the existence of complex but as-yet-undetected microbial assemblages in these geothermally heated, deep-seated petroleum reservoirs. However, they are not taken into consideration further in the present study as it is considered very unlikely that thermophiles would be discovered in Vostok DF, given the frigid temperatures present in the borehole (Table 1) and the industrial origin of the kerosene, which eliminates microorganisms during the fractionation process.

Within oil-polluted soils, representatives of *Sphingomonas* (*Alphaproteobacteria*) and *Pseudomonas* (*Gammaproteobacteria*) dominate (Balkwill *et al.*, 1997; Shen *et al.*, 1998; Aislabie *et al.*, 2000; Yu *et al.*, 2000; Eckford *et al.*, 2002). In contrast, in polluted ground waters and marine reservoirs and sediments it is possible to recover bacteria from any taxonomic division, though *Gammaproteobacteria*, with the exception of the genus *Pseudomonas*, seem to be more abundant in sea reservoirs (Hedlund *et al.*, 1999; Chung & King, 2001; Abed *et al.*, 2002; Iwabuchi *et al.*, 2002; Melcher *et al.*, 2002; Head *et al.*, 2006).

Hydrocarbon-degrading microorganisms isolated from the extreme cold and dry environment of Antarctica are currently of special interest, principally in establishing the extent of anthropogenic influence on both the Antarctic environment and indigenous microorganisms, especially through fuel pollution, but also in the detection of cold-adapted enzymes and developing bioremediation strategies for environmental pollution (Aislabie *et al.*, 1999, 2000; Margesin & Schinner, 2001; Whyte *et al.*, 2002; Ma *et al.*, 2006). The genera *Sphingomonas, Pseudomonas, Rhodococcus, Acinetobacter* and *Mycobacterium* are the most abundant hydrocarbon-degrading bacteria recovered in Antarctica (Aislabie *et al.*, 2000; Bej *et al.*, 2000; Eckford *et al.*, 2002; Ma *et al.*, 2006). Of these genera, only the genus *Rhodococcus* is essentially ubiquitous (Oldfield *et al.*, 1997; Folsom *et al.*, 1999; Ringelberg *et al.*, 2001; Castorena *et al.*, 2002; Iwabuchi *et al.*, 2002).

Even in Antarctica, different hydrocarbon-polluted eco-niches are dominated by different bacteria and our knowledge of bacterial diversity is still far from complete (Amann *et al.*, 1995). Many bacteria are still only known by their DNA signatures (as environmental clones) and constitute a transient operational taxonomic unit called the 'Candidate Division' (Dojka *et al.*, 1998; Abed *et al.*, 2002). It is therefore difficult to predict which bacteria will thrive in Vostok DF.

Comments on our findings

Two of our *Sphingomonas* phylotypes matched (and by this proved to be conspecific in terms of nucleotide substitution

numbers) sequences of bacterial clones or isolates identified in the Vostok ice core [at depth 3593 m (Christner *et al.*, 2001; Christner, 2002) and 3607 m (Bulat *et al.*, 2004)] and Taylor Dome ice core (Christner, 2002). These are two geographically separate regions of Antarctica with two different types of DF by chemical composition (kerosene/forane and *n*-butyl acetate, respectively) and ice type by origin (Lake Vostok accretion ice and deep glacial ice, respectively). One of our phylotypes, *Sphingomonas* sp., was also recovered in the bottom horizon of the GISP2 ice core, Greenland, which had been drilled with *n*-butyl acetate (Miteva *et al.*, 2004). Additionally, this phylotype has been recovered in soils polluted with aviation kerosene near Scott Base, Antarctica (Aislabie *et al.*, 2000) and even in boreal oligotrophic forest soils (Mannisto & Haggblom, 2006). Another phylotype, *Sph. natatoria*, was encountered in a low altitude coastal deep subsurface sample (Fredrickson *et al.*, 1995) as well as in a hospital water pool in the USA (Angenent *et al.*, 2005). All this points to the global occurrence of these two *Sphingomonas* species as they can be encountered everywhere and therefore represent a significant contamination source.

Some isolates conspecific (in a sequence) to our *Sphingomonas* sp. phylotype have been shown to degrade hydrocarbons under cold conditions (Table 3). Thus, *Sphingomonas* sp. Ant20 strain isolated from contaminated Antarctic soil can utilize alkylbenzenes (toluene and xylene) as well as aviation fuel JP-8 and successfully degrade PAH species (naphthalene and phenanthrene) (Aislabie *et al.*, 2000). Another strain, *Sphingomonas* sp. 437D, isolated from the same source, has been shown to utilize phenanthrene (Saul *et al.*, 2005). Another *Sph. natatoria*-relative phylotype has an indication for one conspecific isolate from the deep terrestrial subsurface (clone B0477) which utilizes monoaromatic hydrocarbon compounds such as salicylate and benzoate (Fredrickson *et al.*, 1995) (Table 3).

Some further clones or isolates conspecific to our two *Sphingomonas* phylotypes were recovered from cold environments in Antarctica and Greenland (Table 3). For example, the endolithic isolate *Sphingomonas* sp. eh2 was recovered from Antarctic salt crusts exposed to -10 to $0\,^{\circ}C$ (Hughes & Lawley, 2003). This could suggest possible psychrotolerant or psychrophilic properties in our *Sphingomonas* phylotypes that would allow them to thrive in the deepest horizons of Vostok DF where they would be exposed to *in situ* physiological temperatures of -10 to $-6\,^{\circ}C$ year-round (Table 1) and use alkylbenzenes and PAH species as carbon and energy sources. Preliminary data from GC-MS analyses of Vostok DF points to detectable concentrations of toluene, xylene, naphthalene, fluorene and phenanthrene (Y. Jouanneau, personal communication). However, to prove such a hypothesis, specialized culturing methodologies will need to be implemented.

Contamination issues

We would point out that in the context of Vostok DF, all the bacteria present in the samples analyzed must be considered contaminants. Amongst these we would identify two functional taxonomic groupings, namely those that can metabolize DF chemical components *in situ* and are therefore likely to dominate the DF community structure both qualitatively and quantitatively (biomass) and those that cannot metabolize DF components and are therefore likely to be present only as nongrowing, low biomass populations.

Two of our *Sphingomonas* phylotypes (*Sph. natatoria* and *Sphingomonas* sp.) matched well (99–100% similarity in a sequence) to sequence findings from different ice cores from Vostok, Taylor Dome (both Antarctica) and GISP2 (Greenland). On the basis of our studies and reference to the relevant documentation, it is considered likely that all these ice samples were not fully decontaminated before biological analyses were made, leading to some traces of DF remaining with the samples. The complete removal of DF traces (and, hence, associated contaminant microorganisms) from an ice core dedicated to molecular biological studies still represents a real challenge (Vincent, 1999). New ice decontamination approaches to not just removal of traces of DF, but making it DNA-free, are already being tested (e.g. chemical decontamination with kerosene/forane compatible solutions followed by ozonation).

In the case of our Vostok sample #3607, the phylotype identified as *Sph. natatoria* was originally presumed to be a contaminant, initially on the basis of the PAH-degrading capability of its closest relatives in GenBank (Bulat *et al.*, 2004) and now proved to be a contaminant by finding its counterpart in Vostok DF. This example testifies to the need to establish a microbial database for drilling fluids to provide authentication of ice findings.

The phylotypes of human- and soil-associated bacteria could have been brought into the Vostok DF during kerosene transportation to Vostok and subsequent storage at the station in big commercial tanks as well as during DF handling during its formulation whilst pumping into a borehole. This proposal is supported by the fact that two (of three total) human-associated phylotypes were detected in the uppermost level of the borehole (sample #110), which was the horizon most exposed to human activity. Moreover, this sample was collected within the 120 m coring case, which could provide a further source for contaminants.

Concluding remarks

Our assessment study has shown that Vostok DF obtained from different borehole horizons contained two *Sphingomonas* phylotypes which matched bacteria listed in global databases as capable of degrading oil and its products in

soils. At the same time, the study revealed few contaminants attributable to a human or soil source in the Vostok DF.

Detailed characterization of the microbial composition of the Vostok DF, especially at the bottom of the borehole, is a very significant ecological issue in view of proposed Russian plans to penetrate Lake Vostok via the existing drill hole. Lake Vostok is a pristine and extreme ecosystem whose upper waters, at least, may possibly not support heterotrophic life (Bulat *et al*., 2004), so any possibility of contamination must be addressed and an important step in this process is to characterize the possible contaminants. The present study provides such information as well as biomass estimates and cell distributions within a borehole. The study identifies the dominant populations of chemoorganotrophic *Sphingomonas* bacteria capable of degrading a broad range of substituted aromatic compounds, as well as identifying the occasional occurrence of DF contaminants attributable to human and soil sources. However, to make a comprehensive assessment of Vostok DF the development of a new DF sampling strategy designed specifically for clean sampling for biology and analytical chemistry is essential. In addition, because of the low biomass estimates obtained for the Vostok DF cell/DNA extraction and DNA amplification, protocols need to be appropriately adjusted to give higher sensitivity.

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