ANALYSIS AND CHARACTERIZATION OF MICROBES FROM ANCIENT GLACIAL ICE

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ABSTRACT

Scott O. Rogers, Advisor

The objective of this research was to study the bacterial and fungal composition of ice cores from Arctic and the Antarctic regions to understand their geographical and temporal distribution. Cosmopolitan microbes dominate these environments and have been deposited primarily by winds, birds, Ocean currents and mammals. Atmospheric currents play a significant role in the transport of these microorganisms to the polar regions. We hypothesize that the geographical isolation of the Antarctic region from other land masses, compared to the Arctic region, affects the transport of the microorganisms to the Antarctic region, resulting in lower number of microbes entrapped in the Antarctic ice. Four ice cores each were analysed from the Arctic (GISP2D core) and the Antarctic (Vostok 5G core) regions dating back to 10,000 YBP, 57,000 YBP, 105,000 YBP and 155,000 YBP using culturing, PCR, sequencing and SEM techniques. We report the isolation of fungi and bacteria from six of the eight ice cores analysed. Fungi most closely related to Rhodotorula, Penicillium, Cladosporium, Alternaria, Aspergillus and Cryptococcus, and bacteria related to Caulobacter and Bacillus were identified. The ice cores from Arctic had higher number of microorganisms and species richness than the core sections from the Antarctic region. Phylogenetic studies were done to compare the organisms with one another and also with the conspecifics. Some sequences showed high similarity to contemporary species, while some did not group closely with the present day organisms. Fungi isolated from the different locations, and closely related to the same genus, did not show high similarity with one another.
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CHAPTER I: INTRODUCTION AND LITERATURE REVIEW
Glacial ice- formation and significance

Microorganisms have been found to survive and even thrive in some of the most harsh and extreme environments present on Earth. They have been isolated from hot springs, which have extremely high temperatures, and also from cold environments including permafrost, sea ice, glacial ice and subglacial lakes. Study of the microbes that can survive and remain active in extreme environments is interesting and significant because these conditions are comparable to some extraterrestrial environments. Study of life in the permanently cold environments of glaciers in the Arctic and Antarctic regions is especially significant because of the discovery of ice beyond Earth. An icy cover has been discovered on Europa (a moon of Jupiter) and recent studies point towards traces of ice on Mars (Chyba 2000). Glacial ice in the polar regions on Earth has been accumulating for hundreds of thousands of years and in some cases for several million years. The process of formation of glacial ice from snow is a complex phenomenon controlled by different factors and taking a long time. Snowfall is the first step in this process, followed by the conversion of snow to firn (wet snow). Glacial ice is formed when the interconnecting air passages between the grains (crystal or a group of crystals) in firn are sealed, leaving gas in bubbles (Paterson 1994).Therefore, glacial ice holds a valuable record of the past climate along with a continuous record of microbial life. The study of microbes in glacial ice helps us to understand the survival mechanisms of these organisms under extreme conditions, and to understand how they compare to contemporary organisms from present day environments.
Glacial ice and the study of climate

The surface ice caps and ice sheets of Earth have trapped the atmospheric constituents from different times in chronologic order. Study of the physical and chemical composition of these ice cores and the resulting data can help us to understand the past climatic conditions and the global changes in the climate. Correlation of the change in climate (e.g., increases in temperature) with the atmospheric composition (e.g., CO₂ levels) during those times may help us to understand the current and future climate changes, especially with the global climate change taking place currently. Various components of the ice cores help us to understand different aspects of the past climate. The O₁₈/O₁₆ ratio is dependent on the temperature, atmospheric chemistry is reflected by anion to cation concentrations, sulphates in the ice give a record of the volcanic eruptions, dust concentrations indicate the turbidity of the atmosphere, trace element concentrations can be used to measure atmospheric emissions and the expansion and contractions of local vegetation can be understood by measuring the levels of nitrate concentrations (Patterson 1994).

There are other records in nature that provide useful information about past climate change like coral reefs, tree rings, and deep sea sediments. However, each of those methods has drawbacks. Compared to the other records, proxy records from the ice cores hold a continuous and more detailed record of the various paleoclimatic parameters including an accurate record of the gaseous concentration of the ancient atmosphere (Patterson 1994). Using the ice cores retrieved from different parts of the world, scientists have been able to reconstruct past climate records up to several hundred thousand years. Ice cores from Greenland, Antarctica and Tibet have been used to put together the
climate record going back through the last glacial-interglacial cycle (Dansgaard et al 1993; Grootes et al 1993; Lorius et al 1985 and Thompson et al 1997). The study of the Vostok ice core has helped reconstruct the climate record for the past 420,000 years which covers 4 glacial cycles (Petit et al 1999). The study of the ice from polar regions gives a more comprehensive picture of the past climate record compared to the study of ice from other geographic locations. The size and volume of polar glaciers plays a role in regulating global climate changes and patterns. Tropical and subtropical glaciers do not (Thompson et al 2000).

The study of climate patterns of the past helps in understanding the evolution of life on the planet. Earth has seen climate fluctuations resulting in huge changes in the temperatures resulting in ice ages over long periods of time. The cycles called glacial and interglacial cycles have been a common occurrence throughout history, but three such events (2.7-2.8 billion years ago, 2.3-2.5 billion years ago and 600-800 million years ago) have been big enough to have played an important role in the evolution of life. The two periods of global glaciation that occurred 600-800 million years and 2.3-2.5 billion years ago resulted in a condition called “snowball Earth”, when the Earth was covered in ice completely (Hoffman et al 1998 and Kirschvink et al 2000). These periods of extremely low temperatures and extensive ice cover were followed by periods of rapid warming caused the the release of huge amounts of CO₂ into the atmosphere from volcanic sources and the end of some of these cold periods coincided with large meteorite impacts. However, the condition of snowball Earth might have taken 4-30 million years to reverse and such prolonged periods of freezing conditions and modified geochemistry might have had serious implications for the various biological ecosystems of the time (Hoffman et al
1998). The period of warming after the second snowball Earth might have been responsible for the Cambrian explosion, a period when all the animal phyla rapidly evolved (Hoffman et al 2000). The study of the physical and chemical properties of the ice cores helps us to understand the past climate and how it influenced the evolution of life on Earth.

**Preservation of life in ice**

Environmental ice acts as a protective matrix for microorganisms over extended periods of time, and provides a valuable record of microbial evolution and ancient biodiversity (Ma et al. 2000). Organisms trapped in ice may remain viable in a dormant state, or are active and able to carry out a low level of metabolism. Thymidine and leucine incorporation experiments on *E.coli* at -15°C and -70°C showed metabolic activity in cells at -15°C but not at -70°C (Carpenter et al 2000). Liquid water is present in ice at low temperatures, and is essential for the metabolic activity of the cells. Water is present at the interface of ice crystals and also as a film on air bubbles, and has been shown to exist in ice up to temperatures of -60°C (Ostromou and Siegert 1996; Price 2000). The absence of liquid water (water activity almost zero) may be responsible for the absence of metabolic activity in *E.coli* at -70°C. Glacial ice has constant temperatures, but higher than -60°C, making it ideal for long term preservation of microorganisms (Willerslev et al 2004). The low temperatures in these environments will stop cell division but retain low levels of metabolic activity. Continuous catabolism in cells with decreased anabolism rates lead to the production of free radicals responsible for damaging DNA and proteins (Aldsworth et al 1999). Low metabolic activity in these conditions prevents the release of free radicals, thereby protecting the cells. The lack of
cell division in these conditions prevents genetic mutations caused by active replication while the low metabolic activity allows for DNA repair of any spontaneous mutations. Small acid-soluble proteins (SASP) have been observed in dormant cells like bacterial endospores and help reduce the DNA damage by binding to the DNA (Willerslev et al 2004). The organisms that get trapped in the environmental ice and survive the freeze-thaw cycles may remain in a dormant or viable state for long periods of time (Rogers et al 2004).

Study of the organisms isolated from environmental ice may give valuable insights into evolutionary processes, and in the case of pathogens may be important for health safety and precautions. Environmental ice carries a huge and diverse mix of microbes, and with the increased rate of glacial melting, ancient microbes have been released in huge numbers. The release of ancient microbes may lead to these microbes entering the contemporary population from which they have been separated for hundreds of thousands of years. The temporal gene flow that may occur because of this mixing of the ancient and modern genotypes has been termed as “genome recycling” (Fig 1). The rate of glacial retreat has increased in the last 1000 years and recent increases in temperatures because of global climate change resulted in an acceleration of this process (Rogers et al 2004). The melting of environmental ice releases an estimated $10^{17}$ to $10^{21}$ viable microbes every year (Smith et al 2004). The release of pathogenic microbes is of even greater concern as they may have an increased virulence because of the lack of immunity or defense to these strains in the host. The appearance of some strains of influenza and calicivirus decades apart may be related to the melting of environmental ice.
**Source:** Rogers et al 2004

**Fig 1: Genome recycling through glacial ice.** Microorganisms transported into the atmosphere by various means like wind and vulcanism are deposited onto the glaciers by precipitation. These organisms get entrapped in the glacier with the formation of ice. These organisms are trapped in glacial ice for thousands to millions of years. The melting of the glacier releases the entrapped microorganisms into the hydrosphere (lakes, streams, rivers and oceans). This helps the microbes released from glacial ice to interact with the extant populations of the same species in the genome mixing zone and provides an opportunity for temporal gene flow.
(Rogers et al 2004). The presence of viable microbes in environmental ice hundreds of thousands of years old, and the threat of the release of older strains of pathogens highlights the need for the study and monitoring of these ice cores.

**Microorganisms in cold environments**

Microorganisms have been identified and isolated from glacial ice samples from many different regions of the world. The global distribution of these microorganisms in snow and glacial ice is most likely the result of wind currents and atmospheric circulation. Bacteria and fungi have been detected and isolated from different kinds of frozen environments such as permafrost, sea ice, glacial ice and accretion ice from subglacial lakes. Cryopegs are layers of unfrozen ground surrounded by perennially frozen ground. The dissolved solids in the water cause the lowering of freezing point depression, preventing the water from freezing and forming pockets of water in permafrost, with the temperature ranging between -9 and -11°C (Ozerskaya 2004).

Ozerskaya et al (2004) reported the isolation of 40 fungal strains from two cryopegs in Kolyma lowland region of Siberia. The fungi isolated belonged to the genera *Alternaria*, *Aureobasidium*, *Cladosporium*, *Geomyces*, *Penicillium*, *Ulocladium*, *Valsia* and *Verticillium*. More genera were recovered from the MEA medium and at 25°C as opposed to 4°C. Gilichinsky et al (2005) isolated bacteria, fungi and yeast from cryopegs near the East Siberian Sea coast. *Psychrobacter* was the most abundant bacterial isolate. Isolation of bacteria was done from Siberian cryopegs by Bakersman et al (2003), and were most closely related to the genera *Psychrobacter*, *Arthrobacter*, *Frigoribacterium*, *Subtercola*, *Microbacterium*, *Rhodococcus*, *Erwinia*, *Paenibacillus* and *Bacillus*. Fungi have been isolated from the Arctic soils, where water is not available for biological purposes.
because of the low temperatures. Most of the soil bacteria and fungi have were oligotrophs that can survive in the low nutrient conditions (Bergero et al 1999). Fifty four fungal isolates were recovered from Arctic soils by Bergero et al (1999) and belonged to 21 genera. *Acremonium*, *Cladosporium*, *Geomyces*, *Mortierella*, *Phoma* and *Thelebolus* were the most common genera. Nine species belonging to the genera *Geomyces*, *Phoma*, *Mortierella* and *Thelebolus* were classified as psychrooligotrophic fungi. Most of the fungi had dark or hyaline sterile mycelia, indicating survival strategies for fungi. Permafrost is another cold environment, well studied for the presence of microorganisms. Microbes in these environments are exposed to continuous subzero temperatures and background radiation. Lock (1990) reported that a thin film of water surrounds the soil and ice particles in permafrost even at subzero temperatures. The liquid water might facilitate the transfer of nutrients and toxic chemicals, and might be essential for the survival of microbes in these environments. Studies on the permafrost soils from the Arctic and Antarctic regions showed differences in their physical and chemical properties. The Antarctic permafrost has lower temperatures, lower organic matter, higher pH, and a higher reducing environment (Table1).
Table 1: Physical and chemical characteristics of Arctic and Antarctic permafrost. The differences in the physical and chemical characteristics of permafrost samples from the Arctic and Antarctic regions.

<table>
<thead>
<tr>
<th>Location</th>
<th>Depth (m)</th>
<th>Age[a]</th>
<th>Temperature (°C)</th>
<th>pH</th>
<th>Organic carbon[b]</th>
<th>Ice[b] (%)</th>
<th>$E_h$ (mV)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arctic Permafrost</td>
<td>0–100</td>
<td>Present to 5 Ma</td>
<td>−7 to −11</td>
<td>5.6–7.8</td>
<td>0.35–10</td>
<td>17–164</td>
<td>40 to −250</td>
<td>Vorobyova et al. (1997), Gilichinsky (2003), Shi et al., (2004), Vishnivetskaya et al. (2000) Pollard, personal communication or our data, unpublished</td>
</tr>
<tr>
<td>Kolyma lowlands, Siberia</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eureka, Ellesmere Island, Canada</td>
<td>0–15</td>
<td>Present to 20,000 years old</td>
<td>−10 to −17</td>
<td>6.5</td>
<td>2.19</td>
<td>30–100</td>
<td>NA[c]</td>
<td></td>
</tr>
<tr>
<td>Antarctic Permafrost</td>
<td>0–17</td>
<td>150,000 to 2 Ma</td>
<td>−18 to −27</td>
<td>7.8–9.8</td>
<td>0–0.43</td>
<td>NA</td>
<td>260–480</td>
<td>Vorobyova et al. (1997)</td>
</tr>
<tr>
<td>Dry Valley, Taylor Valley, Miers Valley, Mt. Feather</td>
<td></td>
<td></td>
<td></td>
<td></td>
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[a]Ma millions of years  
[b]Percent of dry weight soil  
[c]Data not available

Source: Steven et al 2006.
Diversity in the Arctic and Antarctic environments

The diversity and number of microbes isolated from the permafrost samples are high along with a significant difference between Arctic and Antarctic permafrost. The population and diversity of bacteria isolated from the Arctic permafrost samples was significantly higher compared to those isolated from Antarctic permafrost samples (Table 2; Gilichinsky et al 1995, Shi et al 1997, Vorobyova et al 1997). Fewer fungi have been isolated compared to bacteria from these samples, but the biomass of eukaryotes has been found to be ten times greater than prokaryotes in these environments (Vorobyova et al 2001). Yeasts were the dominant group of eukaryotes isolated, with Cryptococcus, Rhodotorula and Saccharomyces being the most commonly reported genera (Vorobyova et al 1997).

Temperature might not be the key factor in the recovery of viable microbes. The age of the permafrost was inversely proportional to the number and diversity of the isolates. As the age increased, the number and diversity decreased in some cases to zero (sterile ice samples) (Gilichinsky et al 1989, Gilichinsky et al 1992). Vorobyova et al. (2001) reported that the percentage of viable cells recovered from the Antarctic permafrost (0.001-0.01%) was much lower compared to the Arctic permafrost (0.1-10%). Several studies have looked at the presence of microbes in glacial ice from different locations, with most of the samples coming from Arctic and Antarctic regions. Ma et al (1999) isolated bacteria and fungi from various Greenland ice core sections, using culturing and PCR techniques. The isolates belonged to several genera: Cladosporium, Penicillium, Aureobasidium, Rhodotorula, Cryptococcus, Alternaria, and Aspergillus.
**Table 2: Bacterial isolates from Arctic and Antarctic permafrost.**

Differences in species richness of microorganisms isolated from Arctic and Antarctic permafrost.

<table>
<thead>
<tr>
<th>Arctic Permafrost</th>
<th>Antarctic Permafrost</th>
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<tr>
<td><em>Bacillus, Arthrobacter, Micrococcus,</em></td>
<td><em>Arthrobacter, Bacillus and Streptomyces</em></td>
</tr>
<tr>
<td><em>Cellulomonas, Rhodococcus,</em></td>
<td></td>
</tr>
<tr>
<td><em>Flavobacterium, Pseudomonas,</em></td>
<td></td>
</tr>
<tr>
<td><em>Aeromonas, Myxococcus,</em></td>
<td></td>
</tr>
<tr>
<td><em>Exiguobacterium, Nitrobroch,</em></td>
<td></td>
</tr>
<tr>
<td><em>Nitrosononas, Nitrosospira and Streptomyces</em></td>
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Most of the bacterial isolates were closely related to the genera *Sarcina* and *Micrococcus*. Ma et al (2000) reported the isolation of fungi from Greenland glacial ice cores. The study specifically looked for fungi in these ice cores, and isolated organisms belonging to the genera *Penicillium, Ulocladium, Cladosporium, Alternaria, Tricholoma* and *Acremonium*. Most of the studies on glacial ice focused on the identification or isolation of bacteria. Sheridan et al (2003) and Miteva et al (2004) isolated bacteria from a 100,000 year old GISP2 ice core section close to the sediment-ice interface rich with sediments. Recovery time was much shorter for the anaerobic isolates compared to the aerobic isolates. Seven of the twenty-seven unique organisms identified were suggested to represent new genera. A high percentage of isolates belonged to the genera *Pseudomonas, Bacteroides* and *Clostridium*. The number of colonies and unique sequences of aerobic organisms was greater than the anaerobic isolates. Seven of the fifty-six aerobic isolates have been reported as new species. Microbiological studies have been done on the accretion ice of Lake Vostok. This ice offers the closest representation of the lake water. D’Elia et al (2008) reported the isolation of bacteria from Vostok accretion ice samples. Several bacterial colonies were isolated and yielded eighteen unique sequences, closely related to the species in the genera *Bacillus, Carnobacterium, Micrococcus* and *Caulobacter*. Several fungal isolates belonging to the genera *Rhodotorula, Penicillium, Cladosporium, Cryptococcus* and *Ustilago* have been isolated from the accretion ice.
Adaptations of microbes

The organisms present in these conditions follow different strategies to survive the harsh conditions. Some might be dormant for most of the time and return to activity under favorable conditions. These are organisms that perform metabolic activities at minimal rates, and should have adaptations to low temperature, high pressure, high salinity and ground radiation. The organisms achieve physiological and ecological success in these environments due to adaptations in their proteins, membranes and their genetic responses to thermal shifts (Deming 2002).

Enzymes and cold shock proteins

Some enzymes (e.g., xylanases, laminarases, valine dehydrogenase, chitinases and pectate lyases) identified in the cold adapted bacteria have been reported to aid in their survival under cold conditions (Humphrey et al 2001, Oikawa et al 2001, Lonhienne et al 2001, Bendt et al 2001). These enzymes had reduced activation energies leading to catalysis of reactions at low temperatures. Studies compared the enzymes from cold-adapted bacteria to their conventional forms and observed that specific amino acid residues, mostly in the active site domain, were responsible for their activity in the cold conditions (Bendt et al 2001, Irwin et al 2001). Studies on enzymes involved in the protein-synthesizing machinery of an Antarctic methanogen showed similar adaptations (Thomas et al 2001). A relation was also observed between the cold shock proteins of the organism and its survival under high pressure (barophily) (Deming et al 2000). Bacterial genes for cold shock proteins were identified in some members of the Crenarchaeota that might indicate horizontal gene transfer between the different domains (Beja et al 2002).
Protein synthesis in psychrophiles is also a concern as earlier studies showed that ribosomes do not function well at low temperatures. Initiation of protein synthesis is the important step because it is the most susceptible to low temperatures. Ribosomes in psychrophiles have adapted to function at these low temperatures (Russell 1997). Studies further showed that psychrophilic bacteria produce cold shock proteins in response to drops in temperature and cold acclimitation proteins at continuous low temperatures (Roberts et al 1992). CspA is one such cold shock protein that acts as a transcriptional protein for other cold shock genes in *E.coli*. Studies identified homologous proteins performing the same function in *B.subtilis* and some Antarctic psychrotrophic bacteria (Willemsky et al 1992). A DNA-binding protein H-NS identified in *E.coli*, protects the organism from cold shock and also helps the organism grow faster at low temperatures; however, it is not essential for growth at low temperatures (Russell 1997).

**Cell membrane structure and cytoplasm**

An important adaptation observed in microbes for survival at low temperatures is the capability of the cell membrane to adjust to the harsh conditions and protect the cells from freezing. Changes in the membrane lipid composition facilitate this process. The cytoplasm of the cell should be protected from freezing to enable metabolic reactions to occur normally. The cell membrane performs this function by preventing external ice from entering the cell. This phenomenon, where the inside of the cell stays in a liquid state below zero is called super cooling, and this stage can persist to about \(-15^\circ\text{C}\), below which the cytoplasm freezes. Another interesting mechanism observed to prevent the freezing of the cytoplasm is the water fraction of the cytoplasm. Studies reported that lower water fractions in the cytoplasm lowered the chances of freezing. A water fraction
of 10% in the cytoplasm prevents the freezing of cytoplasm even at -40°C (Finegold 1996). Some of the microbes might send water out of the cell at lower temperatures to protect the cytoplasm. Membranes of these microbes avoid the transition from liquid to gel that results at low temperatures. This is achieved by modifications in the fatty acyl chains of lipids. An increase in the proportion of unsaturated acyl chains, shortening of acyl chains and increased methyl branching results in increased fluidity of the membrane. A group of enzymes called desaturases, which convert saturated acyl fatty acids to unsaturated acyl fatty acids also have been proposed to aid the membrane in maintaining its fluidity (Finegold 1996). Fungal cell membranes show similar changes at low temperatures to maintain their fluid state. The degree of unsaturated fatty acids increased at low temperatures in Candida, Leucosporidium, and Torulopsis. The degree of unsaturation increased with lower temperatures (Kerekes et al 1980).

Along with the constant low temperatures, microbes in frozen environments have to deal with low water activity. Studies using low water activity (0.946) enumeration media for the isolation of fungi from glacial ice and sea ice samples reported significantly higher CFU/L, when compared to the enumeration media with normal water activity (1.00) (Sonjak et al 2006).
Pigmentation and other physiological adaptations

Pigmentation was observed in several extremophiles and was suggested as an adaptation to low temperatures. Study of bacteria isolated from Antarctic sea ice showed a predominance of pigmentation (Bowman et al 1997a). Pigments belonging to the chlorophyll group protect organisms from UV radiation. Carotenoid pigments have been reported to play an important role in the maintenance of membrane structure and fluidity of several microorganisms (Armstrong 1997). Accumulation of a C-50 carotenoid Bacterioruberin, was observed in a psychrotrophic strain of *Arthrobacter agilis*, at low temperatures (Fig.2). Bacterioruberin was suggested to play a role in membrane stabilization at low temperatures (Fong et al 2001).

Several physiological mechanisms have been proposed to play an important role in cold tolerance of fungi. Trehalose, a widely distributed disaccharide and an important storage compound in fungi, is a general stress protectant and stabilizer of membranes during dehydration (Goodrich et al 1988). Studies reported that trehalose accumulated in large quantities in fungal hyphae at low temperatures. Niederer et al (1992) showed that trehalose concentration doubled in alpine mycorrhizal roots, on exposure to low temperatures. Tibbett et al (1998a) reported that Arctic strains of *Hebeloma sp.* showed accumulation of trehalose as opposed to temperate strains of the same species. Mannitol also is an important cryoprotectant. Weinstein et al (1997) compared the sugar levels and composition of an Antarctic strain *Huminola marvinii* with a temperate strain, *Huminola fuscoatra*, and reported high levels of mannitol in the Antarctic strain as opposed to high levels of glucose and fructose in the temperate starins, while the total sugar levels were equal.
Various adaptations allow microorganisms to survive in extremely cold, and permanently frozen environments. Some of these environments include: the deep sea aquatic environments (-1\(^0\)C to -4\(^0\)C), glacial ice (-5\(^0\)C), lake ice (-5\(^0\)C) and Arctic and Antarctic marine environments (-1\(^0\)C to -35\(^0\)C) (Deming 2002). Price et al. (2000) proposed that liquid water might exist in veins at the interface of three ice crystals (triple junctions). Even at subzero temperatures in deep glacial and sea ice, these liquid veins may serve as a habitat for surviving microbes. Novel techniques in microscopy have helped identify such brine pockets in Arctic ice at -15\(^0\)C along with microbial inhabitants (Junge et al 2001). Tritiated thymidine and leucine have been used in incubation experiments with Antarctic ice samples and identified metabolic activity to -17\(^0\)C (Carpenter et al 2000). Bacteria can survive in the coldest form of ice. A form of ice (ice-IV), not found on Earth, forms under enormously high pressure and had been observed to support life (Sharma et al 2002). The difference between ice-I (found on Earth) and ice-IV is that the solid phase of water is heavier than the liquid phase in ice-IV (Deming 2002). Liquid water is retained even in this form of ice, between different ice crystals, wetting the surfaces of the crystals. The interface between the solid and liquid water is called the eutectic phase. Microbes survive in this interface and are called Eutectophiles (Sharma et al 2002).

**Fig 2: Variation in pigment production with temperature.** Increase in the production of the pigment Bacterioruberin is observed with a decrease in temperature in *Arthrobacter agilis*. The pigment Bacterioruberin plays an important role in the survival of the organism at low temperatures by playing an important role in the membrane stabilization. Pigmentation is a widespread adaptation observed in several microorganisms in response to low temperatures.
Psychrophilic and Psychrotolerant organisms

Most of the bacteria and fungi isolated from these permanently cold environments were psychrotolerant as opposed to psychrophillic, having optimal growth temperatures well above the freezing temperatures common to these kinds of environments (Table 3). The base for the classification are the optimal growth temperatures and the maximum temperatures at which the organisms grow. Psychrophiles are organisms that grow at 0°C and have an optimum growth temperature below 15°C and a maximum growth temperature below 20°C and psychrotrophs are less likely to grow at 0°C, but will grow at 3-5°C and have optimum and maximum growth temperatures above 20°C (Morita 1975).

Miteva et al (2004) reported similar findings among isolates from GISP2 sediment ice core. A few isolates showed the temperature-growth profile of psychrophiles as opposed to the majority isolates that were classified as psychrotrophs (Fig.3).

Decontamination of ice cores

The authenticity and reliability of any study involving the isolation and identification of microbes or nucleic acids from any ancient geological samples like glacial ice is dependent on the effectiveness of the decontamination protocol. A good decontamination protocol will include effective methods to eliminate the contamination present on the ice core surface while preserving the organisms and nucleic acids inside the ice core sample (Ma et al 2000). Isolation of unique organisms or sequences is one of the primary objectives of the study of ancient ice (Ma et al 2000). Finding a rare or unique sequence or culture may not be sufficient to authenticate it as only about 1-15% of the extant microbial diversity is currently known. The most important issue in the claims
Psychrotrophs outnumber psychrophiles in permafrost. Psychrotrophs are believed to better survive the freeze thaw cycles that occur in most of the cold environments, whereas psychrophiles dominate sea ice which has a constant low temperature throughout.

Table 3: Psychrotrophs outnumber psychrophiles in permafrost. Psychrotrophs are believed to better survive the freeze thaw cycles that occur in most of the cold environments, whereas psychrophiles dominate sea ice which has a constant low temperature throughout.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Description</th>
<th>Minimum growth temperature (°C)</th>
<th>Optimal growth temperature (°C)</th>
<th>Generation time (days)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Psychrobacter cryopegella</td>
<td>Psychrotrophic, halotolerant aerobic Gram-negative heterotroph</td>
<td>−10</td>
<td>22</td>
<td>62.5c</td>
<td>Bakermans et al. (2003)</td>
</tr>
<tr>
<td>Carnobacterium pleistocenium</td>
<td>Psychrotrophic Gram-positive facultative anaerobe</td>
<td>0</td>
<td>24</td>
<td>NA</td>
<td>Pikuta et al. (2005)</td>
</tr>
<tr>
<td>Clostridium algophilum</td>
<td>Psychophilic anaerobic spore-former</td>
<td>−5</td>
<td>5</td>
<td>2.1</td>
<td>Sheherbakova et al. (2005)</td>
</tr>
<tr>
<td>Psychrobacter sp. 273-4</td>
<td>Aerobic Gram-negative heterotroph</td>
<td>−2.5</td>
<td>26</td>
<td>3.5</td>
<td>Ponder et al. (2005)</td>
</tr>
<tr>
<td>Psychrobacter sp. 215-51</td>
<td>Aerobic Gram-negative heterotroph</td>
<td>−2.5</td>
<td>26</td>
<td>3.5</td>
<td>Ponder et al. (2005)</td>
</tr>
<tr>
<td>Exiguobacterium sp. 255-15</td>
<td>Aerobic Gram-positive heterotroph</td>
<td>−2.5</td>
<td>42</td>
<td>5.5</td>
<td>Ponder et al. (2005)</td>
</tr>
<tr>
<td>Virgibacillus sp. nov</td>
<td>Psychrotrophic spore-former, aerobic heterotroph. Capable of growth in 20% NaCl</td>
<td>0</td>
<td>30</td>
<td>2.4</td>
<td>Our data, unpublished</td>
</tr>
<tr>
<td>Sulfobacillus sp. nov</td>
<td>Psychrotrophic facultative autotroph. Sulfur oxidizer</td>
<td>−4</td>
<td>25</td>
<td>3.5</td>
<td>Our data, unpublished</td>
</tr>
</tbody>
</table>

Source: Steven et al 2006
Fig 3: Psychrophilic and psychrotrophic bacteria in Greenland ice. Temperature range of growth for the bacterial isolates from the Greenland sediment ice cores. A majority of the isolates grow in a temperature range of 2-37°C and thereby fit the profile of psychrotrophic organisms.
of ancient DNA or microbes is the authentication of positive results. The widespread presence of microbes and nucleic acids everywhere, makes it difficult to convince critics about the authenticity of such claims, and hence a tested and proven decontamination protocol is of utmost importance in such studies (Willerslev et al 2004). Preventing the contamination of the ice core in the laboratory is an important part of the decontamination process because of the universal distribution of microbes in laboratory settings. A lack of protocol to clearly distinguish between an endogenous DNA sequence or culture from that of a contaminant emphasizes the need to prevent any contamination of environmental ice sample in the laboratories (Hebsgaard et al 2005).

Contamination can be broadly classified as sample-based contamination and laboratory-based contamination. Sample-based contamination occurs during the excavation of ancient materials or drilling of the ice cores. Human DNA is the most common form of contaminant, and is almost impossible to remove. Laboratory-based contamination includes several aspects of the work carried out in the labs. While the commonly spread microbes in the laboratory are a concern, the biggest concern is the PCR-product carryover. The opening of PCR tubes and liquids transfers invisible aerosol droplets ($10^3$-$10^9$ molecules each) into the surroundings. The environmental DNA concentration in glacial ice samples is less than 1pg/ml which is less than the DNA concentration of a single PCR aerosol. Apart from the PCR carryover, free DNA molecules and cells are also spread by lab people, reagents and tools (Hebsgaard et al 2005).

The use of a clean laboratory with an isolated ventilation system, UV-irradiation of surfaces and positive air pressure along with the cleaning of all surfaces and objects in
the room with 5% bleach on a daily to weekly basis helps prevent laboratory-based contamination (Hebsgaard et al 2005). Failure to prevent contamination and PCR carryover results in false positives and will be a major concern in the validation of the results (Willerslev et al 2004).

Laboratory-based contamination can be minimized by following the above mentioned steps, but sample-based contamination cannot be avoided due to all the complicated processes involved in drilling the glacial ice cores. The analysis of the ancient ice cores is difficult due to the need to authenticate and reproduce the results in order to exclude the possibility of the presence of contaminants (Willerslev et al 2004). Therefore the selection of a standardized and proven decontamination protocol is of utmost importance to validate the isolation of viable microbes and DNA from ancient ice samples.

Several decontamination procedures have been developed and used by various groups with varying effectiveness. However, each method has some advantages and disadvantages. Christner et al (2006) used 95% ethanol as the disinfectant after the ablation of the top layer of the ice core using a sterile blade. The procedure worked well in the case of the microbes but 95% ethanol does not kill all the microbes or destroy nucleic acid contaminants. Rogers et al (2004) compared several decontamination protocols and determined that treatment with 5% sodium hypochlorite provides the most effective treatment. Sham ice cores were prepared with *Aureobasidium pollulans* and *E.coli* in the ice cores and *Ulocladium atrum* and *Bacillus subtilis* along with RNA and DNA on the exterior surface of the cores. Different decontamination procedures compared included 5.25% sodium hypochlorite treatment, 95% ethanol treatment, heated
probe method, drilling method, sterile water ablation, mechanical ablation and UV irradiation method. The meltwater from the sham cores was collected in shells, where shell 1 represented the outermost layer of the ice core and shell 5 represented the innermost layer of the core. Culturing and PCR amplification techniques were used to determine the effectiveness of the different decontamination protocols (Fig. 4) (Rogers et al. 2004).

It is not possible to completely exclude contamination based only on the experimental design and sequence identification and analysis (Hebsgaard et al. 2005). Several precautions have to be taken throughout the experimental procedure and tests conducted during the various steps to ensure the authenticity of the results (Willerslev et al. 2004, Rogers et al. 2005). All the work involving the samples should be done in a clean room specified for this purpose (Rogers et al. 2004, Willerslev et al. 2004). The clean room should be regularly sterilized on a daily or weekly basis using UV irradiation and 5% sodium hypochlorite (Rogers et al. 2004, Hebsgaard et al. 2005). A sterile laminar flow hood treated with UV irradiation, 5% sodium hypochlorite and 95% ethanol should be used for processing the ice samples and further work (PCR and culturing) involving the samples (Rogers et al. 2004). All the lab personnel using the clean room should wear gloves and lab coats at all times (Willerslev et al. 2004). All the surfaces, tubes used in the experiments and tools should be treated with 5% sodium hypochlorite before use (Rogers et al. 2004). Various reagents used in the procedures should be autoclaved and sterilized properly. Some of the equipment may need to be autoclaved twice to ensure the
**Fig 4: Summary of the results of different decontamination protocols.** The upper graph shows the number of colonies of organisms in the outer shell and the lower graph indicates the number of colonies of the organisms inside the sham ice cores after decontamination. The 5.25% sodium hypochlorite treatment resulted in effective removal (100%) of the outer organisms (contaminants) while preserving the inner organisms. Other decontamination protocols were unsuccessful in killing all the organisms in the outermost layer (contaminants).
destruction of smaller DNA pieces (Willerslev et al 2004). An effective decontamination protocol needs to be selected using sham ice cores in the lab with known organisms and the surface of the sham cores spiked with known contaminants (Rogers et al 2004). The manipulation of the ice cores in the lab should be kept at a low level (Rogers et al 2004, Willerslev et al 2004). Additionally, blanks should be used as controls, ideally during every step of the procedure to validate the results (Rogers et al 2004, Hebsgaard et al 2005). Control plates need to be placed throughout the procedures to monitor the air flow in the laminar flow hood (Ma et al 2000). If the experiments involved filtration of the samples, a blank filter (used to filter sterile water) is used as a control (Willerslev et al 2004). Negative controls (only reagents and no core sample) should be included during the PCR experiments to rule out any carryover contamination (Hebsgaard et al 2005). Cloning of the PCR product and comparing sequences from multiple clones helps authenticate the findings (Willerslev et al 2004). The corroboration of the results in the same laboratory by a different person and the independent reproducibility of the results by a different laboratory will give greater validity to the results (Rogers et al 2005, Hebsgaard et al 2005).
Isolation techniques

In recent years, a number of microbial ice core studies have employed techniques other than culturing (e.g. PCR and microscopy) for the identification of microorganisms, due to the difficulties involved in culturing these organisms. While these techniques provide faster and more reliable data, other useful information including the study of metabolic properties and survival mechanisms under extreme conditions requires the isolation of viable organisms (Miteva et al. 2005). Because only a small percentage of the known microbial population is culturable, isolation and growth of microorganisms from extreme environments such as glacial ice is even more difficult. Various culturing techniques have been developed by different groups, but the process is made more difficult because of the varying harsh conditions these microbes experience in their environments. These conditions need to be duplicated to facilitate the recovery and growth of the microorganisms in the lab. One of the techniques developed for enumeration of microorganisms from Arctic sea ice samples involves preparing media with low water activity (Gunde-Cimerman et al. 2003). Microorganisms growing in extreme cold conditions are influenced by ice formation resulting in a lack of biologically available water. The freezing and binding of water in the ice crystals results in the expulsion of ions into the remaining liquid water, leading to the increase in their concentration. Low-water activity media with high concentrations of salt or sugar were prepared, and a higher CFU count was observed in the low water activity media (aw 0.946) as opposed to the media with normal water activity (aw 1.000) (Fig.5) (Gunde-Cimerman et al. 2003). The use of low-water activity media prevents osmotic imbalance, providing better recovery and growth of microorganisms (Sonjak et al. 2006).

**Fig 5: Recovery of fungi at low water activity.** Recovery of various fungal isolates from sea water, sea ice and glacial ice samples using normal and low-water activity media. In case of both the ice samples, higher number of colonies were obtained using the low-water activity medium DG-18 (aw 0.946).
Most of the microorganisms entrapped in glacial ice are in an inactive state for prolonged periods of time and may also be starved and damaged. Techniques have been developed to provide the best possible conditions for these cells to recover and grow. Meltwater from ice cores is filtered through 0.2 \( \mu \text{m} \) filters, which are resuspended by vortexing in phosphate-buffered saline. The solution is then plated on different types of media including low-nutrient media. The plates are incubated at varying temperatures under dark conditions for long periods of time (up to several months) (Christner et al 2003). A high percentage of the microbes isolated from extremely cold environments are psychrotolerant rather than psychrophilic, and a wide range of incubation temperatures helps provide the best possible conditions for the growth of the microorganisms. The long periods of incubation aids the cells in synthesizing enzymes needed to repair the damages incurred by the cells during their long inactive or dormant periods (Christner et al 2000). Trying to replicate the closest conditions existing in their environment increases the chances of recovery of microorganisms from extreme environments.

Microorganisms with diameters less than 0.3 \( \mu \text{m} \) are called ultra small microorganisms or ultramicrobacteria, and comprise more than 70% of the soil microorganisms, and less than 0.2% of these are culturable (Miteva et al 2005). An abundance of ultramicrobacteria (size less than 1\( \mu \text{m} \)) have been found in deep Greenland ice cores (Miteva et al 2004). These ultra small microorganisms may be a distinct class of intrinsically small organisms, or may be the dormant and starved forms of normal sized microorganisms (Miteva et al 2005). The small size of the organisms confers upon them several advantages in extreme environments, such as more efficient nutrient uptake in oligotrophic conditions as a result of larger surface to volume ratio, easier occupation
of microenvironments and protection from predators. A technique developed to selectively isolate and culture ultramicrobacteria involved the selective filtering of melt ice samples through different pore size filters (0.4 µm, 0.2 µm and 0.1 µm) successively and incubating the filtrate in anaerobic, low nutrient media at low temperatures. The technique helps isolate psychrophilic ultramicrobacteria and incubation temperatures range between -2°C and 5°C. The filtration steps help separate the larger bacteria from the smaller cells, thus preventing overgrowth by the larger, fast growing organisms that may be inhibitory to the smaller cells. Anaerobic conditions help avoid the oxidative stress to the resuscitating cells and helps in maintaining conditions similar to the glacial environment (Miteva et al 2005). Most of the culturing techniques involving filtration, that excludes the ultramicrobacteria. The technique developed by Miteva et al (2005) helps look for a class of microorganisms that may be present in high numbers in extreme environments (Fig.6).

The dilution-to-extinction culturing technique is another technique developed to culture psychrotolerant bacteria from lakes in the McMurdo region of Antarctica. The technique is especially useful to culture microorganisms that have remained uncultured using the more traditional methods (Stingl et al 2008). The method uses sterilized and nutrient-amended lake water as the incubation media. Lake water samples are diluted in chilled media to a concentration of 3 to 10 cell per ml, which is then added to 2 ml of media in microtiter plate wells. The plates are incubated at 4°C for 24 weeks. After the incubation period, 200 µl of sample from each well is stained with DAPI, fixed with formalin and transferred to a polycarbonate membrane that is screened by epifluorescence microscopy. Cell concentrations of greater than 10^5 cells mL^-1 are
Fig 6: Isolation of ultramicrobacteria using enrichment by filtration. Filtration through a 0.2 µm filter resulted in the concentration of ultramicrobacteria in the filtrate and separated the larger bacteria. This might have enabled the successful isolation of the ultramicrobacteria from Greenland ice cores. The graph shows the difference in the number of ultramicrobacteria isolates from filtered and non-filtered samples and stresses the importance of enrichment by filtration.
considered positive for growth and the cells are analysed by PCR amplification of the 16S rDNA sequences (Stingl et al. 2008). The technique permitted the isolation and culturing of abundant bacteria including novel strains of groups that were previously uncultured from specific environments (Stingl et al. 2008). This new culturing technique highlighted the success of modified lake water as a medium compared to the artificial media. The lake water media replicates the natural environment of the microorganisms more closely than any of the artificial media and may be responsible for the isolation of previously uncultured bacteria. The dilution-to-extinction technique exemplifies the importance of developing isolation techniques to replicate the natural environmental conditions of the microorganisms.

Although fungi account for a significant proportion of the microbes in cold environments, the study of fungi as opposed to the study of bacteria in the extreme ice environments has been significantly lower. Different techniques have been developed to isolate and culture fungi from the cold environments. Bergero et al. (1999) used nutrient-rich and nutrient-poor media to culture a broad spectrum of fungi isolated by soil dilution and soil washing techniques from the Arctic soils. Fungi have been known to withstand low nutrient conditions and grow well in media with very low concentrations of carbon, and are generally considered to possess oligotrophic capabilities. Malt extract agar (MEA), potato-carrot agar (PCA) and carboxymethylcellulose agar (CMC) were used to culture fungi from the soil samples. The psychro-oligotrophic capabilities of the fungi were tested by growing the fungi on silica gel with N (Nitrogen) but no C (Carbon) and at 0°C. Fungi isolated from the Arctic soil have shown good growth on nutrient rich-media
and nutrient-poor media along with good growth at low and normal temperatures (0 and 20\(^\circ\)C), which indicate psychrotrophy (Bergero et al 1999).

Another technique used to isolate and culture fungi used eight different media: Sabouraud’s (SAB), yeast-malt extract agar (YMA), acidified YMA, nutrient agar (NA), malt extract agar (MEA), potato dextrose agar (PDA), mycobiotic agar (MA) and oatmeal agar (OMA). The plates were inoculated with 200 µl of the meltwater from Greenland ice cores and incubated at 8\(^\circ\)C for 6 weeks followed by 15\(^\circ\)C for 2 weeks (Ma et al 2000). The use of several different media proved to be a successful technique to culture fungi from cold environments.

Ozerskaya et al (2004) used a different technique to isolate fungi from cryopeg samples. Water samples were concentrated by filtration through a sterilized 0.3 µm filter which was cut and transferred to a tube with sterile water. The tube was vortexed to enable the separation of CFUs and inoculated on organic (MEA) and synthetic (Czapek) media. To replicate the high salt concentrations in cryopegs, NaCl was added to the media in different concentrations (1.5 and 20%). This method was useful in isolating halophilic fungi in these environments (Ozerskaya et al 2004). The various techniques developed to isolate fungi from the cold environments take into account various conditions including temperature range, nutrition conditions and salt concentrations. These techniques emphasize the necessity to replicate the conditions of the natural environments of the fungi in order to isolate a broad spectrum of fungi from these extreme environments.
Objective of the study

Microorganisms from glacial ice have been isolated and identified dating to hundreds of thousands of years, from several locations in the Arctic and the Antarctic regions. Cosmopolitan microbes dominate these environments and have been deposited primarily by winds, birds, Ocean currents and mammals. Atmospheric currents play a significant role in the transport of these microorganisms to the polar regions. We hypothesize that the geographical isolation of the Antarctic region from other land masses compared to the Arctic region affects the transport of the microorganisms to the Antarctic region, resulting in lower number of microbes entrapped in the Antarctic ice. The GISP2D (Greenland Ice Sheet Project) core from the Arctic region and the Vostok 5G core from the Antarctic region have been selected for this purpose. The GISP 2D core has been drilled to 3053 m deep, and has been dated by D.A.Meese et al who determined the depth-age scale for the GISP2D core using a combination of parameters including visual stratigraphy, oxygen isotopic ratios, electrical conductivity and major ion chemistry. Ice core sections from the GISP2D core corresponding to depths of 1600 m, 2501 m, 2776 m and 3014 m have been selected for this investigation. Using the depth-age scale, the ages of the selected cores have been determined to be 10,442 ybp, 57,018 ybp, 105,000 ybp and 157,112 ybp, respectively. The Vostok5G core has been drilled to a depth of 3650 m. The glacial ice ends at 3538 m, and the deeper ice is accretion ice, which formed when water from subglacial Lake Vostok froze to the bottom of the glacier. Salamatin et al dated the Vostok core using isotope records and icesheet flow models. The Vostok5G ice core at a depth of 3528 m has been dated back to more than 1 million years. Four different depths have also been selected from the Vostok5G core, including 302 m, 900
m, 1529 m and 2149 m. The age of these cores from the depth-age scale are 10,000 ybp, 57,000 ybp, 105,000 ybp and 155,000 ybp, respectively.

The aim of this study was to evaluate the fungal and bacterial species richness in ice cores from two geographically distinct locations. Ice core sections from Arctic and Antarctic regions that represent similar time periods in geological history were used for the microbiological assays. An important step in isolation of organisms from ancient ice cores is the decontamination process. To prevent contaminants, the decontamination protocol developed by Rogers et al (2004) was followed. Culturing, PCR (Polymerase chain reaction), amplification and sequencing were used for the isolation and identification of bacteria and fungi from the ice sections. Along with the growth media for fungi and bacteria, low-nutrient media were also used in the culture method, taking into consideration the starved conditions of most of the isolates in these environments.

Phylogenetic analysis needs to be done to understand the significance of the results in an evolutionary perspective by trying to understand the relationship between:

- isolates from the same ice core section
- isolates from the 4 ice core sections from the same geographic location
- isolates from ice cores representing similar time scales but different geographic locations
- isolates from the ice cores and closely related organisms from the current time period, belonging to different environments.
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CHAPTER II: ANALYSIS AND CHARACTERIZATION OF MICROBES FROM ANCIENT GLACIAL ICE
Abstract

The microorganisms entrapped in glacial ice provide a continuous record of the microbial life within and allows the study of microbial evolution by comparison of ancient bacteria and fungi with their contemporary conspecifics. The Arctic and the Antarctic ice cores represent two geographically distinct locations with varying landscapes and precipitation patterns. The Antarctic region is far more remote compared to the Arctic region. The goal of this research is to isolate bacteria and fungi from the Arctic and Antarctic ice cores belonging to similar time periods, and to do a comparative analysis of the organisms with one another and with contemporary organisms. Four ice cores each were analysed from the Arctic (GISP2D core) and the Antarctic (Vostok 5G core) regions dating back to 10,000 YBP, 57,000 YBP, 105,000 YBP and 155,000 YBP using culturing, PCR, sequencing and SEM techniques. Several bacteria and fungi were isolated and identified using 16S rDNA sequencing. Microorganisms were isolated from Greenland ice cores dating back to 155,000 years. Phylogenetic analysis was performed to compare the similarity of the organisms to the present day organisms. Some of the fungi isolated from the Arctic and the Antarctic regions closely related to the same genus did not show close resemblance with one another. More organisms were isolated from the GISP2D ice core compared to the Vostok 5G ice core and the species richness decreased with the increase of age in both the ice cores. Some sequences showed high similarity to contemporary species, while some did not group closely with the present day organisms. Fungi isolated from the different locations, and closely related to the same genus, did not show high similarity with one another.
**Introduction**

Microorganisms survive and even thrive in some of the most harsh and extreme environments present on Earth. They have been isolated from hot springs with extremely high temperatures, and also from cold environments including permafrost, sea ice, glacial ice, and subglacial lakes. Study of microbes that can survive and remain active in extreme environments is interesting and significant due to the fact that these are the conditions comparable to some extraterrestrial environments. Study of life in the permanently cold environments of glaciers in the Arctic and Antarctic regions is especially significant because of the discovery of ice beyond Earth. An icy cover has been discovered on Europa (a moon of Jupiter) and recent studies point towards traces of ice on Mars (Chyba 2000). Glacial ice in the polar regions on Earth has been accumulating for hundreds of thousands of years and in some cases more than 8 million years. The lengthy process of glacial ice formation from snow is a complex phenomenon controlled by many factors. Snowfall is the first step in this process, followed by the conversion of snow to firn (wet snow). Glacial ice is formed when the interconnecting air passages between the grains (crystal or a group of crystals) in firn are sealed, leaving the gases in bubbles (Paterson 1994). Therefore, glacial ice holds a valuable record of the past climate along with a continuous record of microbial life. The study of microbes in glacial ice helps us to understand the survival mechanisms of these organisms under extreme conditions and how they compare to contemporary organisms from present day environments.

Microorganisms have been identified and isolated from glacial ice samples from many different regions of the world. The global distribution of these microorganisms in snow and glacial ice is most likely the result of wind currents and atmospheric
circulation. Bacteria and fungi have been detected and isolated from many different frozen environments such as permafrost, sea ice, glacial ice, and accretion ice from subglacial lakes. Viable bacteria, fungi and yeasts also have been isolated from marine cryopegs averaging -9 to -11°C during all times of the year (Gilichinsky et al 2005). The presence of fungi in frozen environments from both polar regions has been documented. Studies have looked at the presence of microbes in glacial ice from different locations, including samples coming from Arctic and Antarctic regions. Ma et al (1999) reported the isolation of bacteria and fungi from various Greenland ice core sections, using culturing and PCR techniques. Bacteria and fungi have been isolated and identified using a combination of culturing, PCR and microscopy techniques from the glacial and accretion sections from Lake Vostok ice core (D’Elia et al 2008). Most of the bacteria and fungi isolated from these permanently cold environments were psychrotolerant as opposed to psychrophilic, having optimal growth temperatures well above the freezing temperatures common to these kinds of environments.

How do these organisms survive in ice? Price et al. (2000) proposed that liquid water might exist in veins at the interface of three ice crystals. Even at subzero temperatures in deep glacial and sea ice, these liquid veins may serve as a habitat for hardy microbes. Novel techniques in microscopy have helped to identify such brine pockets in Arctic ice at -15°C along with their microbial inhabitants (Junge and Deming et al. 2001). Tritiated thymidine and leucine have been used in incubation experiments with Antarctic ice samples and identified metabolic activity to -17°C (Carpenter et al 2000). Bacteria have been found that can survive in Ice-IV, the coldest form of ice (which is not found naturally on Earth).
Different microbial survival strategies have been identified. Several enzymes, (e.g. xylanases and chitinases) remain active at low temperatures by having reduced activation energy. A special class of cold-adapted proteins, called cold shock proteins, play an important role in aiding various functions including protein synthesis. Cellular adaptations have been identified which prevent membrane rigidity at low temperatures and help keep the cytoplasm in liquid phase. Specific pigments in cell membranes have been identified that aid in maintaining biological activity in membranes at lower temperatures. The sugars trehalose and mannitol play important roles as cryoprotectants in fungi. Antifreeze proteins also are important for fungal survival at low temperatures. These proteins induce the extracellular freezing of water that prevents damage inside the cell caused by ice crystal formation.

The percentage of culturable organisms declines sharply with the increase in depth of the ice cores (Abyzov 1993, Ma et al 2000). The age of permafrost was inversely proportional to the number and diversity of the isolates. As the age increased, the number and diversity decreased in some cases to zero (sterile ice samples) (Gilichinsky et al 1989, Gilichinsky et al 1992). This supports the theory that the organisms are either dormant or have slow metabolic rates sufficient only to support repairs in the cell but are not for cell growth and division. However living cells have been isolated from ice, millions of years old from Antarctica (D’Elia et al 2008).

Glacial ice has constant temperatures, making it ideal for long term preservation of microorganisms (Willerslev et al 2004). Environmental ice acts as a protective matrix for microorganisms over extended periods of time and provides a valuable record of microbial evolution and ancient biodiversity (Ma et al. 2000). Microorganisms from
glacial ice have been isolated and identified dating back to hundreds of thousands of years, from locations in the Arctic and the Antarctic regions. Study of the organisms isolated from environmental ice has given valuable insights with regard to the evolutionary aspects of microbes. However, studies have not been done to compare microorganisms belonging to similar timescales entrapped in glacial ice from the two polar regions. The Arctic and the Antarctic regions apart from their geographically distant positions, represent distinct landscapes. The Antarctic region is a dry desert with little precipitation, while the Arctic region has high snowfall and is geographically less isolated compared to the Antarctic region. The study of microbes isolated from these two distinct locations, dating back to similar time periods may give an insight into the cold adaptation and survival of microbes and the processes of microbial evolution.

The GISP2 (Greenland Ice Sheet Project 2) ice core and the Vostok ice core from the Antarctic region have been selected for the study. The GISP2 core has been drilled to 3053 m and has been dated using a combination of parameters including visual stratigraphy, oxygen isotopic ratios, electrical conductivity and major ion chemistry. The Vostok core has been drilled to a depth of 3650 m. The glacial ice ends at 3538 m and the rest of the core is accretion ice which is formed when water from subglacial Lake Vostok freezes to the bottom of the glacier. Four different depths have been selected from the GISP2 and the Vostok cores dating back to similar time periods. We employed culturing, PCR and microscopy techniques for the isolation and identification of bacteria and fungi from the decontaminated ice cores (decontamination protocol by Rogers et al 2004). Phylogenetic analyses were performed on all the bacterial and fungal sequences to
compare the isolates with one another and also with conspecific organisms of the contemporary population.
Materials and Methods

Ice Cores:

A total of eight different ice core sections were analysed. Four of the sections were from the GISP2D core in Greenland at the following depths: 1600, 2501, 2776 and 3014 m. The other four sections were from the Antarctic Vostok 5G at the following depths: 302, 900, 1529 and 2149 m. The sections from GISP2D (1600 m) and Vostok5G (302 m) are, approximately 10,000 years old, GISP2D (2501 m) and Vostok5G (900 m) are approximately 57,000 years old, GISP2D (2776 m) and Vostok5G (1529 m) are approximately 105,000 years old and GISP2D (3014 m) and Vostok5G (2149 m) are approximately 157,000 years old.

Decontamination of ice cores:

The outer surfaces of the ice cores were decontaminated before melting. Clorox (a 5.25% Sodium hypochlorite solution) was used as the decontaminating agent. The decontamination protocol developed by Rogers et al (2004) was used for this procedure. A 6 cm long piece of ice was cut from the ice core. The core section was stored at 4°C for 1 hour and the sodium hypochlorite solution was prechilled to 4°C before decontamination to avoid thermal shock of the ice. The decontamination procedure was carried out in a sterile class II cabinet (biosafety laminar flow hood) in a sterile room. The laminar flow hood was sterilized with 70% ethanol, 5.25% sodium hypochlorite and then UV irradiated for 1 hour prior to use. The sterile room also was UV irradiated for 1
hour prior to use. Inside the laminar flow hood, the ice core section was immersed in 500 ml of 5.25% sodium hypochlorite for 10 seconds. The section was then transferred to a sterile funnel and then rinsed with 200 ml of prechilled sterile water (18.2 Mohm, <1ppm total organic carbon). The ice core was subjected to 2 more rinses and each time was transferred to a new sterile funnel. Then the section was transferred to a sterile funnel and melted at room temperature. The meltwater was collected in aliquots of 30-50 ml. After each aliquot, the ice core section was transferred to a sterile funnel for the next aliquot. A total of 5 such aliquots were collected. The first aliquot represents meltwater from the outer core surface and the last aliquot represents the innermost portions of the core. Parts of the aliquots were immediately used for culturing and the remainders were further split into smaller portions and were stored at -20°C for further use.

**Culturing:**

The meltwater (200 µl) from each shell of the ice cores was spread onto several types of solid media in duplicate, and incubated at 8°C to test for the presence of viable microorganisms. The following media were used: malt extract agar [1.28% maltose, 0.27% dextrin, 0.24% glycerol, 0.08% peptone, 1.5% agar (pH 4.7)], potato dextrose agar [0.4% potato starch, 2% dextrose, 1.5% agar (pH 5.6)], rose bengal agar [0.5% soytone, 1% dextrose, 0.1% monopotassium phosphate, 0.005% rose bengal, 1.5% agar (pH 7.2)], nutrient agar [0.3% beef extract, 0.5% peptone, 1.5% agar (pH 6.8)], oatmeal agar [6% oatmeal, 1.25% agar (pH 6.0)], Sabouraud dextrose agar [1% enzymatic digest of casein, 2% dextrose, 2% agar (pH 7.0)], yeast extract agar [3% yeast extract, 3% malt extract, 0.5% peptone, 1% dextrose, 2% agar (pH 6.2)], acidic yeast extract agar [3% yeast extract, 3% malt extract, 0.5% peptone, 1% dextrose, 2% agar (pH 6.2)],
extract, 3% malt extract, 0.5% peptone, 1% dextrose, 2% agar (pH 4.5)], meat-liver agar [2% meat liver base, 0.075% D(+)-glucose, 0.075% starch, 0.12% sodium sulfite, 0.05% ammonium ferric citrate, 1.1% agar (pH 7.6)], blood agar [1.5% pancreatic digest of casein, 0.5% papaic digest of soybean meal, 0.5% sodium chloride, 5% sheep’s blood, 1.5% agar (pH 7.3)], R2A [0.05% yeast extract, 0.05% proteose peptone No.3, 0.05% casamino acids, 0.05% dextrose, 0.05% soluble starch, 0.03% sodium pyruvate, 0.03% dipotassium phosphate, 0.005% magnesium sulfate, 1.5% agar (pH 7.2)], Luria-Bertani agar [1% tryptone, 0.5% yeast extract, 0.5% sodium chloride, 1.5% agar (pH 7)], water agar [2% agar]. They were incubated for 2 weeks, monitored for any growth and transferred to 15°C for 2 weeks. The plates then were incubated at 22°C and monitored periodically for microbial growth. The cultures obtained were recorded and subcultured for future use. Throughout the decontamination and culturing procedures, control plates (2 MEA and 2 LBA) were employed in the laminar flow hood. Each plate was placed at one corner and exposed to the environment in the hood. They were incubated along with the culture plates and observed regularly for any growth or contamination.

**PCR amplification:**

The fungal and bacterial isolates obtained by culturing were subjected to PCR amplification. ITS4 and ITS5 primers were used to amplify the ribosomal DNA (rDNA) internal transcribed spacers (ITS1 and ITS2) and the 5.8S gene in fungi (White et al 1990). For bacterial isolates, primers 16S-2 and 23S-7 were used to amplify the rDNA intergenic spacer region (ITS1), along with portions of the small subunit and large subunit genes. A GeneAmp PCR Reagent kit (Applied Biosystems, Branchburg, New
Jersey) was used for amplification. The composition of each 50 µl reaction was as follows: 5 µl of cell suspension, 50 pmol of each primer, 10 pmol of each dNTP, 2U Taq DNA polymerase, 50 mM KCl and 1.5 mM MgCl₂. The program cycle used for amplification was: 95°C for 8 min, 40 cycles of 94°C for 1 min, 54°C for 1 min 30 sec and 72°C for 2 min followed by 72°C for 8 min, and finally cooled to 4°C.

Additionally, PCR amplification was performed with the meltwater from the ice cores. Several reactions were performed with the fungal and bacterial primer sets. Semi-nested PCR was used with fungal primers, ITS4 and ITS5 for the first round reaction, and then using either ITS2 and ITS5 or ITS3 and ITS4 for the second round reaction. For amplification of bacterial cells or DNA, primers 16S-2 and 23S-7 were used with native Taq DNA polymerase (Fermentas Inc., Hanover, MD). The composition of 50 µl of each reaction was: 50 pmol of each primer, 10 pmol of each dNTP, 2U Taq DNA polymerase, 50 mM KCl and 1.5 mM MgCl₂. Different amounts of meltwater were used in the reactions and varied from 1 µl to 10 µl. Also, for some reactions, 1 ml of meltwater was centrifuged at 8000 rpm for 15 min, 800 µl of supernatant collected and the pellet (invisible) resuspended with the remaining 200 µl of water. This was an attempt to concentrate any cells that were present in the meltwater, thereby increasing the chances for PCR amplification. Different program cycles were used for amplification:

Cycle Set 1: 95°C for 8 min, 40 cycles of 94°C for 1 min, 54°C for 1 min 30 sec and 72°C for 2 min followed by 72°C for 8 min.

Cycle Set 2: 95°C for 8 min, 40 cycles of 94°C for 1 min, 54°C for 4 min and 72°C for 4 min (ramp- 1.25°/sec) followed by 72°C for 8 min.
Cycle Set 3: 95\(^{\circ}\)C for 8 min, 40 cycles of 94\(^{\circ}\)C for 1 min, 54\(^{\circ}\)C for 2 min and 72\(^{\circ}\)C for 4 min (ramp- 1.25\(^{\circ}\)/sec) followed by 72\(^{\circ}\)C for 8 min.

PCR reactions were subjected to electrophoresis on 1% agarose gels with TBE (90 mM Tris-Borate, 2 mM EDTA, pH 8.0) and 0.5 µg/ml ethidium bromide was used to view the amplification using UV light. For PCR amplification, sterilized water was used as a negative control. *Rhodotorula mucilaginosa* was used as the positive control for PCR with fungal primers and *Bacillus subtilis* as positive control for bacterial PCR.

**Cloning and Sequencing:**

A TOPO TA cloning kit (Invitrogen, Carlsbad, CA) was used for cloning the amplified products. The amplified DNA from the fungal and bacterial isolates was ligated into the PCR 4-TOPO vector. The ligation reaction included: 2.5 µl of PCR product, 1.0 µl of salt solution (200 mM NaCl, 10 mM MgCl\(_2\)) and 1.5 µl of vector (10 ng µl\(^{-1}\)). The ligation reaction was carried out following the manufacturer’s directions. One Shot\(^{\circledR}\) TOP10 Competent *E. coli* cells were transformed with the ligation reaction. The plasmid DNA was isolated from transformed cells using the Cyclo-Prep Plasmid DNA isolation kit (Amresco, Solon, OH). The plasmids then were tested for the presence of inserts using *EcoR1* digestion. The products were subjected to electrophoresis on 1% agarose gels with TBE and 0.5μg/ml ethidium bromide to analyse the digestion products. Plasmids containing the inserts then were sent to Gene Gateway LLC (Hayward, CA) for sequencing.
Scanning Electron Microscopy:

An aliquot (meltwater from ice cores) of 5 ml was filtered through a 0.2 μm polycarbonate filter using a sterile syringe. The filter was transferred to a Petri dish with 2.5% gluteraldehyde in 0.1 M phosphate buffer (pH 7.2). The filter was fixed in this solution for 1 hour, followed by three 10 min rinses in 0.1 M phosphate buffer (pH 7.2). The filter was dehydrated with 40%, 60%, 80% and 95% ethanol solutions, sequentially for 10 min each and finally with 100% ethanol 3 times (10 min each). After dehydration, the filter was dried using a Samdri 780A critical point drier. Then the filter was cut into four pieces to be mounted into a coating unit. A Polaron E500 SEM coating unit was used to sputter coat the filters with a 5 nm thick gold-palladium coat. The filters then were observed in a scanning electron microscope (Hitachi S-2700, SEM). Control filters were prepared for SEM using sterilized water, taken through the fixation and dehydration procedure and observed.

Phylogenetic Analysis:

The ribosomal DNA sequences obtained from both the bacterial and fungal isolates were used in BLAST searches of the GenBank NCBI-database to identify sequences of related taxa. Sequence alignments were created using ClustalX 2.0 for bacteria and fungi using the isolate sequences and related GenBank sequences (Taylor et al 1997). Alignment files were used to generate maximum parsimony phylogenetic trees using the program PAUP (Swofford 1999). The phylogenetic trees were created using the heuristic search option, and gaps were treated as a fifth base. Bootstrap support using 1000 replications also was
determined using the same criterion. The fungal tree was midpoint rooted and the outgroup *Aquifex pyrophilus* was used to root the bacterial tree.
Results

Revival and Molecular Identification of Glacial Isolates:

There are geographical and temporal differences in the microbial composition of ice core samples (Table 1). The youngest ice analyzed was 10,000 years old from the GISP2D core at a depth of 1600 m and the Vostok5G core at 316 m. The highest number of fungal isolates recovered was from the GISP 2D core at 1600 m. Seven isolates were recovered from this ice core. In the ITS sequence analysis, isolates GI866 and GI867 from GISP2D 1600 m shared 97 and 98% sequence similarity with *Rhodotorula mucilaginosa* sequences in the NCBI database. Sequences also were obtained from isolates that showed 98% similarity with *Penicillum corylophilum* (GI862), 97% similarity with *Cladosporium tenuissimum* (GI861), 100% similarity with *Alternaria alternata* (GI860) and 98% similarity with *Fusarium culmorum* (GI859). Only one bacterial isolate (GI865) was found in GISP2D 1600 m, and it shared 94% similarity with *Caulobacter* spp. Only two isolates were recovered from the Vostok ice core section at 316 m, which corresponds in age with GISP2D 1600 m. Fungal isolate GI877 was 99% similar to *Davidiella tassiana*. The remaining isolate from Vostok 316 m (GI878) showed 100% ITS sequence similarity with *Bacillus amyloliquefaciens*.

The second group of ice core sections investigated came from 57,000 year old ice from GISP2D (2501 m) and Vostok (900 m) cores. The GISP2D core section provided a total of 6 isolates, three of which also were found in GISP ice from a depth of 1600 m (Table 1). A single isolate, GI873, was most closely related to *Rhodotorula mucilaginosa* when the ITS sequences were compared.
Table 1: Isolates from GISP2D and Vostok 5G ice cores. Lists all the isolates (bacterial and fungal) from the GISP2D and Vostok 5G ice cores. The core ID, along with the depth and age are shown for each isolate. The medium of isolation and the color of the isolate are also recorded along with the temperature at which growth was first observed. The closest related genera and species were obtained using the NCBI blast search and are shown along with the percentage of sequence similarity. Twenty two isolates have been recovered of which seventeen were fungi and five bacteria. Eighteen isolates were from the GISP2D cores while four isolates were from the Vostok 5G cores.
<table>
<thead>
<tr>
<th>Isolate</th>
<th>Core ID</th>
<th>Depth</th>
<th>Age (years)</th>
<th>Isolation media</th>
<th>Color of Colony</th>
<th>Incubation Temp. (°C)</th>
<th>Organism with highest sequence similarity</th>
<th>% query coverage</th>
<th>% sequence similarity</th>
</tr>
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<tbody>
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<td>GI 859</td>
<td>GISP2D</td>
<td>1600m</td>
<td>10,442</td>
<td>MA</td>
<td>White</td>
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<td>98</td>
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<td>OMA</td>
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<td><em>Alternaria alternata</em></td>
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<td>100</td>
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<td>10,442</td>
<td>YMA</td>
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<td>22</td>
<td><em>Cladosporium tenuissimum</em></td>
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<td>97</td>
</tr>
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<td>1600m</td>
<td>10,442</td>
<td>YMA</td>
<td>White</td>
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<td><em>Penicillium corylophilum</em></td>
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<td>98</td>
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<td>GI 865</td>
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<td>NA</td>
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<td><em>Caulobacter crescentus</em></td>
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<td>94</td>
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<td>GI 866</td>
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<td>10,442</td>
<td>OMA</td>
<td>Yellow</td>
<td>15</td>
<td><em>Rhodotorula mucilaginosa</em></td>
<td>79</td>
<td>97</td>
</tr>
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<td>1600m</td>
<td>10,442</td>
<td>BA</td>
<td>Red</td>
<td>15</td>
<td><em>Rhodotorula mucilaginosa</em></td>
<td>100</td>
<td>98</td>
</tr>
<tr>
<td>GI 868</td>
<td>GISP2D</td>
<td>2501m</td>
<td>57,018</td>
<td>R2A</td>
<td>White</td>
<td>15</td>
<td><em>Aspergillus restrictus</em></td>
<td>91</td>
<td>99</td>
</tr>
<tr>
<td>GI 869</td>
<td>GISP2D</td>
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<td>57,018</td>
<td>AYMA</td>
<td>White</td>
<td>22</td>
<td><em>Aureobasidium pullulans</em></td>
<td>100</td>
<td>99</td>
</tr>
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<td>57,018</td>
<td>LBA</td>
<td>Yellow</td>
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<td><em>Caulobacter crescentus</em></td>
<td>79</td>
<td>86</td>
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<td>R2A</td>
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<td>MLA</td>
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<td>105,000</td>
<td>YMA</td>
<td>White</td>
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<td><em>Cryptococcus magnus</em></td>
<td>94</td>
<td>99</td>
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<td>GISP2D</td>
<td>2777m</td>
<td>105,000</td>
<td>YMA</td>
<td>Pink</td>
<td>15</td>
<td><em>Rhodotorula mucilaginosa</em></td>
<td>98</td>
<td>99</td>
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<td>GI 875</td>
<td>GISP2D</td>
<td>3014m</td>
<td>157,112</td>
<td>YMA</td>
<td>Green</td>
<td>15</td>
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<td>98</td>
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<td>GISP2D</td>
<td>3014m</td>
<td>157,112</td>
<td>PDA</td>
<td>Red</td>
<td>15</td>
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<td>316m</td>
<td>10,000</td>
<td>LBA</td>
<td>Black</td>
<td>22</td>
<td><em>Davidiella tassiana</em></td>
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<td>99</td>
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<td>GI 878</td>
<td>Vostok 5G</td>
<td>316m</td>
<td>10,000</td>
<td>OMA</td>
<td>Yellow</td>
<td>15</td>
<td><em>Bacillus amylo liquefaciens</em></td>
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<td>105,000</td>
<td>SAB</td>
<td>Pink</td>
<td>15</td>
<td><em>Rhodotorula mucilaginosa</em></td>
<td>84</td>
<td>99</td>
</tr>
</tbody>
</table>
Rhodotorula mucilaginosa also was recovered in the shallower and younger ice from GISP2D at a depth of 1600 m. Two isolates were recovered and identified that belong to the genus Aspergillus. High sequence similarities classified isolate GI868 as *A. restrictus*, and isolate GI871 as *A. conicus*. The revival of two *Aspergillus* isolates is the only time that more than one isolate in any single genus was found in the same ice core, with the exception of *Rhodotorula* isolates. The remaining two isolates from GISP2D 2501 m were bacterial. These isolates, GI870 and GI872, both shared 86% sequence similarity with the genus *Caulobacter*. A significant difference was observed between these two cores that came from the same geological time but different geographical locations. No bacterial or fungal isolates were recovered from the Vostok5G core at 900 m.

The number of isolates obtained from the GISP2D ice core sections decreased with increase in age of the ice cores (Fig 1). Ice core sections from GISP2D 2777 m yielded only three isolates. No filamentous fungi were recovered from this depth, but two yeast isolates were successfully cultured, including one *R. mucilaginosa* isolate (GI876) and one isolate that shared 99% ITS sequence similarity with *Cryptococcus magnus* (GI875). This was the only *Cryptococcus* recovered in this study. Two fungal cultures were isolated from ice of the same age, [105,000 years, 1529m)] in the Vostok 5G core. Isolate GI879 was identified as *Alternaria tenuissima* (100% similarity), and isolate GI880 was identified as *R. mucilaginosa*. Members of both of these genera were also found in the GISP2D ice from 1600 m in depth. The oldest ice analyzed from both polar ice cores contained the fewest number of isolates (Table 1). No isolates were revived from the Vostok5G core at a depth of 2149 m (155,000 ybp) whereas GISP2D ice from
Fig 1: Bar graph showing the number of isolates from the GISP2D cores.

The number of microorganisms isolated decreased with the increase in the age of the ice cores.
3014 m, (155,000 years old) provided two fungal isolates. Isolate GI855 was identified as *Penicillium chrysogenum* (98% similarity) and isolate GI858 was identified as *R. mucilaginosa* (98% similarity).

Overall, the GISP2D ice core contained a higher number of microorganisms than the Vostok5G core. Each depth analyzed from the GISP2D core provided fungal and bacterial isolates. Furthermore, each core section contained unique isolates not found at any other depth, or in the Vostok5G core. There was overlap in the taxa isolated between the two polar regions examined. Isolates of *Rhodotorula* were found in both GISP2D (GI858, GI866, GI867, GI873 and GI876) and Vostok5G (GI880) ice core sections. Isolates of *Alternaria, Cladosporium* and *Rhodotorula* were isolated from both polar regions. Within the GISP2D ice core, there were two recurring genera which included *Penicillium* (GI855 and GI862) and *Rhodotorula*. Bacterial isolates most closely related to *Caulobacter* also were isolated from two different depths of the GISP core (1600 m and 2501 m). The only bacterial isolates recovered from both the GISP2D (2777 m) and Vostok5G (316 m) cores were isolates GI874 and GI878 belonging to the genus *Bacillus*.

All of the isolates from both cores were either isolated under psychrotrophic conditions or related to psychrotolerant taxa from polar regions. Many of these organisms were initially isolated at 15°C, except for four isolates that first grew at 22°C. The isolates identified as *Cladosporium sp.* (GI 861, 1600 m), *Aureobasidium sp.* (GI 869, 2501 m) and *Aspergillus sp.* (GI 871, 2501 m) were from the Arctic and *Davidiella sp.* (GI 877, 316 m) was from the Vostok core. The incubation periods for all the isolates varied from a month to more than one year.
**Phylogenetics:**

Sequences of 17 fungi isolated from the GISP2D and Vostok5G cores were combined with 61 sequences from NCBI GenBank for phylogenetic analysis. The resulting phylogram allowed us to compare these isolates with other fungi from cold environments and to contemporary taxa (Fig 2). All of the ice isolates grouped either with ascomycetes or basidiomycetes. The ascomycete group had fewer individual isolates, but the distribution was across a broader range of genera. Eight of the 14 fungal isolates from Greenland cores, were ascomycetes. *Penicillium* sp. (GI 855, GISP2D-3014 m and GI 862, GISP2D-1600 m) and *Aspergillus* sp. (GI 868, GISP2D-2501 m and GI 871, GISP2D-2501 m) were the only ascomycetes isolated more than once in the GISP2D ice. Ascomycetes were less abundant in the Vostok5G ice core compared to the GISP2D core. Isolate GI 877 grouped within the *Cladosporium* subclade and shows a close relation to a *Cladosporium* previously isolated from the GISP2D ice core (Ma et al 2000). Isolate GI 879 from the Vostok core showed low similarity to ITS sequences from other members within the *Cladosporium* group. The *Alternaria* group contained one of the instances that an isolate of the same genus was found in both the GISP2D (GI 860) and Vostok (GI 879) core although the sequences differed from one another and did not group closely with one another. Organisms belonging to the genera *Rhodotorula, Alternaria* and *Cladosporium* have been isolated previously from the GISP2D and the Vostok 5G ice cores.
**Fig 2: Phylogenetic analysis of fungal rRNA sequences.** Phylogram of fungal rRNA ITS sequences (with maximum parsimony on PAUP, Swofford 1999) from the GISP2D and Vostok 5G ice cores. Sequences that start with GI are the isolates from the ice sections. Ice core location and depth (meters below the glacial surface) are also given. Taxonomic grouping of the organisms are showed on the right side. Organisms belonging to the genera Rhodotorula, Alternaria and Cladosporium have been isolated from the GISP2D and the Vostok 5G ice cores. The closest sequences to the unknown samples were selected by NCBI blast search and used for the analysis. NCBI accession numbers (in parentheses) and source of the isolate (square brackets) are also included. Bootstrap values (1000 replications) are given for branches with support greater than 50%.
Of the six isolates classified as basidiomycetes, five belonged to the genus *Rhodotorula* (GI 858, GI 866, GI 867, GI 873 and GI 876). *Rhodotorula* sp. was also the most common fungal isolate observed (six of the 17 fungal isolates belong to this genus). Three *Rhodotorula* isolates (GI 867, GI 873 and GI 858) grouped closely together and were from three different depths of the GISP2D ice core. The *Rhodotorula* isolates also were related to other *Rhodotorula* isolates collected from Lake Vostok accretion ice, deep sea and marine environments.

Phylogenetic analysis also was performed on the five bacterial isolates (Fig 3). Three of the four bacterial isolates from the GISP2D core were grouped within the alpha-proteobacteria. These isolates, GI 865, GI 870 and GI 872, classified as *Caulobacteriales*, and are similar to isolates previously recovered from Vostok accretion ice. The remaining isolates grouped within the Gram-positive clade, as Firmicutes. Isolate GI 878 from the Vostok ice from 316 m (10,000 ybp) grouped very closely with isolate GI874 from GISP2D ice of a depth of 2777 m (105,000 ybp).

**Scanning Electron Microscopy:**

Scanning electron microscopy (SEM) was used to verify the low concentration of cells revealed by culturing and PCR techniques. Scanning electron microscopy also helped in the study of the morphological diversity of the microbial cells in the ice samples. Microbial cells were observed in all the ice core sections analysed with the exception of the Vostok 5G section at 2149 m. We also were unable to isolate bacteria or fungi from this ice core (Table 1).
Fig 3: Phylogenetic analysis of bacterial sequences. Maximum-parsimony phylogram of portions of the small subunit genes of the bacterial isolates. The closest related sequences were collected using NCBI blast search and used for the analysis. Sequences were aligned using ClustalX and a maximum parsimony tree was constructed using PAUP (Swofford 1999). Taxonomic groupings are indicated on the right. The sequences starting with GI are the isolates from the lab and the location and depth (meters below the glacial surface) is indicated. The NCBI accession numbers and locations of the closest related taxa from the NCBI blast search are provided. Bootstrap values (1000 replications) are given for branches with support greater than 50%.
Microbes of various morphologies were observed in the ice cores including spherical spore like structures, rod shaped cells, spirally elongated forms, filaments, filaments with bulbous structures in the middle and diplococcoid forms (Figures 4 and 5). Diplococcoid cells were observed in several Greenland cores but only in one Antarctic core (Figures 4 and 5). Rod-shaped cells were commonly found in all the Greenland core sections but not in the Antarctic cores (Figure 4). Elongated helically-coiled cells were observed in the GISP2D 2777 m ice core (Fig 4) and the Vostok 5G 316 m ice core (Fig 5). Overall, Scanning electron microscopy confirmed the low concentration of microbes in the ice cores analysed and revealed the diversity of shapes and structures of the cells.
Fig 4: SEM micrographs of organisms in the GISP2D ice cores. (1-6, 1600 m; 7-11, 2501 m; 12-18, 2777 m; 19-22, 3014 m). Micrographs 1, 3, 5 and 6 are rod shaped bacteria. The microbes in 1 and 6 have a sheath-like cover over the exterior and is similar to the microbes previously observed in Vostok accretion ice (red arrow). The organisms in 3 look identical to the rod shaped bacteria observed in previous GISP2 sediment ice studies (white arrow) (Miteva et al 2005). Micrographs 7 and 11 are similar to long rod shaped bacteria observed in the Vostok ice cores (green arrow). The spherical shaped particle in 9 has been observed in several previous studies including the Vostok ice (blue arrow). The spherical structure is similar to that of a fungal spore but is not conclusive. Organisms in 14 and 15 look like rod shaped bacteria and have been previously isolated from Vostok and GISP2 ice cores (blue arrow). The organism in 16 has a diplococcoid form (yellow arrow) and has been observed in several studies of the Vostok and GISP2 ice cores and from several other cold environments including the Siberian permafrost. This microbe looks similar to *Psychrobacter*, a diplococcoid bacteria commonly found in the cold environments. The microbe in 18 has an unusual spiral structure (orange arrow) similar to organisms isolated from the Vostok core (D’Elia et al 2008). These may be related to the order spirochaetales, which have long helically coiled cells.
Fig 5: SEM micrographs from Vostok 5G ice cores. (23-25, 316 m; 26-27, 900 m; 28-30, 1529 m). No organisms were observed in the Vostok 5G 2149 m ice core. Diplococcoid organisms similar to the ones observed in the GISP2D cores were observed (yellow arrow). The microbe in 24 has a spiral structure and may be related to Spirochaetales. Spore like structures were observed commonly (red arrow). A fungal hyphae like structure was observed in the Vostok 5G 900 m ice core (27).
Discussion

Species of microorganisms entrapped in Arctic versus Antarctic ice during the same time period differed from one another. The Arctic and Antarctic ice cores used in this study come from different depths but represent similar time periods. However, the fungi and bacteria isolated from the ice cores show disparities in species richness and taxa. These differences are small in the cores from different depths of the same site, but are large in the cores geographically separated. Fourteen of the 17 fungal isolates and four of the five bacterial isolates were from the Greenland cores. The disparity in species richness can be attributed to different reasons reflecting possible differences due to geography and time. Most of the organisms isolated from glacial ice samples in Antarctic and Greenland regions have been deposited from various regions, primarily by wind and to a lesser extent by birds and other animals. The high numbers of cosmopolitan species isolated in Arctic and Antarctic regions along with spore trap data add credence to the point (Vincent 2000). The prevalence of psychrotolerant organisms as opposed to psychrophilic organisms in these environments also supports hypothesis that microbes are deposited in polar regions from other environments (Vincent 2000). The seasonal extremes in temperature in polar regions act as a greater selective force than the cold temperatures per se, and as a result psychrotolerant organisms adapted better to these environments. Geographically, Antarctica is a remote environment compared to Greenland and this may explain the differences in numbers and diversity of isolates between the two regions. Another important factor responsible for the low number of microbes, especially in the Vostok ice core may be the lack of precipitation in Antarctica. Antarctica is a cold desert and receives much less snowfall compared to Greenland. This
might explain the lower number of organisms isolated from the Vostok ice core compared to the GISP2 cores. An important selective force in these environments along with the length of entrapment (age of ice core) is the physical pressure on the organisms trapped in glacial ice. As the depth of the core increases, so does the pressure. This might explain the decrease in the number of organisms isolated at increasing depths in both the Vostok and Greenland ice cores.

The higher number of microbes isolated from the Greenland ice cores may also be related to the geographic location and much warmer climate in the recent history compared to the Antarctic cores. During the Eemian interglacial, which started around 130,000 years ago, the northern hemisphere had warm temperatures comparable or higher to the temperatures of the Holocene period. During this period, part of Greenland (southernmost part of Greenland) was covered with forests including a diverse array of conifers (Willerslev et al 2007). The warmer temperatures along with the higher precipitation and the geographically closer location to forests may be responsible for the higher number of organisms isolated from the Greenland ice cores.

The organism most widely distributed among all ice cores was *Rhodotorula mucilaginosa*. Also, it was the only organism isolated from both the Arctic and Antarctic cores. This result is consistent with earlier studies that showed *Rhodotorula* was widely distributed in cold and aquatic environments. All of the *Rhodotorula* isolates were pigmented, which is an important survival strategy in cold environments, and might explain its abundance in the cores analyzed (Marshall 1997). The different isolates of *Rhodotorula* from ice cores were closely related to one another and to isolates from other environments, suggesting that similar species are distributed in both polar regions. Two
isolates each closely related to *Penicillium* and *Aspergillus* were isolated from the Greenland core sections. Isolate GI862 (98% similar to *Penicillium corylophylum*) was isolated from the 10,000 year old ice core and GI855 (98% sequence similarity with *Penicillium chrysogenum*) was isolated from 157,000 year old ice core. Filamentous *Penicillium* was isolated in high numbers and diversity from Arctic subglacial ice, and *Penicillium chrysogenum* was one of the most frequently isolated species. Several species of *Penicillium* are tolerant to cold conditions and are known to survive for long periods of time (Sonjak 2007). Three of the isolates from Greenland ice cores were closely related to the species of *Cladosporium*, *Cryptococcus* and *Aureobasidium*. All three organisms were frequently isolated from cold environments, e.g. Antarctic moss which is one of the richest microhabitats for fungi (Tosi et al 2002). *Cladosporium* spores have been found in high numbers in the air and constitute about 5% of the total fungal spore load in the Arctic region as compared to much higher numbers in the temperate and tropical environments (Marshall 1997). Spore counts in the Antarctic atmosphere varied with the seasons, and highest spore counts were observed during the summer months, attributed to winds from northern regions (Marshall 1997). One of the isolates from the Vostok ice core was closely related to *Alternaria* sp. isolated from cryopegs in Siberia. Bacterial species isolated from the ice cores had high sequence similarity to the genus *Bacillus* and *Caulobacter*. Organisms belonging to these genera are tolerant to low temperatures and have been isolated from the glacial ice and accretion ice samples of Lake Vostok (D’Elia et al 2008). All of the organisms isolated from the Arctic and Antarctic ice cores were closely related to species that are known to survive and thrive in cold environments and
have been isolated from similar environments, indicating that the isolates probably are
from the glacial ice and not contaminants.

All of the bacterial and fungal isolates were initially isolated at 15\(^{\circ}\)C with the
exception of four cultures (GI861, GI869, GI871 and GI877), which were first observed
at 22\(^{\circ}\)C. All of the cultures showed growth at 22\(^{\circ}\)C. These isolates do not fit the profile of
psychrophiles, whose optimal growth temperature is below 15\(^{\circ}\)C and do not grow at
temperatures above 20\(^{\circ}\)C. The isolates can be classified as psychrotolerant, as they can
survive and grow at low temperatures while having the optimal and maximum growth
temperatures above 20\(^{\circ}\)C. These cold environments are dominated by psychrotolerant
organisms as opposed to psychrophiles, which are assumed to thrive in these cold
conditions. However, earlier studies also indicate that most of the polar ice habitats are
dominated by psychrotolerant organisms (Vincent 2000). Psychrophiles thrive and
flourish in continuously cold environments like the sea ice where the long periods of
sustained cold temperatures act as the major selective pressure. However, in the polar
regions, sharp and rapid changes in seasonal temperatures act as the main selective
pressure. Significant environmental changes may kill many true psychrophiles, while
generalists such as psychrotolerant species have better chances to survive. The organisms
in these environments should be able to survive freeze thaw cycles that occur during the
seasonal changes. Sharp changes in the surface temperatures have been observed in the
Antarctic soils and have been recorded to go as high as 30\(^{\circ}\)C (Franzmann 1996). These
conditions favor the psychrotolerant species that can withstand the temperature
fluctuations. If fungi and bacteria from these surrounding environments are deposited in
the glacial ice, we would expect to find psychrotolerant species among our isolates.
*Rhodotorula mucilaginosa* remains viable after many freeze thaw cycles, which explains their abundance in the ice cores analyzed.

We revived fungi and bacteria from ice cores that dated back to 105,000 and 155,000 years. Previous studies by Ma et al (1999 and 2000) and D’Elia et al (2008) also resulted in the revival of fungi from ancient ice cores in the Arctic and Antarctic regions up to approximately 2 million years old. Organisms have been isolated from the deepest and oldest glacial ice core section from the Vostok core. Apart from surviving the really harsh conditions in these environments, the time period of survival is even more interesting. Bacteria and fungi that survive in these conditions employ a host of survival techniques. The membrane constitution is altered to maintain the fluid state at low temperatures by increasing the proportion of unsaturated fatty acids in several yeasts like *Candida* and *Leucosporidium* (Karekes et al 1980). Sugars like Mannitol and Trehalose were found to act as cryoprotectants in fungi at low temperatures (Goodrich et al 1988). Antarctic strains of the fungi *Humicola* produced high levels of mannitol compared to the temperate strains, though the total amounts of sugars were equal (Weinstein et al 1997). Antifreeze proteins (AFP’s) were identified in several fish and plants and play an important role in their survival by preventing the crystallisation of ice. Studies have identified AFP’s in several species of fungi belonging to the genera *Typhola*, indicating the possibility of the presence of AFP’s in other fungi in cold environments. Cold shock proteins and cold acclimitation proteins are important for survival of psychrotrophic bacteria like *Bacillus subtilis* at low temperatures (Roberts et al 1992).

We were able to isolate organisms from six of the eight ice cores analysed. The number of isolates decreased with the increase in the age of the ice core and was
consistent with earlier studies. Ice cores from the Arctic region showed more species richness compared to the Antarctic ice cores and may be the result of the geographic location and warm climate in the Arctic region in the recent past. All the isolates from the GISP2D and the Vostok cores have been isolated from various cold environments and have been known to survive in these conditions. SEM micrographs of the microbes were similar to microbes observed by different groups in different cold environments and supported the results from the sequence analysis.

In general, the species that were isolated from Greenland and Antarctic ice cores were different. Furthermore, when members of the same genus and species were isolated from both polar regions, the sequences differed from one another. Therefore, it appears that the two polar regions entrap microbes only from geographically local regions. This supports the hypothesis that microbes at the poles are isolated from one another, indicating that there is little or no mixing or distribution from pole to pole.
Literature Cited


APPENDIX A
Isolation of Microbes from Lake Vostok Accretion Ice

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Bacteria from seven Lake Vostok accretion and two deep glacial Vostok ice core sections were characterized. The cell concentrations were low, but many of the cells were viable. From the hundreds of cultures, 18 unique bacterial rRNA gene phylotypes were determined. Lake Vostok may contain a complex microbial ecosystem.

Subglacial Lake Vostok, the eighth largest lake on Earth (area = 14,000 km², volume = 5,600 km³) (9, 16), is covered by a 4-kilometer-thick layer of glacial ice. As the glacier traverses the lake over a period of 18,000 years, ice freezes (or accretes) to the bottom surface of the glacier, eventually forming a 200-m layer of accretion ice that has retained a linear and temporal record of the contents of the upper surface of the lake. The glacier passes over a shallow embayment, near an island (or peninsula), and then over part of the main lake basin. As the glacier passes through the embayment, initially it is grounded on the lakebed, and partly because of this, it collects mineral inclusions, making the ice silty (termed type I accretion ice) (13). Melting and freezing in this area, as well as a possible influx of material from a river system and/or from hydrothermal activity, may contribute to the characteristics of the type I ice (3, 14). The glacier is suspended over open water in portions of the embayment and over most of the main parts of the lake. The ice that forms over open water contains far fewer inclusions and lower concentrations of ions, organic carbon, and biomass (6, 10, 14). This ice is very clear ice and has been termed type II accretion ice (13). The top section (from 3,538 to 3,595 m), which accreted within and near the embayment, primarily consists of type I ice, although there are some regions of type II ice (2). Bacteria from this ice, including potentially psychrophilic and psychrotolerant species as well as the molecular signature of a thermophilic bacterium, have been reported (1, 3, 4, 5, 7, 10). Within the lake, temperatures average 2°C, pressures approach 400 atmospheres, high oxygen levels exist, there are low nutrient levels, and it is completely dark.

We isolated and characterized microbes from Lake Vostok type I and II accretion ice from the embayment and the main basin as well as from glacial ice immediately above the accretion ice layers. Sequence results from the rRNA small subunit genes and internal transcribed spacers indicate that at least 18 species are represented in the accretion ice. All are psychrotolerant in that they grew at 4°C, although optimal growth was often at higher temperatures.

Descriptions and cell concentrations. Nine Vostok 5G ice core sections were assayed. Five sections represented ice that accreted over the shallow embayment (depths of 3,540, 3,563, 3,582, and 3,584 m, all type I ice, and 3,591 m, type II ice) (2) approximately 3,800 to 5,100 years ago (2, 6, 13, 14), two accreted 3,400 to 3,500 years ago over the main lake basin (3,606 m, type I ice, and 3,610 m, type II ice), and two were glacial ice cores near the bottom of the glacier (3,501 and 3,520 m, approximately 1 to 2 million years old) (13, 14). The surfaces of all of the ice core sections were decontaminated prior to melting, as described previously (12). Ice core meltwater initially was analyzed using a live/dead stain (BacLight viability kit; Molecular Probes, Eugene, OR) to count cells from 10 ml samples for each core section by using fluorescence microscopy. The concentrations (means ± standard deviations) of viable and nonviable cells ranged from 2.33 ± 0.29 to 12.33 ± 600 cells/ml.
FIG. 2. Fluorescence and scanning electron micrographs of cells found in the glacial (3,501 and 3,520 m) and accretion ice (3,540, 3,563, 3,582, 3,584, 3,591, 3,606, and 3,610 m) core sections. Cells in the fluorographs were stained with BacLight (Molecular Probes, Eugene, OR). Green fluorescence indicates possible viable cells, based on membrane integrity. Red fluorescence indicates possible dead cells based on intracellular staining. Bars represent 5 μm on fluorographs and 1 μm on electron micrographs. 3501, cells characteristic of eubacteria, possibly eukaryotes (in one case, middle right); 3520, probably bacteria (upper two cells; upper right potentially viable), germinating spore (lower left; bacterial or fungal), and unknown (lower right); 3540, all apparently coccoid bacteria; 3563, all apparently bacteria (all nonviable); 3582, filamentous bacteria or fungi (upper portion; some structures may be oogonia [or similar structures]), coccoid bacteria (middle), and a mix of Bacillus-type cells, a spiral-shaped cell, and three linear and angular cells (lower portion; the one on the lower right might be related to one type of green algae, and the two angular cells are similar to those reported to occur in core section 3593 [5]); 3584/5 (the cell on the far left in the fourth row may represent a small Caulobacter-like cell); 3591, all three apparently bacteria; 3606 and 3610, cells are similar to those in 3582 and 3584/5.
Considering the partition coefficient (0.56) (6), for ice versus water, the concentrations in the lake are approximately 1.78 times higher than these values (4.15 to 21.95 cells/ml). The number of viable cells in each ice core section varied from nearly 0 to a mean of 6.56 cells/ml (Fig. 1). The mean concentrations of nonviable cells were from 1.28 to 5.58 cells/ml. The concentrations of viable cells in glacial ice primarily were lower, between 1.00 and 2.00 cells/ml. This is expected, given the fact that the glacial ice that was examined was between 1 and 2 million years old, while the accretion ice was only 3,400 to 5,100 years old (6, 13, 14).

A 5-ml sample from each core section also was examined by scanning electron microscopy (SEM) (Fig. 2). After filtration, the filter was fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2), rinsed, dehydrated with ethanol, dried (Samdri 780A critical point dryer), sputter coated with gold-palladium (Polaron E500 SEM coating unit), and viewed using an SEM (Hitachi S-2700). Many of the cells in the glacial ice were distorted (Fig. 2), which is consistent with the low cell viabilities indicated by the fluorescence microscopy assays (Fig. 2). Cells in the shallow embayment type I ice also exhibited damage. However, cells in type II ice of the embayment exhibited lower levels of distorted cells, and the diversity of cell shapes was higher. The same pattern was observed for type I and type II ice from the main lake basin, but the numbers of cells and the level of cell diversity were lower than in the ice from the embayment.

Cultures and sequence analyses. Approximately 2,000 meltwater aliquots (200 μl each) were spread on agar plates containing 13 different media and were incubated for weeks to months at four temperatures (4, 8, 15, and 22°C). Portions of the rRNA loci were amplified by PCR (as in reference 12, with primers described in reference 11) and sequenced, followed by BLAST searches, CLUSTAL alignment, and phylogenetic analysis (as in reference 8). A total of 665 colonies resulted from the seven Vostok 5G accretion ice core sections, and an additional 22 were isolated from the glacial ice immediately above the accretion ice. The bacteria isolated represent a va-
riety of taxa (Fig. 3), all of which are psychrotolerant (data not shown). All are related to taxa that are aquatic and/or live in lake sediments, soils, or rocks (Fig. 3). In addition to the bacteria, a dozen unique fungi were isolated (data not shown).

Conclusions about Lake Vostok. The assembly of microbes that were found in the Lake Vostok accretion ice samples indicates that the lake has a diverse population of microorganisms and potentially a complex ecosystem. Nonetheless, the concentrations of microbes in the subglacial lake are lower than those in most environments on Earth (7). Some have suggested that Lake Vostok is sterile, since parts of the lake may be extremely oligotrophic (3). However, all of our data indicate that the lake supports a diverse microbial assembly, as has been concluded elsewhere (6, 7, 10, 15). There appear to be distinct ecological zones, either spatially or temporally, since different sets of microbes were isolated from each of the four zones (type I and II ice from the shallow embayment and type I and type II ice from the main basin), representing different ages of ice (Fig. 1). Our results indicate that the highest concentrations of viable cells are located close to the transition zones between type I and type II ice, which would correspond to the shoreline of the lake near the grounding line of the glacier. While most research on the accretion ice has focused on bacteria, some fungi have been described (3), and fungi were isolated and photographed in this study (Fig. 2). Therefore, heterotrophs may be present in Lake Vostok. If so, the Lake Vostok ecosystem is more complex than previously thought.

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APPENDIX B
Isolation of fungi from Lake Vostok accretion ice

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Abstract: Here we report the characterization of fungi from 10 accretion ice sections (3300–5100 y old) as well as two deep glacial ice sections that are close to the bottom of the glacier (1 000 000–2 000 000 y old) from the Vostok, Antarctica, 5G ice core. Fungi were characterized by fluorescence microscopy culturing and sequence analyses of ribosomal DNA internal transcribed spacers. A total of 27 fungal colonies were cultured from the accretion ice of subglacial Lake Vostok and an additional 14 from the glacial ice immediately above the accretion ice. Mean concentrations were 0–4.42 cells mL−1 ice meltwater of which 0–100% exhibited viability (as determined by fluorescence microscopy). Thirty-one unique fungal ribosomal DNA sequences (28 from accretion ice and three from glacial ice) were determined and compared to recent taxa. The results, plus tests for growth at low temperatures, indicated that Lake Vostok contains a mixture of heterotrophic psychrotolerant fungal species. This indicates that the lake is not sterile but contains a unique ecosystem.

Key words: Antarctica, heterotrophs, ITS, subglacial lake

INTRODUCTION

More than 140 subglacial lakes are known to exist in Antarctica (Siegert et al 2005), complete with river systems (Wingham et al 2006). The first discovered was Lake Vostok (Kapista et al 1996, Siegert et al 2001). With a volume of 5400 km³ and a surface area of 14 000 km², Lake Vostok is the largest of all subglacial lakes (Siegert et al 2001, 2005) and is the eighth largest lake on Earth. The average depth of Lake Vostok is 400 m, with a maximum depth of 1200 m. The lake has been isolated from the atmosphere approximately 15 000 000 y (Siegert et al 2003), making any life within the lake ideal for exploring evolutionary processes related to extended isolation and extreme conditions.

The ice sheet that covers Lake Vostok is composed of discrete regions based on the origin of the ice and the flow of the glacier. Data collected from the Vostok drilling station has accurately reconstructed the paleoclimatic record of the past 420 000 y by analyzing the upper 3310 m ice core (Petit et al 1999). The deepest part of the Vostok core of meteoric origin (3538 m) has been estimated at 1 000 000–2 000 000 y old (Salamin et al 2004), although at 3310–3338 m the ice is disorganized because of its flow over a ridge as it enters the lake. The composition of the ice changes at depths greater than 3539 m and represents lake water that has frozen, or accreted, to the bottom of the ice sheet (Petit et al 1999). The final 210 m of the Vostok core has been termed accretion ice, which is characterized by having a chemistry and crystallography distinct from the glacial ice (Jouzel et al 1999). Usually glacial ice has low concentrations of ions but carries bubbles from the atmosphere. Some of the accretion ice has higher concentrations of ions, no gases and often has large ice crystals.

The flow of the ice sheet across Lake Vostok has been calculated at 16 000–20 000 y (Bell et al 2002). Thus the accretion process has preserved a spatial and temporal record of the upper contents of the water from the lake. The ice flows onto Lake Vostok from Ridge B ice divide in a west to east direction (Kapista et al 1996). The path of the ice sheet from the shoreline to Vostok station leads to ice accretion from distinct areas of the lake, allowing a comparison of microorganisms from different regions. Ice core sections at 3539–3609 m, primarily consisting of ice that formed in a shallow embayment (Bell et al 2005, Salamin et al 2003), are characterized by having inclusions (ice with silt, termed type I ice) and high salt concentrations that might be the result of the temporary grounding of the glacier on the lakebed as it enters a shallow embayment (Royston-Bishop et al 2005). Accretion ice at depths of 3609 m and deeper is formed over the open-central region of the lake (termed accretion ice type II) and contains almost no inclusions and lower concentrations of ions, organic carbon and biomass (Christner et al 2006, Priscu et al 1999, Salamin et al 2003). Often individual ice crystals are more than a meter long (de Angeles et al 2004, Salamin et al 2003). Because the shallower accretion ice is older, analysis of this ice requires consideration of a temporal component. It therefore is possible to collect ice core sections that represent...
the distinct regions of the lake from different times and determine whether there is a relationship between the diversity and concentration of microorganisms based on the origin of the accretion ice.

Microbiological investigations of Lake Vostok have provided results on the biological conditions that might be present in the lake. Freshwater is supplied to Lake Vostok by melting glacial ice near the shoreline and at the ice-water interface in the north (Siegert et al 2000, Studinger et al 2004). In addition, subglacial streams and rivers might add water (Wingham et al 2006). Glacial melting is a source of oxygen and nitrogen, as well as nutrients and organic matter. For example, concentrations of dissolved oxygen in the lake water column are estimated to be as high as 50 times greater than air-equilibrated water (Christner et al 2006, McKay et al 2003). However, gases supplied from the meltwater are excluded from the accretion process, as is evident in the fact that accretion ice is essentially gas free compared to glacial ice (Jouzel et al 1999). Reported dissolved organic carbon (DOC) levels range from adequate for growth of microbial heterotrophs (Christner et al 2006, Priscu et al 1999) to insufficient to support heterotrophic life (Bulat et al 2004). Initial estimates of microbial concentrations in Lake Vostok accretion ice samples have varied from zero to nearly $10^6$ cells mL$^{-1}$ (Bulat et al 2004, Priscu et al 1999). Another early study of an ice core section from 3603 m indicated $2 \times 10^2$ cells mL$^{-1}$ (Karl et al 1999). Current estimates for the surface of Lake Vostok, using a partitioning coefficient to account for cells excluded during the refreezing process, are 140–770 cells mL$^{-1}$ (Christner et al 2006). However our previous results from the accretion and glacial ice sections indicate that the total cell concentrations range from 2.33 ($\pm$ 0.29) to 12.33 ($\pm$ 9.58) cells mL$^{-1}$ and the mean viable cell concentrations range from 0 to 6.56 ($\pm$ 3.56) cells mL$^{-1}$ (D’Elia et al 2008). The discrepancies among biological assays are most likely due to natural heterogeneity in accretion ice core sections, as well as the methodologies used in analysis of microbes in the ice.

Fungi have been isolated from a variety of cold environments and have developed several mechanisms to withstand cold temperatures and low nutrient availability. The vast majority are psychrotolerant, able to survive below freezing temperatures but with optimal growth temperatures greater than 15 C. Few are true psychrophiles, which die if exposed to temperatures above 20 C, with optimal growth temperatures below 15 C. Subglacial ice from arctic glaciers provided the first reports of the isolation of yeasts (Butinar et al 2007) and a new species of *Penicillium* (Sonjak et al 2007) from these unique environments. Viable fungi have been recovered after 140,000 y in Greenland glacial ice, which represents a significant amount of time separated from contemporary populations (Ma et al 2000, 2005; Starmer et al 2005). Mycelial fungi have been isolated from Siberian cryopegs, despite subzero temperatures and high salinities (Ozerskaya et al 2004). The fungal component of antarctic environments also has been widely studied. These investigations have identified fungi in glacial ice (Abyzov et al 2004, Poglazova et al 2001), soil (Arenz et al 2006), airborne spore traps (Marshall 1997) and mosses sampled in Antarctica (Tosi et al 2002). Most are reported to be psychrotolerant. Few are true psychrophiles. Several general physiological adaptations are used by fungi to survive in extremely cold environments. Membrane fluidity is maintained by increasing the unsaturated lipid content (Weinstein et al 2000). Additional physiological mechanisms that enhance cold adaptation include an increase in intracellular trehalose and polyol concentrations, secretion of antifreeze proteins (Snider et al 2000) and the use of cold adaptive enzymes (Robinson 2001).

Fungi have been reported in the deep, ancient glacial layers of the Vostok core. Yeasts of genus *Rhodotorula* and *Cryptococcus* have been identified by molecular methods, and fungal mycelia have been observed by direct epifluorescence microscopy (Abyzov et al 2004). Initial reports on accretion ice have detected fungal hyphae and conidia from the 3585 m core section (embayment) and yeast cells from the 3611 m core section (main basin), which were observed by fluorescence microscopy (Poglazova et al 2001). Here we report the isolation and identification of fungi from Lake Vostok accretion ice. Ice core section meltwater samples were used for enrichment cultures, direct PCR and microscopic analysis (fluorescence and scanning electron).

**MATERIALS AND METHODS**

*Description of ice core sections.—* Ten accretion ice core sections were examined for biological contents (as described below). Five (at 3540, 3563, 3582, 3585 and 3591 m) represented ice that had accreted over a shallow embayment and were approximately 3800–5100 y old (Bell et al 2005, Christner et al 2006). Five (at 3606, 3610, 3613, 3619, and 3621 m) accreted over the main lake basin and were approximately 3300–3500 y old. Two additional cores were examined from deep glacial ice (3501 and 3520 m), which were approximately 1,000,000–2,000,000 y old (Salamatin et al 2003, 2004). All were examined for the presence of living and dead fungi. Sections from 3613, 3619 and 3621 m were from the Russian allocation and were kindly shared by Prof Sergey Bulat and colleagues. All other ice core sections were obtained from the National Ice Core Laboratory (NICL) in Denver, Colorado.
Decontamination.—The outer ice core surfaces were decontaminated with 5.25% sodium hypochlorite with a protocol shown to be effective at removal of all external microbial and nucleic acid contamination (Rogers et al 2004, 2005). Quatered ice core sections (6 cm long) were warmed at 4 C at least 30 min before surface decontamination. Work surfaces in a room isolated from the working laboratory were sterilized with 5.25% sodium hypochlorite (undiluted Clorox), 70% ethanol and UV irradiation 1 h before handling of the ice core sections. Inside a sterile Class II cabinet (biosafety laminar flow hood) ice core sections were surface decontaminated by immersion in 500 mL 5.25% sodium hypochlorite solution (prechilled to 4 C) 10 s followed by two rinses with 200 mL sterile water (4 C, 18.2 MΩ, <1 ppb total organic carbon). The core section was transferred into a sterile funnel and was melted at room temperature by collection of 30–50 mL aliquots. This process allows for collection of “shells” of meltwater corresponding to initially the outer portion and sequentially more interior portion of the ice core section. Up to five shells were collected for each ice core section (based on size of core section). A portion of the meltwater was used immediately for culturing, and the remaining water was frozen for subsequent molecular and microscopic investigation. During the handling process four agar plates (nutrient agar and malt extract agar, see below) were placed in the laminar hood. The lids were removed to allow continuous exposure to the working environment. These controls allowed for monitoring air quality within the laminar flow hood.

Fluorescence microscopy.—Ice core meltwater samples were viewed by fluorescence microscopy with the LIVE/DEAD BacLight™ Viability Kit (Molecular Probes, Eugene, Oregon). Using aseptic techniques in a laminar flow hood, meltwater samples were concentrated 10-fold by preparing 1 mL aliquots meltwater that were centrifuged in sterile 1.5 mL microfuge tubes for 10 min at 10 000 g. The supernatant was removed and saved. The remaining pellet was resuspended in 100 L supernatant. A total of 10 mL meltwater for each core was used for cell counts and viability examination under the same condition (negative controls). Sterilized water preparations also were examined by epifluorescence microscopy (Ziess Axiophot Epifluorescence Microscope, FITC long pass filter set) at final magnification. The samples were not filtered for examination to avoid false negatives (filtering might kill viable cells or introduce contaminating cells), accumulation of debris and multiple manipulations that could introduce contaminants. Sterilized water preparations also were examined under the same condition (negative controls).

Scanning electron microscopy (SEM).—Meltwater samples were prepared for SEM by filtering 5 mL water through a sterile 0.2 µm polycarbonate filter. The filter was fixed with 2.5% gluteraldehyde in 0.1 M phosphate buffer (pH 7.2) 1 h and rinsed three times (10 min each) in 0.1 M phosphate buffer followed by dehydration 10 min each in 40%, 60%, 80%, 95% and 100% (three times) ethanol. All solutions used for processing meltwater samples were filter sterilized by passage through a 0.2 µm filter before use. A Samdry 780A critical point dryer was used to dry the fixed/dehydrated filters. Sputter coating of the mounted filters was performed by adding a 5 nm gold-palladium coat with a Polaron E500 SEM coating unit. Samples were observed under SEM (Hitachi S-2700) for the presence of microorganisms. Control filters were processed in identical manners with sterilized water and were examined for the presence of any microorganisms that indicated contamination.

Culturing.—Meltwater collected from the surface-decontaminated ice core sections was used to assay for viable microorganisms by distribution and inoculation on a variety of agar media to encourage the growth of fungi. Culturing was performed by spreading 200 µL meltwater from each shell onto duplicates of each media. The media included malt extract agar (1.28% maltose, 0.27% dextrin, 0.24% glycerol, 0.08% peptone, 1.5% agar [pH 4.7]), potato dextrose agar (0.4% potato starch, 2% dextrose, 1.5% agar [pH 5.6]), rose bengal agar (0.5% soytone, 1% dextrose, 0.1% monopotassium phosphate, 0.005% rose bengal, 1.5% agar [pH 7.2]), nutrient agar (0.3% beef extract, 0.3% peptone, 1.5% agar [pH 6.8]), oatmeal agar (6% oatmeal, 1.25% agar [pH 6.0]), Sabouraud dextrose agar (1% enzymatic digest of casein, 2% dextrose, 2% agar [pH 7.0]), yeast extract agar (3% yeast extract, 3% malt extract, 0.5% peptone, 1% dextrose, 2% agar [pH 6.2]), acid yeast extract agar (3% yeast extract, 3% malt extract, 0.5% peptone, 1% dextrose, 2% agar [pH 4.5]), meat-liver agar (2% meat-liver base, 0.075% D(+)-glucose, 0.075% starch, 0.12% sodium sulfite, 0.05% ammonium ferric citrate, 1.1% agar [pH 7.6]), blood agar (1.5% pancreatic digest of casein, 0.5% papain digest of soybean meal, 0.5% sodium chloride, 5% sheep’s blood, 1.5% agar [pH 7.3]) and water agar (sterile distilled water with 2% agar). The inoculated agar plates were incubated at 8 C at least 2 wk, followed by 15 C at least 2 wk and then maintained at 22 C. Plates were examined for fungal growth weekly. Any isolates obtained from meltwater culturing were immediately subcultured to obtain pure cultures for molecular identification. Subcultures were tested for growth at 4, 8, 22 and 37 C. Triplicates of each isolate were cultured on MEA plates and incubated at the respective temperature. Mean growth rates were calculated based on colony diameter measurements after 2 wk incubation.

PCR amplification of isolates.—Nuclear ribosomal DNA (rDNA) internal transcribed spacers (ITS1 and ITS2) and 5.8S gene were amplified with primers ITS4 and ITS5 (White et al 1990). DNA was amplified with a GeneAmp PCR Reagent Kit (Applied Biosystems, Branchburg, New Jersey). Each reaction consisted of 5 µL cell suspension, 50 pmol each primer, 10 pmol each dNTP, 2U Taq DNA polymerase, 50 mM KCl, 1.5 mM MgCl₂, in 50 µL total volume. In some cases native Taq DNA polymerase (Fermentas Inc., Hanover, Maryland) was used. The thermal cycler (Mastercycler gradient, Eppendorf, Westbury, New York) program used was 95 C for 8 min, 40 cycles of 1 min at 94 C, 1 min 30 s at 54 C and 2 min at 72 C, followed by an incubation for 8 min at 72 C. PCR reactions...
Fig. 1. Micrographs of cells found in the glacial (3501 and 3520 m) and accretion (3540, 3582, 3584, 3606, 3610, 3613, 3619 and 3621) ice core sections. No cells consistent with fungi were found in sections 3563 or 3591. Cells in the fluorographs were stained with a LIVE/DEAD BacLight Viability Kit (Molecular Probes, Eugene, Oregon). Green fluorescence indicates possible viable cells, based on membrane integrity. Red fluorescence indicates possible dead cells based on intracellular staining.
were viewed on 1.0% agarose gels, with TBE (90 mM Tris-borate, 2 mM EDTA, pH 8.0), and 0.5 μg mL⁻¹ ethidium bromide, illuminated with UV and photographed with a digital camera.

**Cloning and sequencing.**—PCR amplified ITS rDNA from the fungal isolates were inserted into a pCR 4-TOPO vector (TOPO TA Cloning Kit for Sequencing, Invitrogen, California) following the manufacturer’s instructions. The reaction was set up as follows: 2.5 μL PCR product, 1.0 μL salt solution (200 mM NaCl, 10 mM MgCl₂) and 1.5 μL vector (10 ng μL⁻¹). The reaction was used to transform One Shot® TOP10 Competent E. coli cells as described by the manufacturer. Plasmid DNA was isolated from transformed cells with the Cyclo-Prep Plasmid DNA isolation kit (Amresco, Solon, Ohio) and analyzed for inserts by restriction digestion with EcoRI. Digested plasmids were analyzed by electrophoresis through a 1% agarose gel and viewed by ethidium bromide (0.5 μg mL⁻¹) staining and UV irradiation and fluorescence. Plasmids containing positive inserts were diluted to 50 ng μL⁻¹ and sequenced commercially (Gene Gateway LLC, Hayward, California).

**Phylogenetic analysis.**—ITS DNA sequences were used in BLAST analyses of the NCBI (National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov) database to identify sequences of related taxa. The sequences from the isolates and related taxa then were aligned with Clustal X 2.0 (Thompson et al 1997) and manually adjusted. A maximum parsimony tree was created (heuristic search) from the alignment with PAUP (Swofford 1999). Gaps were treated as a fifth base, and the tree was midpoint rooted. Bootstrap analysis (1000 replications) was used to determine amount of support for the branches.

**RESULTS**

**Meltwater analysis by microscopy.**—Fluorescence microscopy revealed a wide variety of morphologically distinct microbial cells, and the low cell concentrations and diversity of shapes were confirmed by SEM and culturing (Figs. 1, 2). Many spores were found in the meltwater (Fig. 1), including spherical, oblate and oblong forms. Some appeared to be germinating. Melting the ice might have triggered this process. In one core section (from 3582 m) many filaments were observed. Some appeared to be emanating from bulbous ends that resembled spores. Occasional bulbous sections were observed in the middle of the filaments as well. Some of the filaments formed aggregates consisting of a dozen or more individual filaments. Walls between cells in the filament were observed in some cases (Fig. 1, arrows). The cells found in section 3619 m (that accreted over open water in the main lake basin) consisted almost exclusively of long filaments that resembled hyphae. Some appeared to be multinucleate. The total number of cells always was low (averaging 1.66 cells mL⁻¹). The curves for the number of viable cells, colonies and unique sequences were similar (Fig. 2). All peaked near the boundaries between type I ice with silt and type II (clear) ice. The silt in type I is thought to come from grounding of the glacier on the lake bottom, water turbulence and/or geothermal activity below the lake. Type II accretes over calm open water, and cell characteristics differed from those in type I.

**Cultured isolates and DNA sequence analysis.**—Culturing of meltwater from the two glacial ice and 10 accretion ice samples resulted in 284 colonies of fungi (Table I). No growth was observed on any control plates (sterile water or culture plates opened in the hood) that were incubated 12–24 mo. Only 14 colonies in total were isolated from deep glacial ice
core sections, 3501 m and 3520 m. For eight of the accretion ice cores the number of total colonies was 0–11. The remaining two accretion ice sections from 3610 m and 3582 m respectively had the highest number of colonies, 72 and 169. The high number of colonies from core section 3582 was mainly distributed among three culture plates with the remaining plates having fewer colonies. This indicates the heterogeneity in these samples and might reflect a high concentration of microbes adhering to mineral inclusions entrapped in the ice. One of these accretion ice cores (3610 m) is from open water, while the other (3582 m) is in a transition zone that contains both type I and type II ice and is associated with the shallow embayment.

A total of 38 fungal cultures were selected for sequence analysis of ribosomal ITS regions. All sequences were obtained directly from cultured isolates (all attempts to PCR amplifying fungal ITS DNA directly from ice core meltwater were unsuccessful). Of these 30 were from at least four genera of basidiomycetes (27 from accretion ice and three from glacial ice), while eight were from at least five genera of ascomycetes (all from accretion ice). Sequences closest to *Rhodotorula mucilaginosa* were the most frequently observed. A total of 26 *Rhodotorula* ITS sequences were obtained for isolates from every accretion ice core section except 3540 m and 3585 m (isolates GI822, GI902-904, GI908-911, GI914, GI926-929, GI931, GI933, GI939, GI945-949, GI951, GI952, GI959, GI962 and GI966). Three additional isolates were collected from the deep glacial ice core, section 3501 m (GI966, GI962 and GI959). Isolates related to *Rhodotorula* were most abundant in the 3582 m core section, which formed over the embayment.Percent similarity scores for the *Rhodotorula* isolates were 93–100% compared to taxa in NCBI. Isolates of *Rhodotorula* were the only fungi found in the glacial ice as well as the accretion ice. All other isolates were found only in the accretion ice. Isolates initially were collected from a variety of media, including minimal nutrient media, after incubation of 15 d to more than 9 mo. *Rhodotorula* isolates showed optimal growth at 22 C but also grew well at 15 C and 4 C (TABLE II), indicative of psychrotolerance. Other basidiomycetes included a single isolate (GI913) belonging to genus *Cystofilobasidium* (96% similarity to GenBank) and two isolates (GI817 and GI944) that are most closely related to members of genus *Cryptococcus*. A final basidiomycete was isolate GI985 that was closest to species of *Pseudocyma* and *Ustilago* and was psychrotolerant (TABLE II).

A total of seven ascomycete isolates were identified in six (3563, 3582, 3585, 3610, 3613 and 3619 m) of the 10 accretion ice core sections (TABLE I). A single isolate (GI920) from 3585 m was determined to be most closely related to genus *Penicillium*, with 88% ITS sequence similarity to *P. chrysogenum*, and displayed psychrotolerant growth characteristics (TABLE II). (Isolate GI737 was from the Greenland Dye-3 ice core section that was approximately 5500 y old. It was included for comparison.) Accretion ice samples that formed over the embayment (3582 m) and open main basin of the lake (3610 m) each contained few isolates (GI924 from 3582 m, and GI900 and GI901 from 3613 m) with closest sequence similarities to unknown fungi that are most likely within or close to genus *Cladosporium*. Isolate GI932 also originated from ice core section 3582 m and shares 95% ITS sequence similarity with *Aerobasidium pullulans*. One isolate (GI898) was closest to *Aspergillus* species but differed significantly from all sequences in the NCBI database (maximum of 84% similarity). A single ascomycete was collected from accretion ice representative of the grounding line of the ice sheet in the embayment. This isolate (GI951) from 3563 m showed low sequence similarity to taxa in GenBank (highest similarity of 69%), although it is closest to *Cerella androprogonis* and *Phoma* spp. However, because of the low sequence similarity it is likely that it represents a different genus and possibly is in a different family. Tolerance to low growth temperatures was observed for all isolates classified as ascomycetes (TABLE II). All isolates originally were detected after incubation at 15 C. No ascomycetes were isolated from the glacial ice core sections, estimated to be 1 500 000 and 2 000 000 y old (Salamin et al 2004).

Phylogenetic analysis.—Phylogenetic analysis was performed on ITS sequences obtained from the 36 ice core isolates (plus isolate GI737 from Greenland; Ma et al 2000), and an additional 39 fungal sequences obtained from the NCBI database related to contemporary taxa and isolates from polar and frozen environments (FIG. 3). The phylogenetic analysis revealed two major groups comprising ascomycetes and basidiomycetes. The basidiomycete group contained four major subclades including genera *Rhodotorula*, *Cryptococcus*, *Cystofilobasidium* and *Pseudozyma*. The majority of sequences isolated from accretion ice cores grouped within the *Rhodotorula* subclade with strong bootstrap support. These sequences were closely related to other species of *Rhodotorula*, including species isolated from Antarctica and deep sea environments. The phylogram shows the presence of multiple genotypes within genus *Rhodotorula* and their frequency in different ice core sections. Isolates classified as *Rhodotorula* from
accretion ice core section 3582 m grouped closely together, while isolates from core sections 3606 m and 3501 m were more widely distributed throughout the Rhodotorula subclade. Another basidiomycete, isolate GI913 (3606 m), was closest to genus Cystofilobasidium, grouping with the cold-adapted yeast Cystofilobasidium capitatum. Isolates GI817 and GI944 grouped with antarctic isolates of Cryptococcus and GI895 was closest to antarctic isolates of Pseudozyma.

The ascomycetes in the phylogram are fewer in total number (Fig. 3). Isolate GI920 displayed strong affinity to species of Penicillium from Antarctica, glacial and deep sea environments and was in the same clade as a Greenland glacial isolate (P. griseoroseum). Isolates GI817 and GI944 grouped with marine isolates of Cryptococcus and GI895 was closest toantarctic isolates of Pseudozyma.

The ascomycete group was identified as Cladosporium sp. The isolates were from ice core sections that represent distinct regions of Lake Vostok. Also grouped in this subclade was a Cladosporium herbarum isolated from 140 000 y old Greenland glacial ice (Ma et al 2000) as well as other Cladosporium isolates from Antarctica.

DISCUSSION

Accretion ice from Lake Vostok enables a unique opportunity to investigate the microbiology of an extreme subglacial environment that has been ice-covered for millions of years. Glacial and accretion ice core sections have been extensively studied in terms of the bacterial component and chemistry (Abyzov et al 2004; Bulat et al 2004; Christner et al 2006; D’Elia et al 2008; Karl et al 1999; McKay et al 2003; Priscu et al 1999; Royston-Bishop et al 2005; Siegert et al 2000, 2001, 2003, 2005). This report is the initial detailed study of fungi in Lake Vostok accretion ice. Subglacial environments offer a potentially good habitat for microorganisms due to the presence of liquid water, nutrients and minerals from glacial melting and weathering of bedrock and protection from UV exposure and temperature fluctuations (Sharp et al 1999). The Lake Vostok ecosystem is of particular interest because of the length of isolation from the atmosphere and the extreme conditions. It is hyperbaric (approximately 350 atmospheres), cold (average temperature is -2 to -3 C), dark, oligotrophic and possibly hyperoxic. Isolation and identification of fungi from accretion ice adds new evidence to the range of microbial diversity in the ice and supports the likelihood of a complex ecosystem within the lake.

Seasonal extremes in Antarctica, along with the variety of habitats on the continent, leads to the selection of microorganisms that use a generalist survival strategy that includes psychrotolerance (Vincent 2000). The majority of the isolates obtained from all ice core sections were isolated originally at 15 C, and all isolates tested were determined to be psychrotolerant (TABLE II). Psychrotrophic fungi capable of growth at 0–25 C have been isolated from a variety of polar habitats (Bergero et al 1999, Tosi et al 2002, Ozerskaya et al 2004, Ma et al 2000). Studies of prokaryotic isolates from Antarctica have found that the optimal growth temperature is often higher than expected for the environment but is typically lower compared to similar taxa from nonpolar environments (Franzmann et al 1996). This is consistent with fungi isolated from Vostok accretion ice. The fungal component from the accretion ice is composed of psychrotolerant species (TABLE II), based on the examination of the isolates obtained from accretion
<table>
<thead>
<tr>
<th>Region</th>
<th>Depth (m)</th>
<th>Percent viable cells</th>
<th>Number of colonies</th>
<th>Unique sequences</th>
<th>Closest taxon in BLAST analyses</th>
<th>Characteristics of closest taxon</th>
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<td><em>Rhodotorula mucilaginosa</em> [95]</td>
<td>ant, asc, atm, sno, soi, tol</td>
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<td></td>
<td><em>Aspergillus</em> [84]</td>
<td>ant, atm, gla soi</td>
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<td><em>Unclassified soil fungi</em> [92-94]</td>
<td>ant, asc</td>
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<td><em>Cryptococcus</em> [89]</td>
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<td><em>Penicillium</em> [85]</td>
<td>ant, gla, lks, pnd, soi</td>
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<td><em>Penicillium</em> [85]</td>
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*Based on cell counts from 10 mL of concentrated ice meltwater with LIVE/DEAD staining.*

*b Cultures of fungi on all media.*

' The 3' end of the rDNA SSU and the 5' end of the rDNA SSU, as well as the internal transcribed spacers (including the 5.8S rDNA in fungi) were used in BLASTN analyses of GenBank on the NCBI Website. If the similarity was 95% or greater, species names are provided. For lower percentages, genus or lower taxonomic classifications are used. Similarity percentages are in square brackets. If more than one sequence was compared, the range of percentages is provided.

* A list of some characteristics within the taxon represented by the sequences that are consistent with conditions in Lake Vostok. Abbreviations: ant = antarctic; aqu = aquatic; asc = ascomycetes (listed for unknowns); atm = common in the atmosphere; frw = fresh water; gla = glaciers; lak = lakes; lks = lake sediments; oce = oceans; per = permafrost; pol = polar; pnd = ponds; psy = some species are psychrophilic; sal = saline and salt marshes; sno = snow; soi = soil; tol = some species are psychrotolerant; unk = unknown.

* Partial sequence, not included in phylogenetic analysis.
ice. Even though accretion ice isolates were found related to ubiquitous genera, such as *Penicillium* and *Rhodotorula*, a distinction is observed in the phylogenetic separation and BLAST percent similarities compared to other taxa (Table I, Fig. 3).

Lake Vostok and the surrounding subglacial sediment likely were seeded with a wide range of fungi before becoming isolated by the antarctic ice sheet. In addition, biological and nonbiological materials constantly are deposited in the lake by melting glacial ice and possibly by subglacial rivers and streams. Both the ITS sequence analysis (Table I, Fig. 3) and psychrotolerant characteristics of the isolates (Table II), along with the consistently negative controls, support the conclusion that the isolates are actual members of the Lake Vostok microbial community and not modern contaminants. Some of the fungi may be permanent residents, while others (e.g. the isolate of *Rhodotorula* from the glacial ice) may be constantly seeded into the lake by the overriding glacier.

All isolates identified are related to polar taxa from a variety of permanently cold environments (Fig. 3). Basidiomycetes were the most abundant group of fungi isolated, although only four genera were represented within the ice, with *Rhodotorula* being the dominant genus. Examination of high arctic subglacial meltwater also revealed a prevalence of basidiomycetes (85% of the isolates) with *Rhodotorula mucilaginosa* as a frequently occurring species (Butinar et al. 2007). The pervasiveness of *Rhodotorula* in the accretion ice is not surprising. *Rhodotorula* has been identified in the glacial ice of the Vostok core (Abyzov et al. 2004), and isolates have been recovered from various depths of Greenland glacial ice (Starmer et al. 2005). *Rhodotorula mucilaginosa* exhibits a high degree of viability after freezing (Butinar et al. 2007), which might explain the frequency of *Rhodotorula* in the accretion ice from each region of Lake Vostok.

*Rhodotorula* might be transported to the lake via freeze-thaw cycles of the accretion ice and lake water, from melting of the deep glacial ice or by deposition from subglacial river systems. *Rhodotorula* species are considered well adapted to cold environments due to a wide range of temperature tolerances and the production of carotenoid pigments for cold protection (Starmer et al. 2005). *Rhodotorula* isolates from the accretion ice were psychrotolerant (Table II), and all displayed pink pigmentation. Two additional yeasts isolated from the accretion ice core (GI944 and GI913) were related respectively to *Cryptococcus magnus* and *Cystofilobasidium* sp. Species of *Cryptococcus* have been isolated from Greenland GISP-2D (Greenland Ice Sheet Project) ice core (Ma 1999) and Vostok glacial ice (Abyzov et al. 2004). They have been determined to be the most frequently isolated yeast genus from antarctic mosses (Tosi et al. 2002). *Cryptococcus magnus* and *Cystofilobasidium* also have been isolated from high arctic glacial ice (Butinar et al. 2007).

Ascomycetes isolated from the accretion ice also were found to be similar to taxa from other polar environments. Two isolates that grouped phylogenetically with *Cladosporium* were obtained from 3610 m and 3582 m. *Cladosporium* is a prevalent genus of fungus worldwide. Analysis of 140,000 y old GISP-2D glacial ice recovered viable *Cladosporium* isolates (Ma et al. 2000). *Cladosporium* is the second most frequently trapped airborne fungal spore in Antarctica (Marshall 1997). It is possible therefore that *Cladosporium* spores were aerially deposited in the overlying glacier and have been able to remain viable over the long period of delivery to the lake. Isolate...
Fig. 3. Phylogram of rRNA ITS sequences (with maximum parsimony on PAUP, Swofford 1999) from glacial and accretion ice fungal isolates (alignment and tree on TreeBase, accession numbers S2238, M4443). Taxonomic groupings are indicated on the right. The primary basidiomycetes are species of *Rhodotorula*. This is the only genus that was isolated from both glacial and accretion ice sections. All sequences that have the prefix GI are isolates from the ice core sections. NCBI accession numbers (in parentheses) and depths (in meters below the glacial surface) are provided for each. The sequences closest to the unknown isolates in this study were chosen for inclusion in the phylogenetic analyses. GI37 was isolated in our lab from a Greenland ice core (Dye 3) at a depth corresponding to ice that was 5500 y old and is included for comparison. Sources and environments are included in square brackets (when available). Bootstrap values (1000 replications) are provided for branches with greater than 50% support.
GI920 was closest to *Penicillium* spp. (88% ITS similarity) and was from 3585 m meltwater. Species belonging to *Penicillium* have been isolated from polar glacial ice (Ma et al 1999, 2000), and many species have been found in arctic glacial and subglacial environments (Sonjak et al 2006, 2007). Considering the long period of time that Lake Vostok has been isolated, the finding of a viable *Penicillium* isolate with low percent sequence similarity indicates that the lake potentially is harboring a set of *Penicillium* species that may be uniquely adapted to this extreme subglacial environment. Accretion ice isolate GI950 grouped within the subclade containing *Phoma* (FIG. 3). *Phoma* species have been identified in glacial ice (Ma et al 1999) and isolates from arctic soils have been characterized as psychro-oligotrophic (Bergero et al 1999). The ability to tolerate both the cold and oligotrophic conditions in Lake Vostok make species of *Phoma* potentially compatible with conditions in the lake. Another ascomycete isolated from 3582 m (GI932) was identified as *Aureobasidium pullulans*. This species has been isolated from arctic and antarctic environments (Starmer et al 2005, Tosi et al 2002).

Isolation of fungi from accretion ice core sections supports the heterotrophic potential of Lake Vostok. The numbers of colonies and fungal isolates were highest in regions of the shallow embayment (3582 m and 3585 m) and the main lake basin (3606 m and 3610 m). Accretion ice from 3572–3612 m has indicated the capacity to support heterotrophic metabolisms and contain diverse compositions of microorganisms (Christner et al 2006). This range of ice core depths includes the passage of the ice sheet from the embayment to the main lake basin. The concentration of organic carbon and cell density are highest in the transitional region relative to the open lake and deep glacial ice (Christner et al 2006). Total and viable cell counts (for all microbes) also were shown to be highest for accretion ice from 3582 m (D’Elia et al 2008). Respiration of 14C-labled organic substrates has been detected in accretion ice meltwater from this region, including 3572 m and 3612 m (Christner et al 2006) and 3603 m (Karl et al 1999). The total number of colonies and diversity of fungi obtained in this study was highest in accretion ice from 3582, 3606 and 3610 m. Accretion ice from adjacent core sections also revealed fungi and other eukaryotic microorganisms. Fungal hyphae and conidia were observed in ice from 3585 m, and yeast and unicellular algae were found in 3611 m (Poglazova et al 2001, Mitskevich et al 2001). These results indicate that the ecological conditions within these regions are sufficient to support heterotrophic metabolism and a high diversity of microorganisms, including fungi. This indicates that Lake Vostok might be a complex ecosystem instead of a sterile body of water.

**ACKNOWLEDGMENTS**

We thank Prof Sergey Bulat and Jean-Robert Petit for useful comparisons and collaborations on some of the assays and for providing sections of the Russian allocation of Vostok accretion ice. Most of the laboratory work was performed by TD and RV. VT worked on the 3619 m ice core section. We thank Marilyn Cayer for her excellent help with fluorescence and electron microscopy. This study was supported by a grant from NSF (ANT 0536870).

**LITERATURE CITED**


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