ISOLATION OF BACTERIA AND FUNGI FROM LAKE VOSTOK ACCRETION ICE

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ABSTRACT

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Lake Vostok, located below the 4000 m thick Antarctic Ice Sheet, is the largest subglacial lake in Antarctica. The lake water has not been sampled due to concerns of forward contamination. However, ice cores available from the Vostok 5G well of depths greater than 3538 m represent lake water that has accreted to the overhead glacier from various regions across Lake Vostok. Here, we report the characterization of bacteria and fungi from ten accretion ice sections (15,000 to 18,000 years old), and two deep glacial ice sections (1 to 2 million years old) from the Vostok, Antarctica 5G ice core. Fungi, as well as bacteria, were characterized by fluorescence microscopy, scanning electron microscopy, culturing, and sequence analyses of ribosomal DNA. A total of 703 bacteria and fungi were cultured from the accretion ice of subglacial Lake Vostok. Mean cell concentrations were from 2.3 to 12.3 cells per ml of ice meltwater, of which 5 to 84% were viable. These cell counts are substantially lower than previously reported values. A total of 54 unique ribosomal DNA sequences were determined and compared to recent taxa, of which 36 were fungi and 18 were bacteria. Fungi most closely related to *Rhodotorula*, *Aureobasidium*, *Cryptococcus*, *Cladosporium* and *Penicillium*, and bacteria related to *Carnobacterium* spp., *Bacillus* spp., *Caulobacter* and *Micrococcus* were identified based on sequence analysis from cultured isolates. These findings, plus tests for growth at low temperatures, indicate that Lake Vostok contains a mixture of heterotrophic psychrotolerant and psychrophilic species. The results revealed new genera of microorganisms not previously reported in Lake Vostok accretion ice, lower abundances of microorganisms, and demonstrated the necessity to use multiple methods to thoroughly characterize environmental ice.
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CHAPTER I

LITERATURE REVIEW

The cold biosphere

The Earth has been described as primarily a cold biosphere. Composed of a wide range of cold and frozen environments, the Earth’s cold biosphere is inhabited by a wide diversity of cold adapted organisms. Approximately 90% of the oceans water is at a temperature of 5°C or colder, and the majority of the Earth’s surfaces are cold (Methe et al 2005). Frozen terrestrial environments, in the form of permafrost, glaciers, polar sea ice and snow, account for 20% of possible land habitats (Methe et al 2005). The temperature of the environment varies depending on the specific environmental conditions and geographic location. Deep ocean water can range in temperature from -1 to -4°C, polar marine water reaches -1°C, sea ice brine veins can be as low as -35°C, and glacier and lake ice average about -5°C (Deming 2002). Surface temperatures both the arctic and Antarctic can reach dozens of degrees below zero. These cold temperature environments are inhabited by a variety of organisms ranging from microscopic to large vertebrates. However, in terms of species diversity and biomass, these environments are dominated by microorganisms (Bakermans et al 2007). In addition to serving as habitable environments, the Earth’s snow and ice also act as a refuge for microorganisms for hundreds of thousands of years (Christner et al 2003, 2006, Poglažova et al 2001, Abyzov et al 2004, Miteva and Brenchley 2005). Viable fungi have been recovered after 140,000 years in Greenland glacial ice, which represents a significant amount of time separated from contemporary populations (Ma et al 1999, 2000, 2005). Due to the
extreme nature of cold environments, the full extent of the implications and importance of microbial life in these habitats is still not fully understood.

The study and investigation into Earth’s cold biosphere allows for a more complete understanding of the limits of life on Earth and insight into the driving forces behind species diversity and evolution of microorganisms. The microbial ecosystems of Antarctica are of particular importance in terms of evolutionary ecology because of their relative isolation and severe biological stresses (Vincent 2000). Examination of these environments may allow for the development of evolutionary ecology models of microbial populations that have been removed from the influence of gene flow from the outside. Environments such as Lake Vostok are a potential source of viable microorganisms that may be studied to determine the influence of having been isolated from the global gene pool for periods of time that are evolutionarily significant (Vincent 2000). Implications of studying these environments include elucidating forces that drive microbial speciation, diversity and evolution. Microbes adapted to cold environments also possess uniquely cold-adapted enzymes which may prove to be important for a variety of industrial and environmentally significant applications (Methe et al 2005, Nakagawa et al 2004). Apart from general identification of microbiota present in these habitats, examination of the adaptation, activity and interspecies interactions will lead to a more thorough understanding of the intricate ecology of life in the severe conditions in permanently cold and frozen environments.
Description of cold environments

There are several major types of cold environments that differ in their chemical and physical make up, which subsequently influences the diversity and abundance of the microbial communities present. Arctic sea ice, which may range in temperature from -1 to -35°C (Deming 2002), may contain up to $1 \times 10^5$ to $15 \times 10^5$ cells ml$^{-1}$ (Brinkmeyer et al 2003) and Antarctic sea ice bacterial counts are as high as $1.02 \times 10^{12}$ cells m$^3$ (Sullivan and Palmisano 1984). Microorganisms found in these environments are faced with the combined stresses of low temperatures and low water activity due to the increase concentration of solutes required to maintain liquid water (Bakermans et al 2007). Despite these harsh conditions, cultivable microorganisms make up large proportions of the total population in sea ice (Deming 2002). Cryopegs in the Siberian tundra are subzero in temperature (-9 to -11°C) and have high salt concentrations (150-200 g l$^{-1}$). Data from these environments has revealed fungal colony forming units (CFUs) as high as $4 \times 10^2$ CFU ml$^{-1}$ (Ozerskaya et al 2004). Glacial ice is considered a large reservoir for microorganisms. Glacial ice is formed by the compaction of snow precipitation, and contains very low nutrient concentrations and subzero temperatures (Miteva and Brenchley 2005). Total cell counts for Greenland glaciers have been recorded as high as $>10^8$ microbial cells/cm$^3$ (Tung et al 2006) for ice near the base of the glacier, and cell counts of glacial ice from the East Antarctic ice sheet at Vostok Station range from $1.3 \times 10^2$ to $6.8 \times 10^3$ cells ml$^{-1}$ (Poglazova et al 2001, Abyzov et al 2004). Fluorescence microscopy analysis of the deepest portions of the Vostok glacial ice, ranging in depth from 3501 m to 3520 m (dated to 1 to 2 million years) have revealed cell numbers of less
than 10 cells ml$^{-1}$ and included the detection of potentially viable cells (D’Elia et al 2008).

Subglacial environments represent a permanently cold environment that differs from glacial ice by the presence of dynamic interactions with the sediment, the input of subglacial water and freeze-thaw cycles that may occur. Subglacial environments offer a good habitat for microorganisms because of the presence of liquid water, nutrients, electron acceptors, and insulation from ultraviolet irradiation and temperature fluctuations that are found at the glacial surface (Sharp et al 1999). Dissolved organic carbon (DOC) and nutrients such as nitrate and sulfate, are released during subglacial weathering of bedrock and sediment, and are considered to be more abundant in the subglacial environments compared to supraglacial, where atmospheric deposition is the main source (Bhatia et al 2006). The input of allochthonous carbon is important for subglacial habitats that may have relatively low levels of primary production (Bhatia et al 2006). Microorganisms that live in subglacial environments have to overcome the permanently dark and cold environments, along with the oligotrophic conditions (Skidmore et al 2005). Additionally, there may be seasonal variations in oxygen concentrations and freeze/thaw cycles (Skidmore et al 2005). Results from anaerobic incubation experiments utilizing basal ice samples revealed the ability of the microbial community to utilize alternative electron acceptors (nitrate and sulfate) despite the fact that the subglacial water is an aerobic environment (Skidmore et al 2005). This finding implies the presence of anaerobic microhabitats in subglacial ice. Furthermore, as oxygen levels decrease over the winter, due to microbial activity and reduced input of melt water, other bacteria become active, including nitrate and sulfate reducers (Tranter
et al 2005). These investigations into subglacial environments show the intricate interactions that occur in these relatively isolated environments, and reveal the importance of active microorganisms that are capable of adapting to these extreme conditions.

Abundant and diverse microbial communities have been isolated from subglacial environments around the world. Bacterial isolates identified from Lake Vostok accretion ice belong to a wide range of phylogenetic groups (Priscu et al 1999, Christner et al 2001, 2006, D’Elia et al 2008). Isolates related to the Gram positive genus *Carnobacteria*, and to the endospores forming bacterial group *Paenibacillus*, are specific isolates which have been independently isolated by different investigators (Christner et al 2001, D’Elia et al 2008). Heterotrophic microorganisms, capable of mineralizing radio-labeled glucose to $^{14}$CO$_2$, have also been detected in accretion ice (Christner et al 2006, Karl et al 1999). Iron and sulfur oxidizing bacteria have been identified in subglacial samples (Christner et al 2006, Skidmore et al 2005), indicating that diverse metabolisms may be present depending on nutrient and carbon availabilities. Phylogenetic analysis of subglacial ice has resulted in the identification of anaerobic bacteria, including iron and sulfate reducers (Christner et al 2006). The mycological composition of subglacial environments has also received an increasing amount of attention in recent years. Yeasts have been detected in New Zealand subglacial ice at concentrations of up to 4,000 CFU ml$^{-1}$ (Butinar et al 2007). A unique group of *Penicillium* strains were also detected in the same ice that were not detectable in the surrounding environment (Sonjak et al 2007). The finding of both high concentrations and diverse groups of fungi in subglacial ice adds to the complexity of these extreme environments. The microbial diversity detected in subglacial ice is a
result of adaptation to gradients in nutrient, carbon and oxygen availability in these environments, along with the ability to withstand permanently cold temperatures, high pressures and total darkness.

There are several differences between subglacial environments compared to surface ice that have strong influences on the microbial populations. Glacial ice is formed by the compression and recrystallization of snow, whereas basal ice forms by the freezing of subglacial water which entraps sediments and debris (Foght et al 2004). Microorganisms are either endemic to these environments, or may be enter via several processes. Microorganisms enter subglacial environments through glacial meltwater, vertical transport via glacial movement and incorporation through basal sediments and soil communities (Bhatia et al 2006). The compositions of the communities that become established in subglacial environments are influenced by a variety of factors. Physical conditions that are common to all subglacial environments, such as temperature and darkness likely influence microbial communities that are more likely to be found in a wide range of cold and polar environments (Skidmore et al 2005). The majority of microbial isolates from cold environments are actually psychrotolerant, capable of growth at a wide range of temperatures. The availability and presence of nutrients, carbon and oxygen are considered factors more likely related to the establishment of endemic subglacial communities (Skidmore et al 2005).

**Survival mechanisms**

Microorganisms have developed many adaptations to overcome environmental stressors that are found in the various types of cold habitats. Survival and metabolic
activity at low temperatures requires the capacity to overcome thermodynamic constraints associated with cold environments. At low temperatures, microorganisms must adjust to lowered rates of enzyme catalysis, reduced membrane fluidity and potential damage caused by ice crystal formation (Cavicchioli et al. 2000). Preservation of membrane stability is of particular importance at low temperatures in order to maintain active and passive transport, nutrient uptake, electron transport and for responding to the environment (Georlette et al. 2004). The effects of cold temperatures on membrane fluidity and transport are also considered to be responsible for differential carbon source utilization and antibiotic sensitivity at low temperatures (Ponder et al. 2005). The preservation of transport systems that can function optimally at low temperatures is critical for counteracting the reduced rates of diffusion and passive transport across the membrane at these temperatures (Bakermans et al. 2007). Membrane fluidity is maintained at low temperatures by the incorporation of polyunsaturated lipids, which allow for the continued transport of substrates and nutrients under conditions that would otherwise be too rigid (Deming 2002, Methe et al. 2005). Membranes are further stabilized by the incorporation of cryoprotectants and antifreeze proteins (Wilson et al. 2006). Cryoprotectants provide protection from damage caused by ice crystals by lowering the freezing point of water in contact with the outer surface of the cell (Mindock et al. 2001). One example of a cryoprotectant is extracellular polysaccharide (EPS), which has been well described in the marine psychrophile Colwellia psychrerythraea and is responsible for keeping liquid pockets around the cell to temperatures of -20°C (Junge et al. 2006, Huston et al. 2004). EPS has also been linked to many other cold adapted
responses including the enhanced activity of an extracellular aminopeptidase at low temperature (Huston et al 2004) and aiding in attachment to surfaces (Junge et al 2004).

**Enzymatic and proteomic response**

Cold temperatures require a specific response from microorganisms in terms of protein expression and enzymatic activity. In general, cold adaptive enzymes are characterized by reduced activation energies and high catalytic efficiency. These enzymatic adaptations are related to the use of specific amino acids in the active site, or in other distant regions, that effect the overall conformation and flexibility of the protein at lower temperatures (Deming 2002). The result of maintaining enzyme flexibility, or molecular plasticity, at low temperatures is the ability to conserve the proteins overall architecture and improvement of the catalytic efficiency (Methe et al 2005). There are a large number of proteins that are uniquely expressed at low temperatures, compared to mesophilic conditions. In fact, the proteome of the psychrophile *Psychrobacter cryohalolentis* K5 was determined to be substantially reprogrammed in response to temperature decrease, having 303 of 619 proteins examined showing temperature variation (Bakermans et al 2007). A subset of these proteins, 27 in total, belonged to a class of proteins called cold inducible proteins (CIPs). CIPs are proteins that are preferentially expressed at low temperatures and are essential for survival and activity at low temperatures (Bakermans et al 2007). CIPs are further classified in regards to the timing of their expression after exposure to cold temperatures. Cold shock proteins (CSP) are induced in psychrophiles as the temperature decreases and show peak expression shortly after exposure to reduced temperatures (Deming 2002, Bakermans et
al 2007). CSPs are responsible for aiding the microorganism in overcoming the initial effects of low temperatures and are an essential adaptation to cold shock. One of the most crucial stresses upon cold shock is the maintenance of transcription and translation. This is due to the fact that the processes of replication, transcription and translation are inhibited at low temperatures, which stabilize nucleic acid secondary structures (Cavicchioli et al 2000). Therefore, CSP function to maintain cellular protein synthesis and act as chaperones that prevent the formation of secondary structures in mRNA that would inhibit translation (Georlette et al 2004). CspA, a well characterized CSP, has two cold shock domains which bind RNA and destabilize secondary structures (Jiang et al 1997). Cold shock expression of CspA is evidenced by the fact that CspA accounts for 10% of total cellular protein expression following a temperature drop from 37 to 10°C. CspA mRNA half-life can be as long as 20 minutes at 15°C, compared to <12 seconds at 37°C (Goldenberg et al 1996). Thus, control of CspA is at the level of transcription and is responsible for the temperature dependent expression by the increased stability of CspA mRNA at low temperatures.

Apart from CSPs, other classes of proteins become more important after the initial shock of temperature downshift has occurred. Cold acclimation proteins (CAPs) are a group of proteins that are found in higher levels after growth has resumed following the transition to low temperatures (Bakermans et al 2007). One class of proteins that are important for continued survival at subzero temperatures are antifreeze proteins (AFPs). AFPs are either membrane bound or secreted and lower the freezing point and prevent ice crystal formation (Wilson et al 2006). Another strategy for preventing cellular damage by ice crystal formation is the use of ice nucleating proteins (INPs). INPs are outer
membrane protein aggregates that promote the formation of ice crystals extracellularly by providing a template for ice nucleation (Wilson et al 2006). While INPs have been classified as outer membrane proteins in Gram negative bacteria, the expression of an INP in the Gram positive bacteria *Exiguobacterium* suggests that a soluble form of the protein may also exist (Ponder et al 2005). A novel protein has also been discovered in *Pseudomonas putida* which combines an AFP domain and an INP domain, and may thereby prevent damage by initiating extracellular ice crystal formation (INP domain) and then maintaining the crystals at non-damaging size (AFP domain)(Xu et al 1998).

**Adaptation by fungi**

Fungi have been isolated from cold environments and have also been examined to determine unique mechanisms that are used to withstand cold temperatures and low nutrient availability. Viable fungi and yeasts have been recovered from subglacial Arctic ice (Butinar et al 2007, Sonjak et al 2007), and after 140,000 years in Greenland glacial ice (Ma et al 2000, 2005, Starmer et al 2005). Mycelial fungi have also been isolated from Siberian cryopegs, despite the subzero temperatures and high salinities (Ozerskaya et al 2004). Antarctic environments, including glacial ice (Abyzov et al 2004, Poglavova et al 2001), soil (Arenz et al 2006), airborne spore traps (Marshall 1997) and mosses (Tosi et al 2002) have all resulted in the isolation and identification of fungi. Several general physiological adaptations are used by fungi to adapt and survive in extreme cold and frozen environments. Membrane fluidity is maintained at low temperatures 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. These molecules are storage compounds found in the cells of vegetative fungal cells and spores, and are considered general stress protectants in the cytosol. They have also been related to membrane stabilization in response to dehydration, and accumulate in fungal hyphae in response to low temperatures (Robinson 2001). A link between dehydration and cold response in fungi is not surprising. The presence of solute rich liquid veins is a well documented result of ice formation (Junge et al 2004, Price 2000). Fungi isolated from Arctic subglacial ice have displayed characteristics of both halotolerance and psychrotolerance. The ability to tolerate the dual stressors of cold temperature and increased solute concentrations is expected to aid in the ability of fungi to adapt and survive in the extreme environments of glacial ice (Butinar et al 2007 Gunde-Cimerman et al 2003). Fungi may also utilize cold temperature adaptation mechanisms similar to ones identified in bacteria, including the secretion of antifreeze proteins (Snider et al 2000), and the use of cold adaptive enzymes (Robinson 2001). As more environmental ice habitats are examined in terms of their mycological composition, it is likely more fungi will be identified that are well adapted to cold and frozen environments.

**Ecology and evolution**

Many different cold environments have been extensively studied in terms of microbial community abundance and diversity, along with a general understanding of some critical cold temperature stress adaptations. However, their remains a need to better characterize the driving pressures that influence the ecology and evolution of the microorganisms in the cold biosphere. Physically isolated environments of the polar
regions, including the vast East Antarctic Ice Sheet, provide conditions that are ideal for investigating the effects of separation from outside gene flow on microbial speciation and evolution. The polar front in Antarctica formed over 10 million years ago, providing relative isolation and the potential for collecting cultures that have been separated from the global gene pool for periods of evolutionary significance (Vincent 2000). Further supporting the effects of isolation on Antarctic prokaryotes, it has been reported that many genera of bacteria observed from Antarctica have diverged approximately 75 million years ago, which is over 60 million years before stable ice sheet formation occurred (Franzman 1996). Microbes that have become entrapped in the ice formed since the establishment of the polar front may become dormant and rely on survival energy, which is just sufficient for repairing macromolecular damage caused by amino acid racemization, DNA depurination and natural radiation in the environment (Price and Sowers 2005, Christner 2002). In such instances of dormancy and isolation, it is expected that the entrapped microbes should exhibit nucleotide change rates close to zero (Rogers et al 2004). Therefore, unique microorganisms should be present in ancient ice that have not been subjected to evolutionary processes related to active growth and the influences of gene flow compared to active members of the contemporary global microbial population.

While isolation is one important aspect of life in environmental ice, the polar regions still acquire microorganisms by a variety of mechanisms which are subject to selective pressures upon arrival in order to establish communities in the extreme conditions. Microorganisms continually invade Antarctica through atmospheric circulation, ocean currents, birds, fish, marine mammals and humans (Vincent 2000).
These mechanisms of invasion may facilitate transport of microorganisms over large geographical distances, and contributes to the input of cosmopolitan species to the polar regions. The continued inflow of contemporary microorganisms makes the determination of endemic species difficult. There are a variety of habitats that vary drastically in seasonal temperatures and nutrient availability which are present for exploitation by a wide range of ubiquitous microbes that may find their way to Antarctica. There are many selective pressures that effect whether microbes that are transported to Antarctica are able to become established in the microbial communities. Cold temperatures, which may seem to be the most obvious pressure, may in fact be less influential on microbial community dynamics compared to the effect of seasonal extremes that occur in many of the environments. Therefore, generalists may be selected that can tolerate a broader range of niches with intervals of optimal and suboptimal growth (Vincent 2000). The similarity of bacterial phylotypes from both arctic and Antarctic pack ice (Brinkmeyer et al 2003) and the finding of no correlation between bacterial taxa from glaciers of different geographical locations (Foght et al 2004) supports that temperature alone is not the main influence on microbial assemblages.

**Preservation periods in ice**

The capacity of environmental ice to act as a protective matrix for extended periods of time has been well studied. Analysis of microorganisms from glacial ice allows for a unique opportunity to investigate evolutionary processes and ancient biodiversity (Ma et al 2000). In many cases, microorganisms are preserved well enough for identification or even for revival by culturing. In fact, the microbes found in glacial
ice have been called “frozen fossils” due to the amount of information that can be obtained from their analysis (Ma et al 2000). The presence of constant low temperatures makes glacial ice ideal for long term preservation of microorganisms and biomolecules (Willerslev et al 2004). Reaction rates, including damaging degradation, are reduced an order of magnitude for every 10°C drop in temperature, adding to the ability of ice to protect microorganisms for extended periods of time. Long term survival requires the ability to withstand natural genetic mutations which may occur if the cells are actively replicating, and spontaneous mutations that naturally occur over time. Degradation of DNA and RNA in inactive or dormant cells may occur by endogenous and exogenous nucleases or by spontaneous hydrolysis or oxidation (Willerslev et al 2004). These processes are dependent on water availability, oxygen concentrations and pH, which may vary considerably depending on the environment. Dormant cells, such as bacterial endospores, may also utilize small acid soluble proteins (SASP) which bind to DNA and reduce the rate of DNA damage (Willerslev et al 2004). Cells which maintain some level of activity may utilize their DNA repair mechanisms to overcome mutational damages and survive beyond the predicted time scales. Viable microorganisms have been revived from environmental ice ranging from several hundred to >100,000 years old (Christner et al 2003, 2006, Ma et al 2000, D’Elia et al 2008). It has been estimated that a bacterial genome of $3 \times 10^6$ bp could be resist fragmentation for a period of 81,000 yrs at -10°C, and a period of 1.7 million years at -20°C (Willerslev et al 2004). Continued exploration of the microbial composition of glacial ice will allow for a better understanding of ancient biodiversity, the effect of climate on microbial ecology and distribution and evolutionary processes related to microorganisms (Ma et al 2000).
Glacial melting is a natural part of the water cycle. Since the last glacial maxima 25,000 years ago, glaciers have continued to recede, and melting has only increased due to complications related to global climate change. The melting of glaciers provides the opportunity for the release of viable microorganisms which have been isolated from contemporary populations for over hundreds of thousands of years. The possibility for temporal gene flow between ancient microbes released from glacial melting and extant populations has been termed genome recycling (Fig. 1, Rogers et al 2004). It has been estimated that melting of environmental ice releases $10^{17}$ to $10^{21}$ viable microbes annually (Smith et al 2004). The high numbers increase the chances that potential pathogens may be included in the population of microorganisms released from melting ice. The liberation of ancient pathogens is a particular concern due to the possibility of increased virulence which may occur as a result of extended periods of host and pathogen separation (Ma et al 2000). The use of environmental ice as a reservoir may be related to the reappearance of specific strains of pathogens, including influenza A and marine calicivirus, decades apart (Rogers et al 2004, Smith et al 2004). The proven ability of microorganisms to survive for thousands to millions of years, and remain viable after melting indicates that the further studying and monitoring of environmental ice should be a primary concern in regards to world health and safety.

**Isolation methods**

It is well understood that the majority of the microorganisms found in their natural environments cannot be cultured. However, in order to better understand the physiology and adaptation mechanisms to unique environments, isolates of
Fig. 1. Genome recycling via formation and melting of glacial ice. Microorganisms, which are transported by atmospheric circulation, are deposited onto glaciers by wind and precipitation. As the precipitation accumulates and forms glacial ice, the microbes become entrapped. Glacial ice can harbor viable microorganisms for periods of thousands to millions of years. Melting of glacial ice releases water and entrapped microorganisms back into the hydrosphere. The process of glacial melting allows for the microbes previously entrapped in the ice to mix with contemporary populations in the genome mixing zone. The interaction between extant and entrapped microbes offers the possibility for temporal gene flow. Genome recycling figure reproduced from Rogers et al 2004.
microorganisms need to be available. The development of cultivation methods is of particular importance when looking at extreme environments which may have many variables that would need to be duplicated for successful recovery of the isolates. Several techniques have been developed in regards to culturing microorganisms from environmental ice. One common technique attempts to culture bacteria that may be inactive, dormant or damaged from cold environments. In this method, meltwater aliquots from glacial ice are filtered through 0.2 µm filter. The filter is then placed on a wide range of media, including nutrient poor media (R2A or minimal media MMA) and incubated for several months in the dark at several temperatures (Christner et al 2000, 2003). The most common temperature range is from near 0°C to above 30°C. Growth at both ends of this range would indicate psychrotolerance, which is more common than psychrophily in glacial ice samples. The long term incubations allow for sufficient time for the cells to synthesize enzymes which may be needed to repair any damage which may have accrued over the extended time in the ice (Christner 2000). Both aerobic and anaerobic incubations have been used (Christner et al 2003, Miteva and Brenchley 2005). Anaerobic incubations prevent oxidative stress which may inhibit the resuscitation of dormant or damaged cells (Miteva and Brenchley 2005). Subglacial environments are very complex, and gradients in nutrient availability and oxygen concentrations are factors that influence the microbial communities in these ecosystems. Therefore, successful cultivation methods should attempt to reproduce the natural environment as closely as possible. Considering the variations in nutrients and oxygen concentrations, specific classes of bacteria can be targeted for culturing utilizing selective media. A recent investigation of the bacterial composition of a subglacial environment successfully
isolated both nitrate and ferric iron reducers using selective media for each class (Fought et al 2004). Also isolated from the same ice samples were aerobic heterotrophic bacteria. The use of both heterotrophic and selective media gave combined data which supports that the subglacial system may be comprised of microanaerobic regions where bacterial residences utilize alternate electron acceptors in the absence of oxygen.

In addition to the general and selective culturing methods which have been used to isolate general classes of bacteria from many environments, other novel strategies have been developed to isolate bacteria from environmental ice. Ultramicrobacteria are bacteria which are less than 0.1 µm³ in cellular volume, and have been found in high numbers in soil samples. The small size is considered a possible mechanism to increase nutrient uptake in oligotrophic conditions due to the increased surface to volume ratio, or may help in avoiding predation and in inhabiting unique microhabitats (Miteva and Brenchley 2005). The ultramicrobacteria have been previously found to be difficult to culture. A method was developed to isolate these bacteria from the silty ice (deep ice in contact with sediment) from a Greenland glacial ice core. The method utilized a series of filtration steps which removed larger, fast growing bacteria, other inhibitory substances and aided in the detachment of cells from particulate matter. The meltwater that was passed through the 0.2 µm² filter would contain more of the ultramicrobacteria, and most larger cells would be removed. The filtrate was used to inoculate limited nutrient liquid media (R2 broth and MM1) and the cultures were anaerobically incubated at -2°C or 5°C for several months. The anaerobic conditions and minimal nutrient media were used to prevent oxidative damage, and the low temperature was chosen to mimic glacial ice conditions. The cultures were allowed several months for incubation to allow for the
cells to recover from damage or to resuscitate from dormant states. This isolation technique allowed for the determination that the abundant microbial population in the deep Greenland ice core is dominated by ultrasmall cells (Miteva and Brenchley 2005). The development of this unique isolation method resulted in the discovery of a whole class of bacteria that may have gone undetected by traditional methods.

A unique isolation strategy was recently used to recover a total of 148 bacteria from the permanently ice covered lakes in the McMurdo Dry Valleys, Antarctica (Stingl et al 2008). A dilution-to-extinction method was developed which utilized sterilized and nutrient amended lake water as the media. Water samples from the lake were diluted to a concentration of 3 to 10 cells ml\(^{-1}\). The dilutions were then added to 2 ml of the appropriate lake water media in a microtiter plate. The plates were incubated for 24 weeks at 4°C to allow growth of psychrotolerant microorganisms. DAPI stained aliquots from each well were analyzed by direct counts to determine if growth occurred during the incubation period, and were considered positive if more than 1\times10^5 cells ml\(^{-1}\) were observed. The bacteria in the positive samples were identified by extracting the DNA and PCR amplifying and sequencing 16S rDNA. The use of the sterilized and modified lake water was considered a successful alternative to artificial media due to the inability of commercial media to replicate the relevant environmental conditions. The culture conditions adequately reproduced the oligotrophic and low temperature environment in the lake, aiding in the recovery of many isolates. Overall, the dilution-to-extinction method was very successful at culturing a large number of bacterial isolates which have previously been uncultivable. The cultured isolates also showed a high corroboration with culture-independent studies in regards to the phylotypes identified.
The mycological component of environmental ice communities is often overlooked, even though there is increasingly more evidence that fungi comprise an important part of these environments. Several techniques have been developed to culture fungi from ice and other permanently cold environments. A general method that is commonly used is to utilize a wide range of nutrient rich media and incubation at several temperatures. The most common media that have shown positive results in culturing fungi are the nutrient rich media malt extract agar (MEA) and potato dextrose agar (PDA) (Ma et al 2000, Bergero et al 1999, Tosi et al 2002, Ozerskaya et al 2004, Butinar et al 2007). The particular conditions that are unique to each cold environment need to be adequately addressed to recover fungal isolates by culturing. Fungi that may be present in arctic soils are expected to be able to tolerate the oligotrophic and low temperature conditions. Samples from artic soils have been diluted and plated on carbon rich and poor media to ensure recovery of a wide spectrum of soil fungi. These samples, which were incubated at 5°C and 20°C, resulted in the recovery of 40 taxa of fungi representing 21 genera (Bergero et al 1999). The isolates were then subcultured and tested for growth at 0°C and 25°C to test for psychrotolerance. The isolates were also plated on silica gel supplemented with nitrogen to test for tolerance to oligotrophic conditions. For both the temperature and oligothophy tests, colony growth was determined by measuring colony diameter over time. Another characteristic of permanently cold and frozen environments is the decrease in biologically available water as ice crystals form. As a consequence of ice formation, the solute concentration in the available liquid water increases. Therefore, it is common to find fungi in these environments that show tolerance to both the low temperature (psychrotolerance) and to high solute concentrations (halotolerance). Fungi
have been recovered from arctic sea ice, glacial ice and Cryopegs by using culturing parameters consistent with low temperatures and low water activity (Gunde-Cimerman et al 2003, Ozerskaya et al 2004, Butinar et al 2007). A general fungal media is used for culturing and is supplemented with additional sugar (glucose) or salt (NaCl). The plates are also incubated at a wide temperature range. By utilizing a wide range of salt, sugar and temperatures in the culturing procedure, a wider range of fungi may be recovered that include both psychrotolerant and halotolerant species (Gunde-Cimerman et al 2003, Ozerskaya et al 2004).

Microscopic enumeration of microbes in environmental ice

The use of direct microscopic examination is vital to determining a thorough understanding of the microbiology of a given microhabitat. The majority of microorganisms in a given environment are not cultivable by standard methods, and may go undetected by plate count methods. Total cell counts are up to two orders of magnitude higher when obtained by epifluorescence microscopy compared with culture techniques (Atlas and Bartha 1998). Microscopic cell counts also help to determine proportions of live cells that may later be detected by molecular methods, such as PCR and DNA sequencing. Additionally, the use of microscopic investigation allows for visualization of cells in their natural microhabitat. This becomes particularly important when looking for bacterial attachment to particles in environmental ice, which has been related to survival as temperatures decrease (Junge et al 2004). Microscopy, and the ability to distinguish total cell numbers from viable/nonviable, is a valuable tool for analysis of complex microbial communities.
Several methods have been developed which utilize fluorescence microscopy to determine total and viable cell counts. The use of the fluorescent stain 4’,6’-diamidino-2-phenylindole (DAPI) has been routinely used to determine total cell counts in a wide range of environmental samples. DAPI is a nucleic acid specific stain, and fluoresces when bound to DNA in a cell, regardless of viability. DAPI has also been used to study environmental ice samples from a variety of locations. Total microbial counts were determined using DAPI stained samples from subglacial sediments obtained from the New Zealand Alps (Fought et al 2004). Deep glacial ice from Greenland has also been analyzed and total cell counts determined. Approximately $10^8$ microbial cells per cm$^3$ were found to be attached to clay particles of the deep “silty” ice of the Greenland GISP2D ice core. The ability to observe microbial cells attached to sediments allowed for the conclusion that attachment to these particles is essential for survival, and that iron reducing bacteria in this extreme environment are responsible for the excess in carbon dioxide (Tung et al 2006).

Arctic and Antarctic sea pack ice has also been studied using DAPI stained ice samples (Brinkmeyer et al 2003, Junge et al 2004). These studies have combined the ability of DAPI to determine total cell counts with the fluorescence in situ hybridization (FISH) assays which can determine viability. FISH utilizes rRNA oligonucleotide probes that can be designed to be very general (i.e. *Eubacteria, Archea*) or more phylogenetically informative. The probes are considered a sensitive method of determining viability due to the relation of the intensity of the signal and the amount of rRNA present in the cell. The more rRNA, the more protein synthesis is occurring and therefore the cells are considered very active. The analysis of the sea ice using DAPI and
FISH allowed for the conclusion that large percentages, up to 95%, were considered active members of *Bacteria* (Brinkmeyer et al 2003). FISH and DAPI were also used to study bacterial activity in intact ice core section by developing a novel ice microscopy method that was nondestructive to the ice (Fig. 2, Junge et al 2004). This investigation allowed for analysis of microorganisms within liquid veins of sea ice. The FISH-DAPI analysis of artic sea ice found that 18-86% of the rRNA probe-detected cells were active, and that the proportion of active cells that were attached to inclusions in the liquid veins increased with the colder ice examined, down to -20°C (Junge et al 2004). The ability to observe microorganisms in their undisrupted habitat has the potential to provide valuable information that could explain how they adapt to extreme environments.

Lake Vostok accretion ice has been analyzed in terms of cell concentrations and diversity of microbial morphology by utilizing several epifluorescence microscopic techniques. A large set of both deep glacial ice and accretion ice has been examined using the fluorescent dye fluorescamine (Fig. 3, Poglazova et al 2001, Mitskevich et al 2001). Fluorescamine has the ability to interact with the amino group of proteins and other biomolecules. Viability is not directly determined, however, cells that are very active have high levels of protein synthesis compared to nonviable cells. Also, cells which may have lysed and released the majority of their intracellular constituents are observed as cellular contours (cell membrane proteins) by fluorescamine staining (Abyzov et al 2004). This method has been successfully used to determine the microbial composition of glacial and accretion ice, based on cellular morphology from the Vostok core. The stain is not limited by the community composition, as both prokaryotes and eukaryotes are susceptible to staining. The ice core sections analyzed revealed a wide
Fig. 2. Microscopic analysis of bacteria in sea ice liquid brine pockets. Sea ice from the Chukchi Sea, Alaska, was prepared for microscopy in a non-destructive matter at -5°C (without melting the ice). Top panel shows the liquid brine pocket (red box) that has formed between ice crystals. Bottom panel shows the enlarged brine pocket under transmission light (left) and DAPI stained epifluorescence (right). The DAPI stained bacteria (blue) is clearly visible within the brine pocket microhabitat. Adapted from Junge et al 2004 and Deming 2002.
Fig. 3. Epifluorescence microscopy of Vostok glacial and accretion ice. Meltwater from glacial and accretion ice was stained with fluorescamine and visualized by epifluorescence microscopy. A) Analysis of meltwater from an accretion ice core section from 3565 m. Bacteria resemble members of the genus *Caulobacter* (*bar* = 5µm). B) Filamentous structures found only in glacial ice (*bar* = 5µm). C) Eukaryotic unicellular organisms detected in glacial and accretion ice. Representative micrographs of diatoms (top) and pollen (bottom) from glacial and accretion ice. (*bar* = 5µm). Figures and descriptions adapted from Poglazova et al 2001.
range of morphologically diverse microbes, including bacteria, fungi, microalgae and pollen of higher plants (Fig. 3, Poglazova et al 2001). Also, by utilizing microscopic analysis, a possible correlation of between particle attachment and the number and diversity of microbes was suggested (Poglazova et al 2001).

LIVE/DEAD® BacLight™, manufactured by Molecular Probes, is a two color fluorescence assay of bacterial viability (Molecular Probes 2004). The kit consists of two fluorescence-based dye components, propidium iodide (PI) and SYTO9. PI is a red intercalating stain that is membrane impermeant, and is therefore excluded from healthy cells with intact membranes (Stocks 2004). SYTO9 is a green-fluorescent nucleic stain that labels all cells in a population that contain DNA (Mol Probes). The live/dead viability assay is based on the fact that PI has a stronger affinity to nucleic acids than SYTO9. Therefore, when both stains are present, SYTO9 will be displaced from the DNA and cells will fluoresce red and be labeled nonviable (Stocks 2004). Viable cells, which should have a healthy intact membrane, are only penetrable by SYTO9, and will fluoresce green, an indication of viability (Mol Probes). The use of BacLight offers several advantages. Depending on the nature of the sample, there is little to no incubation period required and results can be obtained in less than one hour (Stocks 2004). Also, the two dye component can adequately determine viability of different regions within multicellular fungal spores (Chen and Seguin-Swartz 2002) and has the ability to spatially resolve viability heterogeneities within mycelia and hyphae (Stocks 2004). An additional benefit of the BacLight system is that there is virtually no background fluorescence, allowing for easy identification of cells (Mol Probes). Potential drawbacks include the possibility of simultaneously staining cells with both PI
and SYTO9, making the results difficult to interpret (Stocks 2004). This could be of a particular concern when observing microbial samples from extreme environments that may be damaged, but still viable. The dyes have also been determined to be lethal to spores of the fungi *Sclerotinia sclerotiorum* after incubation periods of 5 to 8 minutes, but had no negative effect on spores of other genera (Chen and Seguin-Swartz 2002). It is therefore very important that the *BacLight* viability assay be thoroughly investigated and validated for each specific use (Stocks 2004).

The *BacLight* viability assay has been proven successful at determining viability on a wide range of microbial samples. Bacteria from drinking water (Boulos et al. 1999) to deep-sea sediments (Queric et al. 2004) have been analyzed with *BacLight*. Viable bacterial endospores that could not be recovered by plate count methods have also been correctly identified with *BacLight* (Laflamme et al. 2004). Although listed as a bacterial viability assay, the fluorescence nucleic acid dyes used in the kit have also been used to quantify yeast viability (Zhang and Fang 2004). The viability of fungal spores from several genera, including thick-walled spores of *Alternaria brassicae*, has also been assayed using the *BacLight* kit (Chen and Seguin-Swartz 2002). The most recent microscopic analyses of Lake Vostok accretion ice have utilized the fluorescence viability stain kit (Christner et al. 2006, D’Elia et al. 2008). By effectively distinguishing viable and non-viable cells, the use of the *BacLight* system allowed for a more complete analysis of microorganisms in the Vostok accretion ice than previous microscopic analyses. The presence of diverse microorganisms, including the first reports of viable fungi, were also confirmed by the *BacLight* assay of accretion ice (Fig. 4, D’Elia et al. 2008).
Fig. 4. Fluorescence microscopy of Vostok ice using BacLight viability stains. The two component fluorescence-based dye kit, BacLight (Molecular Probes, Eugene, OR), contains propidium iodide (PI) and SYTO9. PI is a red intercalating stain that is membrane impermeant, and stains nonviable cells red (Stocks 2004). Viable cells, which have a healthy intact membrane, are only penetrable by SYTO9, and fluoresce green (Mol Probes). Vostok glacial and accretion ice meltwater was stained with BacLight and
visualized by fluorescence microscopy to determine potential viability. Viable (top, green) and nonviable (bottom, red) coccoid (A) and rod shaped (B) bacteria. C) Representative filamentous structures consistent with fungal hyphae from accretion ice. Both viable and nonviable bacteria and filamentous structures were observed based on the microscopic analysis presented in this report (Chapters II and III).
**Decontamination and aseptic handling of ice cores**

One of the most challenging and important obstacles to overcome when studying environmental ice is establishing effective methods which eliminate contaminants already present on environmental ice and prevent further contamination (Ma et al 2000, Willerslev et al 2004, Bulat et al 2004, Lavire et al 2006, Hebsgaard et al 2005). The analysis of ancient ice is also made more difficult by the need to authenticate results and show reproducibility to confirm that the findings are not a result of contamination. The lack of both standardized decontamination protocols and reproducibility suggest that the wide reports of conflicting results in the literature are more likely due to contamination than to differences in methodological efficiencies (Willerslev et al 2004). In general, decontamination is important due to the ubiquitous nature of microorganisms and microbial nucleic acids in all environments and laboratory settings (Willerslev et al 2004). Finding rare or unique isolates is one objective of microbial analysis of environmental ice (Ma et al 2000). However, identification of rare microbes alone is not sufficient to rule out the possibility of contamination, based on the estimate that less than 5% of the microbial diversity is currently known (Willerslev et al 2004). Confirmation of results is made even more difficult by the fact that there is currently no way to clearly distinguish an endogenous DNA sequence or culture from that of a contaminant (Hebsgaard et al 2005). The current methods used for isolation and identification of microorganisms from environmental ice are also very susceptible to contamination. Both culture dependent and independent identification techniques may produce false positives if proper controls and methods are not closely followed. General non-selective growth media has been routinely used for culturing, and culture independent methods rely on
extremely sensitive PCR techniques which may amplify very trace amounts of contaminant DNA (Willerslev et al 2004). Carryover of PCR contamination is a particularly vulnerable step where contamination could be introduced. Invisible aerosol droplets that are produced by opening PCR tubes may contain $10^5$ to $10^9$ molecules (Hebsgaard et al 2005). If careful attention is not made towards PCR carryover and contamination, false positives may be produce (Willerslev et al 2004). The issue of decontamination is a very real concern when validating results. To date, there has been very little standardization of methods, which makes comparing results in order to authenticate findings nearly impossible.

Several decontamination procedures have been developed utilizing a variety of methods. Based on a comprehensive analysis of multiple decontamination methods, Rogers et al. (2004) determined that submersion in 5.25% sodium hypochlorite, followed by rinsing with sterilized water and melting of subsequent interior shells, offered the best results for decontaminating ice cores (Fig. 5). Other methods tested in this report that were less effective included several techniques which have been routinely used in decontaminating environmental ice samples. These included submersion in 95% ethanol, heated probes, drilling interior samples, ablation of outer surfaces and UV irradiation. Ethanol at a concentration of 95% is considered a bacteriostatic agent, and does not destroy DNA or RNA which may be detected by PCR (Willerslev et al 2004). While ethanol alone may not be sufficient, the combination of physically removing the outer 5 mm of ice, followed by submersion in 95% ethanol, and subsequent rinsing with water followed by two melting periods has been reported to adequately account for possible contaminants (Christner et al 2006). The method also was capable of removing
Fig. 5. Comparison of ice core decontamination methods. Sham ice cores were created and spiked with specific concentrations of microorganisms (*Ulocladium atrum* and *Bacillus subtilis* on the surface, *Aureobasidium pullulans* and *Escherichia coli* on the interior). The cores were surface decontaminated with each respective treatment, and then melted in five shells consisting of successively interior aliquots of melted ice water. The graphs indicate number of colonies observed from each melt water shell for outer organisms (upper graphs; *U. atrum* [white bars] and *B. subtilis* [black bars]) and inner organisms (lower graphs; *A. pullulans* [white bars] and *E. coli* [black bars]) after each treatment. Error bars indicate standard deviation based on triplicate experiments.

Treatment with 5.25% sodium hypochlorite was the only method which was effective at
eliminating contamination by surface microbes, while not significantly reducing the number of interior microbes. Each of the other methods was incapable of complete surface decontamination, evident by the recovery of colonies in shells 1-3. Bar graphs and decontamination analysis from Rogers et al 2004.
contaminants that were added to the ice, which included *Saretia marcescens*, plasmid DNA and the fluorescence stain rhodamine 6G (Christner et al 2006). The inability of ethanol to kill microorganisms and degrade DNA leaves the question of adequate decontamination, specifically when isolates are identified by molecular (PCR) methods that can detect trace amounts of DNA that may not have been physically removed by scraping, ethanol submersion, water rinsing or melting. Additionally, ethanol treatment that does not physically degrade cells or DNA may result in higher total cell counts determined by microscopic analysis. UV-irradiation is effective at killing many microbes and damaging DNA or RNA. However, UV dosages 12 times higher than those required to kill 100% of *Penicillium commune* spores were not capable in causing any reduction in the number of *Ulocladium atrum* germinating spores (Rogers et al 2004). Many environmental ice studies do not attempt to isolate fungi. However, many investigations have found heterotrophic metabolic activity in ancient ice (Christner et al 2006, Karl et al 1999). In order for future investigations to confidently report that the observed microbial metabolic activity was by indigenous microbes and not contaminants, thorough decontamination needs to be performed that includes treatments robust enough to eliminate fungal and bacterial spores.

The processes of decontamination and sample analysis must be performed with the highest regard to aseptic and sterile techniques. Several general requirements should be meet in order to be able to confidently report findings as authentic. All lab equipment and reagents should be assumed to be contaminated (Willerslev et al 2004). Tubes and containers used to hold samples, and all tools and work surfaces should be treated with 5% sodium hypochlorite and UV-irradiation (Willerslev et al 2004, Christner et al 2000,
Ma et al 2000). All reagents used should be passed through ultrafiltration for sterilization before use (Willerslev et al 2004, Christner et al 2006). Autoclaving of equipment alone is not sufficient. Short pieces of DNA <100bp may remain and be amplified by PCR (Willerslev et al 2004). When autoclaving is used, equipment and tools should be autoclaved twice, and treated with other sterilants (Christner et al 2000, Bhatia et al 2006). The efficiency of the decontamination should be empirically tested utilizing sham ice cores (Rogers et al 2004), or by simultaneous treatment of mock ice cores as negative controls in the melting process (Christner et al 2006). Additionally, surfaces of ice cores may be spiked with known contaminants to gauge successful surface decontamination (Willerslev et al 2004, Christner et al 2006, Rogers et al. 2004). Manipulations of all samples should be kept to a minimum to prevent contamination (Willerslev et al 2004, Christner et al 2006, Rogers et al 2005). Experimental analysis of ice samples should be performed in a sterilized (UV-irradiation, 5.25% sodium hypochlorite and 95% ethanol) laminar flow hood. Personnel should wear sterilized gloves and lab coats. Further confidence in the results obtained is possible by utilizing a series of controls for every processing step to identify contaminants. These include controls which monitor air flow within the hood (air exposed agar plates), filter controls if samples are filtered for concentration or microscopy, culture controls containing only media, and DNA extraction and PCR controls which contain only reagents and no ice core sample (Willerslev et al 2004). Unfortunately, due to the sporadic nature of contamination, negative controls, or blanks may not detect contamination. To help increase the chances of identifying all contaminants, many negative controls should be used (Hebsgaard et al 2005) along with identifying common contaminants in the working area (Bulat et al 2004). The findings of
any report are strengthened significantly when the results are capable of being independently corroborated in two or more different laboratories (Willerslev et al 2004, Lavire et al 2006, Rogers et al 2005). Future analysis of ancient ice should include plans for independent analysis by separate investigators.

**Lake Vostok accretion ice: Current state of microbiological understanding**

Initial estimates of microbial cell concentrations in Lake Vostok accretion ice samples have varied from nearly zero to nearly $10^6$ cells ml$^{-1}$ (Table 1, Bulat et al 2004, Priscu et al 1999). Another early study of an ice core section from 3603 m indicated 2-3 x $10^2$ cells ml$^{-1}$ (Karl et al 1999). More thorough investigations, utilizing several ice core sections to account for heterogeneity, have resulted in a decrease in the reported cell numbers. Current estimates for the surface water of Lake Vostok, using a partitioning coefficient to account for cells excluded during the refreezing process, range from 140-770 cells ml$^{-1}$ (Christner et al 2006). It is interesting to note that the range of SYBR gold stained cells in 15 glacial ice samples from the Vostok core ranged from 34 (sd ± 10) to 380 (sd ± 53) (Christner et al 2006). These numbers are much lower than the accretion ice, and indicate that there are microorganisms unique to the accretion ice. The different values reported have been attributed to heterogeneity in physical and chemical parameters between ice section depths (Christner et al 2006), differences in methodology and contamination (Willerslev et al 2004). Only direct sampling of lake water will allow for an accurate determination of cell concentrations. However, it is likely that cell numbers will remain among the lowest reported for any environment on Earth due to the extreme conditions. Additionally, validation and authentication of future results will also
Table 1: Cell concentrations based on analysis of Lake Vostok accretion ice.

<table>
<thead>
<tr>
<th>Investigator</th>
<th>Cells ml⁻³</th>
<th>Methods Utilized</th>
</tr>
</thead>
<tbody>
<tr>
<td>Priscu et al 1999</td>
<td>2800-36,000</td>
<td>Fluorescence microscopy</td>
</tr>
<tr>
<td>Karl et al 1999</td>
<td>200-300</td>
<td>Fluorescence microscopy, SEM, flow cytometry</td>
</tr>
<tr>
<td>Poglazova et al 2001</td>
<td>200-1000</td>
<td>Fluorescence microscopy</td>
</tr>
<tr>
<td>Bulat et al 2004</td>
<td>0</td>
<td>Estimate from direct PCR</td>
</tr>
<tr>
<td>Christner et al 2006</td>
<td>70-450</td>
<td>Fluorescence microscopy</td>
</tr>
<tr>
<td>Bulat 2006, (unpublished)</td>
<td>2-10</td>
<td>Fluorescence microscopy, flow cytometry</td>
</tr>
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require duplication of findings by independent laboratories and strict adherence to aseptic techniques established for handling ancient microbial samples (Willerslev et al 2004).

Any microorganisms present in Lake Vostok or the accretion ice samples are subject to extreme conditions that may influence their viability. Viable microbes have been observed in accretion ice samples by metabolic activity experiments (Christner et al 2006, Karl et al 1999) and direct culturing (Christner et al 2001, 2006). Differential cell staining has been used to estimate that as many as 75-99% of cells in melted accretion ice are potentially viable (Christner et al 2006, D’Elia et al 2008). The total cell counts for this same set of ice sections ranged from two to about 500 cells ml⁻¹ (Christner et al 2006, D’Elia et al 2008). Bacterial isolates have also been previously recovered utilizing enrichment cultures (Christner et al 2001, 2006). Very few isolates have been recovered to date. Only three colonies were recovered from accretion ice from a depth 3593 m (Christner et al 2001). The percentage of recovered isolates and the predicted viability of cells determined by fluorescence microscopy are not in agreement, indicating additional attention is needed in development of isolation and culturing techniques specific to the Lake Vostok environment.

There has yet to be a definitive answer to the origin of the microbial diversity observed in accretion ice cores. Several reports suggest that the melt water from glacial ice releases microorganisms into the lake that are then entrapped in the accretion ice (Christner et al 2001, Poglazova et al 2001, Siegert et al 2003). The models used for explaining water circulation also suggest that microbes in the glacial melt water may circulate and join the accretion ice before they actually establish life cycles in the lake (Siegert et al 2003). However, there are some indications that there are microorganisms
unique to the accretion ice compared to the deep glacial ice. Based on finding no similar bacterial species in both glacial and accretion ice, Bulat et al. (2004) concluded that there is a small probability that microbes are released from the glacial ice and refreeze in accretion ice (Bulat et al 2004). Another report has identified large rod shaped cells morphologically similar to soil bacteria in deep glacial ice that are not found in the accretion ice sample, suggesting unique microbiota in the two types of ice (Poglazova et al 2001). The possible origin of microorganism is further complicated by a recent finding which indicates a complex river system underneath eh Antarctic ice sheet that connects subglacial lakes, including Vostok (Wingham et al 2006). As the microbiology of additional subglacial environments in other geographic locations are explored, it is becoming increasing evident that these environments harbor unique and diverse sets of microorganisms (Sonjak et al 2006, 2007, Butinar et al 2007, Fought et al 2004). It is likely that the water of Lake Vostok, as in other permanently ice covered lakes in Antarctica (Stingl et al 2008), harbors a population of microbes adapted to the extreme conditions.

It is currently difficult to determine which selective pressures are influencing the evolutionary processes that may be occurring in the Lake Vostok ecosystem. There are constraints based on temperature, pressure, isolation and nutrient availability. Temperature has been suggested to play a minor role in the overall microbial population within the lake based on the finding that the majority of the identified microorganisms are psychrotolerant versus psychrophilic (Siegert et al 2003). Cold adaptation is a requirement for any microorganism that lives in polar regions. Therefore, other factors such as nutrient availability and high pressure may be more influential selective pressures
on potential life in the lake. Examinations of isolates cultured from accretion and glacial ice cores corroborate that psychrotolerant taxa are more prevalent, while a few potential psychrophiles may be present. The temporal aspect of isolation has also yet to be fully examined. The lake itself has been isolated for approximately 15 million years (Siegert et al 2003) and microbes that are entrapped in the accretion ice may be separated from the lake for up to 16,000-20,000 years (Bell et al 2002) before any are released back into the lake. Depending on the growth rates within the lake, this time frame may be sufficient for evolutionary divergence to occur between lake and ice entrapped microorganisms.

**Objectives of this study**

Lake Vostok, located below the 4000 m thick Antarctic Ice Sheet, is the largest subglacial lake in the world. To date, the lake water has not been sampled due to concerns of forward contamination. Ice cores available from the Vostok 5G well of depths greater than 3538 m represent lake water that has accreted to the overhead glacier from various regions across Lake Vostok, including the shorelines and open lake. A total of 12 ice core sections were analyzed using direct culturing, rDNA sequence analysis and fluorescence and scanning electron microscopy (SEM). Direct culturing was performed using a wide range of both nutrient rich and limited media for bacteria and fungi. The culture plates were initially incubated at 8°C to increase the likelihood of recovering psychrophilic or psychrotolerant microorganisms. Fluorescence microscopy of melt water samples was used to determine total, viable and nonviable cell counts. The total cell numbers and diversity of cellular morphology was then compared to observations made by SEM. Overall, the general aims of the investigation were to identify
microorganisms in Lake Vostok accretion ice, quantify the abundances of the different microorganisms, and demonstrated the necessity to use multiple methods to thoroughly characterize environmental ice. A thorough analysis of accretion ice core sections from various depths is required to determine which species of microorganisms may be present in the lake, their viability and capacity to adapt to extreme conditions. Complete examination of isolated microorganisms and cloned PCR generated DNA sequences may also aid in the understanding of the extent of evolutionary change that occurs over extended periods of isolation.

Accretion ice samples represent the only way currently available to investigate the microbiological conditions in Lake Vostok. Several studies have been completed that looked at the biological conditions of accretion ice, and there have been many conflicting results. The main differences that have arisen are microbial cell concentrations, the percentage of viable cells present and the presence of nucleic acids for polymerase chain reaction (PCR) amplification (Christner et al 2001, 2006, Karl et al 1999, Priscu et al 1999). It is believed that the utilization of various methods will allow for a thorough analysis of each of these parameters. Additionally, for three of the ice sections representing the deepest accretion ice (open lake), the cores have been sectioned and compared in independent laboratories for direct comparison of decontamination and microbiological data. Proper handling, surface decontamination and maintenance of sterile conditions for all manipulations is of the utmost priority in order to report accurate findings. We have chosen a method that has been thoroughly tested and compared with other possible decontamination protocols (Rogers et al 2003, 2004). Furthermore, strict controls were used for all procedures and monitored for the presence of contamination.
While there have been several studies on the microbiology of accretion ice (Table 2), most only investigated one or two depths (Christner et al 2001, 2006, Karl et al 1999, Priscu et al 1999) and only two groups have studied a wide range of depths which include all accretion regions of the lake (Christner et al 2006, D’Elia et al 2008, Poglazova et al 2001). By utilizing ice cores from 10 different depths, it is believed that a full understanding of the microbiology can be adequately addressed. This will allow for a determination of microbial cell concentrations in the different regions, and an estimate on how water circulation and nutrient availability affect the diversity of the microbial populations. Also, by comparing the deepest sections of the glacial ice to the accretion ice, we hope to determine if microbes are naturally found in the open lake, or if they originate from the glacial ice which melts into the lake. The results from cell enumeration and viability, along with phylogenetic analysis of isolates, should help determine the origins of the microbes, their fate when trapped in the accretion ice, and to what extent evolutionary changes occur over extended periods of isolation. The investigation will result in a better understanding of the microbiology of Lake Vostok. This will allow future research to be more narrowly defined and will validate the use of multiple methods to confirm the results. This study will also improve the development of future investigations of microbial communities in other extreme environments on Earth and for future exploration of similar environments expected on Mars and Europa.
Table 2: Biological assays previously performed on the Vostok ice core.

<table>
<thead>
<tr>
<th>Investigator</th>
<th>Number of Core Sections and Depths</th>
<th>Methods Utilized</th>
</tr>
</thead>
<tbody>
<tr>
<td>Priscu et al 1999</td>
<td>1 Section, 3590 m</td>
<td>Direct PCR (16S rDNA), metabolic activity&lt;sup&gt;a&lt;/sup&gt;.</td>
</tr>
<tr>
<td>Karl et al 1999</td>
<td>1 Section, 3603 m</td>
<td>Fluorescence microscopy, SEM&lt;sup&gt;b&lt;/sup&gt;, flow cytometry, LPS&lt;sup&gt;c&lt;/sup&gt; and ATP&lt;sup&gt;d&lt;/sup&gt; detection, metabolic activity&lt;sup&gt;e&lt;/sup&gt;.</td>
</tr>
<tr>
<td>Christner et al 2001</td>
<td>1 Section, 3593 m</td>
<td>Culturing, direct PCR (16S rDNA)</td>
</tr>
<tr>
<td>Poglazova et al 2001</td>
<td>9 Accretion Ice Sections, 3540-3611 m, 1 Glacial Section, 3534 m</td>
<td>Fluorescence microscopy</td>
</tr>
<tr>
<td>Bulat et al 2004</td>
<td>2 Sections, 3551, 3607 m</td>
<td>Direct PCR (16S rDNA)</td>
</tr>
<tr>
<td>Christner et al 2006</td>
<td>20 Sections, 3540-3622 m, (not all used for each method)</td>
<td>Microscopy (cell enumeration and viability), metabolic activity&lt;sup&gt;f&lt;/sup&gt;, direct PCR, amino acid quantification, NPOC&lt;sup&gt;g&lt;/sup&gt;, SEM (for cells and particles)</td>
</tr>
</tbody>
</table>

<sup>a</sup>DOC: Dissolved organic carbon.
<sup>b</sup>SEM: Scanning electron microscopy.
<sup>c</sup>LPS: Lipopolysaccharide.
<sup>d</sup>ATP: adenosine triphosphate.
<sup>e</sup>Respiration of <sup>14</sup>C-labeled acetate and glucose substrates.
<sup>f</sup>Respiration of <sup>14</sup>C-labeled glucose substrate.
<sup>g</sup>NPOC: Nonpurgeable organic carbon.
LITERATURE CITED


subglacial Lake Vostok, Antarctica, assessed using *rrs, cbb* and *hox*. Environ Microbiol 8:2106-2114.


CHAPTER II
ISOLATION OF FUNGI FROM LAKE VOSTOK ACCRETION ICE

ABSTRACT

Here we report the characterization of fungi from ten accretion ice sections (15,000 to 18,000 years old), as well as two deep glacial ice sections that are close to the bottom of the glacier (1 to 2 million years old) from the Vostok, Antarctica 5G ice core. Fungi, as well as bacteria, were characterized by fluorescence microscopy, culturing, and sequence analyses of ribosomal DNA. A total of 703 bacteria and fungi were cultured from the accretion ice of subglacial Lake Vostok. Mean cell concentrations were from 2.3 to 12.3 cells per ml of ice meltwater, of which 5 to 84% were viable. Fifty four unique ribosomal DNA sequences were determined and compared to recent taxa, of which 36 were fungi (the remainder were bacteria, reported elsewhere). The results, plus tests for growth at low temperatures, indicate that Lake Vostok contains a mixture of heterotrophic psychrotolerant and psychrophilic species. This indicates that the lake is not sterile, but contains a unique ecosystem.
INTRODUCTION

The number of subglacial lakes discovered in Antarctica continues to increase as more of these unique extreme environments are investigated. To date, more than 140 subglacial lakes are known to exist (Siegert et al 2005), complete with river systems (Wingham et al. 2006). The first subglacial lake discovered was Lake Vostok (Fig. 1A, 1B, 1C; Kapista et al 1996, Siegert et al 2001). With a volume of 5400 km$^3$ and a surface area of 14,000 km$^2$, Lake Vostok is the largest of all subglacial lakes (Siegert et al 2001, 2005). The average depth of Lake Vostok measures 400 m, with a maximum depth expected to be 1200 m. The lake has been isolated from the atmospheric environment for approximately 15 million years (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 last 420,000 years by analyzing the upper 3310 m of the ice core (Petit et al 1999). The deepest part of the Vostok core of meteoric origin, 3538 m, has been estimated to be 1-2 million years old (Salamatin et al 2004). 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 (Fig. 1D, 1E; Petit et al 1999). The final 210 m of the Vostok core have been termed accretion ice, which is characterized by having a chemistry and crystallography distinct from the glacial ice (Jouzel et al 1999).
**Fig. 1. Location of Vostok ice core and descriptions of the ice flow and accretion processes.** A. Map of Antarctica indicating the location of Lake Vostok. B. Contour of Lake Vostok based on Bell et al 2005. C. Close-up view of the Vostok ice core site, the flow line of the glacier, and regions of the lake represented by the accretion ice. Grey box indicates cores analyzed to a depth of 3610 m, and remaining dashed line indicates open lake cores 3613, 3619 and 3621 m. D. Schematic cross-section of Lake Vostok. The overlying glacier is 3538 m thick at the Vostok drill site. At that depth, it is estimated to be approximately 2 million years old (Salamatin et al 2004). Microbes are continuously recycled in the glacier through entrapment and subsequent melting. Microbes entrapped
in the glacier are also deposited in the lake as it flows over the ridge into the lake, and by melting. The transit time of the glacier across the lake is approximately 15,000 years. As the glacier moves over the lake, ice accretes onto the bottom of the glacier. The first regions represented in the accretion ice are from a shallow embayment followed by a peninsula (or island), and finally the main lake basin. Ice closest to the bottom of the glacier is approximately 15,000 years old. On the far shore, microbes are released from the accretion ice as some of the ice is ablated by the rocky shoreline. Therefore, there is recycling of microbes via entrapment and release in the accretion ice in cycles of approximately 15,000 years, and in the glacial ice in cycles of approximately one to two million years. E. Age and depth of cores analyzed. The microbes in this study originated from core sections that represent the region from the shallow embayment, past the peninsula, and the main lake basin. Additionally, two glacial ice core sections were assayed, representing ice that may be as old as 2 million years (Salamatin et al 2004).
The flow of the ice sheet across Lake Vostok has been calculated to take between 16,000 to 20,000 years (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 allows for ice to accrete from distinct areas of the lake, allowing a comparison of microorganisms from different regions (Fig. 1). Ice core sections from 3539 m to 3609 m, primarily consisting of ice that formed in a shallow embayment (Bell et al 2005, Salamatin et al 2003), are characterized by having inclusions (silty ice, termed type I ice) that might be the result of the temporary grounding of the glacier on the lakebed as it enters the 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, and contains almost no inclusions and lower concentrations of ions, organic carbon and biomass (Christner et al 2006, Priscu et al 1999, Salamatin et al 2003). Analysis of this ice, termed accretion ice type II (De Angeles et al 2004, Salamatin et al 2003), contains samples from the open regions of southern basin of Lake Vostok. Also, since the shallower ice is older, analysis of this ice requires consideration of a temporal component. It is therefore possible to collect ice core sections that represent the distinct regions of the lake and determine if 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 may be present in the lake. Fresh water is supplied to Lake Vostok by melting of glacial ice near the shore line and at the ice-water interface in the
north (Siegert et al 2000, Studinger et al 2004). Additionally, there might be water input from subglacial streams and rivers (Wingham et al 2006). Glacial melting is a source of oxygen and nitrogen, as well as nutrients, and organic matter. Gasses 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). 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). The concentration of oxygen is influenced by the distance from the source (glacial melting), potential sinks and the saturation limit of the lake water (Siegert et al 2003). Reported dissolved organic carbon (DOC) levels range from adequate for growth of microbial heterotrophs (17–250 μmol NPOC L⁻¹; Christner et al 2006, DOC = 1.2 mg C L⁻¹; Priscu et al 1999) to insufficient to support heterotrophic life (12.5 ppb C; Bulat et al 2004). Initial estimates of microbial cell concentrations in Lake Vostok accretion ice samples have varied from zero to nearly 10⁶ cells ml⁻¹ (Bulat et al 2004, Priscu et al 1999). Another early study of an ice core section from 3603 m indicated 2-3 x 10² cells ml⁻¹ (Karl et al 1999). Current estimates for the surface water of Lake Vostok, using a partitioning coefficient to account for cells excluded during the refreezing process, range from 140-770 cells ml⁻¹ (Christner et al 2006). However, our previous results from the accretion and glacial ice sections analyzed in this study indicate that the total cell concentrations range from 2.33 (sd ± 0.29) to 12.33 (sd ± 9.58) cells ml⁻¹ and the mean viable cell concentrations range from 0 to 6.56 (sd ± 3.36) cells ml⁻¹ (D’Elia et al 2008). The discrepancies between the different biological assays are most likely due to natural heterogeneity in different
accretion ice core sections, as well as the methodologies utilized in decontamination and melting of 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. Recently, 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 and yeasts have been recovered after 140,000 years 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 the subzero temperatures and high salinities (Ozerskaya et al 2004). The fungal component of different 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). Several general physiological adaptations are used by fungi to survive in extreme cold and frozen environments. Membrane fluidity is maintained at low temperatures 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).

The mycological component of the Vostok core, and Lake Vostok accretion ice, has been greatly overlooked. Fungi have been reported in the deep ancient glacial layers of the Vostok core. Yeasts of the 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 (shallow embayment),
and yeast cells from the 3611 m core section (main basin), which were observed by
fluorescence microscopy, but were not identified phylogenetically or isolated by
culturing (Poglazova et al 2001). Here, we report the isolation and identification of fungi
from Lake Vostok accretion ice. Ice core sections were surface decontaminated using a
proven method (Rogers et al 2004) and meltwater samples were used for enrichment
cultures, direct PCR and microscopic analysis (fluorescence and scanning electron). A
total of 36 fungal isolates were identified by molecular methods from the ice core
sections. All isolates are related to polar psychrophilic and psychrotolerant taxa. The
isolation of fungi from Lake Vostok accretion ice indicates the presence of heterotrophic
microbial life within the lake despite the oligotrophic, hyperbaric, cold, and possibly
hyperoxic conditions.
MATERIALS AND METHODS

Description of ice core sections

Ten accretion ice core sections were examined for their biological contents (as described below). Five of them (3540, 3563, 3582, 3584, and 3591 m) represented ice that had accreted over the shallow embayment, and were approximately 3,800 to 5,100 years old (Bell et al 2005, Salamatin et al 2003, 2004, Christner et al 2006). Five (3606, 3610, 3613, 3619, and 3621 m) accreted over the main lake basin, and were approximately 3,300 to 3,500 years old. Two additional cores were examined from deep glacial ice (3501 and 3520 m), which were approximately 1 to 2 million years old (Salamatin et al 2003, 2004). All were examined for the presence of living and dead bacteria and fungi (Table 1; Fig. 3). Sections 3613, 3619, and 3621 m were from the Russian allocation, and were kindly supplied by Prof. Sergey Bulat, and colleagues. All other ice core sections were obtained from the National Ice Core Laboratory (NICL) in Denver, CO.

Decontamination

The outer ice core surfaces were decontaminated with 5.25% sodium hypochlorite, using a protocol previously shown to be effective at removal of all external microbial and nucleic acid contamination (Rogers et al 2004, 2005). Briefly, quartered ice core sections of 6 cm in length were warmed at 4°C for at least 30 minutes before surface decontamination. The work surfaces in a room isolated from the working laboratory were sterilized with 5.25% sodium hypochlorite (undiluted Clorox), 70% ethanol and UV irradiation for one hour prior to handling of the ice core sections. Inside
a sterile Class II cabinet (biosafety laminar flow hood), the ice core sections were surface
decontaminated by immersion in 500 ml of a 5.25% sodium hypochlorite solution (pre-
chilled to 4° C) for 10 s followed by two rinses with 200 ml of 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. This protocol significantly
reduces the risk of contamination of inner ice core meltwater samples. Up to five shells
were collected for each ice core section (based on size of core section). A portion of the
meltwater was immediately used for culturing and the remaining water was frozen for
subsequent molecular and microscopic investigation. During the entire handling process,
four agar plates (nutrient agar and malt extract agar, see below) were placed inside the
laminar hood. The lids were removed to allow continuous exposure to the working
environment. These controls allowed for monitoring of the air quality within the laminar
flow hood.

**Culturing**

Meltwater collected from the surface-decontaminated ice core sections was used
to assay for viable microorganisms by distribution and incubation on 13 different agar
media for fungi (and bacteria). Culturing was performed by spreading 200 µl of
meltwater from each shell onto duplicates of each media. The media used 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, 0.5% 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 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]), and water agar (2% agar). The inoculated agar plates were incubated at 8° C for at least two weeks, followed by 15° C for at least two weeks, and then maintained at 22° C. Plates were checked for microbial growth between each transfer. Any isolates obtained from the meltwater culturing were immediately subcultured to obtain pure cultures for molecular identification. Select subcultures were tested for growth at 4° C, 8° C, 22° C 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 after incubation for two weeks.
PCR amplification of isolates

The nuclear ribosomal DNA (rDNA) internal transcribed spacers (ITS1 and ITS2) and the 5.8S gene were amplified using the primers ITS4 and ITS5 (White et al 1990). DNA from the cells was amplified using the GeneAmp PCR Reagent Kit (Applied Biosystems, Branchburg, New Jersey). Each reaction consisted of 5 µl of cell suspension, 50 pmol of each primer, 10 pmol of each dNTP, 2U Taq DNA polymerase, 50 mM KCl, 1.5 mM MgCl₂, in a total volume of 50 µl. In some cases, native Taq DNA polymerase (Fermentas Inc., Hanover, MD) was used. The thermal cycler (Mastercycler gradient, Eppendorf, Westbury, NY) program used was: 95°C for 8 min, 40 cycles of 1 min at 94°C, 1 min 30 sec at 54°C and 2 min at 72°C, followed by an incubation for 8 min at 72°C. PCR reactions were viewed on 1.0% agarose gels, with TBE (90 mM Tris-borate, 2 mM EDTA, pH 8.0), and 0.5 µg/ml ethidium bromide, illuminated with a UV source, and photographed with a digital camera.

Cloning and Sequencing

PCR amplified ITS rDNAs from fungal and bacterial isolates were ligated into pCR 4-TOPO vector (TOPO TA Cloning Kit for Sequencing, Invitrogen, CA) following the manufacture’s instructions. The ligation 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 ligation reaction was used to transform One Shot® TOP10 Competent E. coli cells as described by the manufacture’s instructions. Plasmid DNA was isolated from transformed cells using 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 visualized 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**

The ITS DNA sequences obtained were used in BLAST searches of the GenBank database to identify sequences of related taxa. The sequences from the isolates and related taxa were then aligned using ClustalX 2.0 (Taylor et al 1997) and manually adjusted. A phylogenetic tree was created from the alignment using the program PAUP (Swofford 1999). The phylogenetic tree was created using maximum parsimony with heuristic searches. Gaps were treated as a fifth base and the tree was midpoint rooted. Bootstrap support (1000 replications) was also determined using the same criterion.

**Fluorescence Microscopy**

Ice core meltwater samples were visualized by fluorescence microscopy using the LIVE/DEAD BacLight™ Viability Kit (Molecular Probes, Eugene, OR). Using aseptic techniques in a laminar flow hood, meltwater samples were concentrated ten-fold by preparing 1 ml aliquots of meltwater that were centrifuged for 10 min at 10000 x g. The supernatant was removed and saved. The remaining pellet was resuspended in 100 µl of the supernatant. A total of 10 ml of meltwater for each core was used for cell counts and viability using this procedure. The 100 µl of concentrated meltwater aliquots were stained with 3 µl of a 1:1 mixture of both stains (3.34 mM SYTO 9 and 20 mM
propidium iodide in DMSO). Slides were prepared with 10 µl of the stained meltwater and examined by epifluorescence microscopy (Ziess Axiophot Epifluorscence Microscope, FITC long pass filter set) at a final magnification of X1000. The samples were not filtered for examination to avoid false negatives (filtering may kill some viable cells, or introduce contaminating cells), build up of debris particles in the meltwater, and multiple manipulations that could introduce contaminants. Sterilized water preparations also were examined under the same condition as the negative controls.

**Scanning Electron Microscopy (SEM)**

Meltwater samples were prepared for SEM by filtering 5 ml of 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) for one hour. The filter was rinsed three times (10 min each) in 0.1 M phosphate buffer followed by dehydration for 10 minutes each in 40%, 60%, 80%, 95% and 100% (3 times) ethanol. All solutions used for processing the meltwater samples were filter sterilized by passage through a 0.2 µm filter prior to use. A Samdri 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 using a Polaron E500 SEM coating unit. Samples were observed in the SEM (Hitachi S-2700) for the presence of microorganisms in the meltwater. Control filters were processed in identical manners using sterilized water, and were examined for the presence of any microorganisms that would indicate contamination.
RESULTS

Meltwater Analysis by Microscopy

Meltwater samples from accretion ice and deep glacial ice were analyzed by fluorescence microscopy and scanning electron microscopy (SEM) to identify microorganisms and to compare morphological diversity between samples. Fluorescence microscopy revealed a wide variety of morphologically distinct microbial cells, and the low cell concentrations and diversity of shapes was confirmed by SEM (Figs. 2 and 3). Many spores were found in the meltwater (Fig. 2), including spherical, oblate, and oblong forms. In addition, some appeared to be germinating. Melting the ice might have triggered this process. In one core section (3582) 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. Some of the filaments formed aggregates consisting of a dozen or more individual filaments. The cells found in section 3619 (that accreted over open water in the main lake basin) consisted almost exclusively of long filaments that resembled hyphae. Some appeared to be multinucleate. Cell counts, numbers of isolates and unique sequences were recorded for each of the ice core sections (Fig. 3). The total number of cells always was low (averaging 8.3 cells ml⁻¹). The curves for the number of viable cells, colonies, and unique sequences were similar. All were low in glacial ice, and peaked near the boundaries between type I (silty) ice and type II (clear) ice. The silt in type I ice is thought to come from grounding of the glacier on the lake bottom, water turbulence, and/or geothermal activity below the lake. Type II ice accretes over calm open water.
Fig. 2. Micrographs of cells found in the glacial and accretion ice core sections. No cells consistent with fungi were found in sections 3563 or 3591. 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. Yellow is from the fluorescence of both dyes, and thus is ambiguous. Bars represent 5 microns on fluorographs and 1
micron on electron micrographs. 3501: A spore and a possible branched hyphae. 3520: Spores and germinating spores. The organism in the upper right of the figure appears damaged, which is consistent with the extreme turbulence and pressure in this zone of the glacier (characterized by cracked, twisted, and crumbled ice) and the low number of viable cells found in this core section (Fig. 3). 3540: Only one possible fungal spore was found in this section. 3582: A large number of filamentous cells were observed in this section. Often, they had larger structures at one terminus (as in upper left micrograph). Some had enlarged portions in the middle of the filaments (as in the lower right micrograph). In a few, discernable cell separations (i.e. possible septa) were seen (upper left, indicated by arrows). One structure that appeared to have multiple nuclei was observed in the fluorescence microscopy study. 3584/5: One of the filamentous structures was large (approximately 10 µm wide and 500 µm long) and appeared to have septa (lower, arrows indicate possible septa). 3606: Only distorted spores were observed. This is consistent with partial grounding of the glacier in this region, causing increased and uneven pressures. Very low cell viabilities were measured in this core section (Fig. 3). 3610: One spore and another organism are shown. The glacier is over open water in this area (as indicated by type II ice), and thus the cells appear to be undamaged compared to those in section 3606. 3613: One spore and one germinating spore are shown. 3619: All of the organisms were filamentous in morphology. The two on the left exhibited many nuclei, as well as concentration of DNA into two foci (leftmost micrograph). These appear to be multinucleate cells. This core section exhibited a spike in the concentration of total cells and viable cells, as well as sequence diversity (Fig. 3). 3621: One spore and a germinating spore.
Fig. 3. Cell concentrations, colonies of viable cells and numbers of unique sequences in the ice core sections. Cell concentrations (upper panel) are based on cell counts of fluorescently stained cells visualized via microscopy from 10 ml of meltwater from two glacial and ten accretion ice core sections. Graph also shows the region of the lake that is represented in each ice core section. The number of colonies and total number of unique
sequences (bacterial [D’Elia et al 2008] and fungal), are displayed in the lower panel. Highest concentrations of cells, viable cells, cultures, and unique sequences are in the areas of open water.
Cultured isolates and DNA sequence analysis

Culturing of meltwater from the two glacial ice and seven accretion ice samples resulted in 703 colonies of fungi, yeast and bacteria (Table 1). Growth was observed on all media tested, and at each temperature. The length of incubation ranged from three weeks, to over one year. No growth was observed on any control plates (sterile water or culture plates opened in the hood). Only 22 colonies in total were isolated from the deep glacial ice core sections 3501 m and 3520 m. For eight of the accretion ice cores, the number of total colonies ranged from 0 to 14. The remaining two accretion ice sections from 3610 m and 3582 m had the highest number of colonies, with 72 and over 500 colonies for each respective core section. The high number of colonies from core section 3582 m were 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 36 fungal cultures were selected for molecular identification by sequence analysis of ribosomal ITS regions (Table 1). All sequences were obtained directly from cultured isolates, as all attempts to PCR amplifying fungal ITS DNA directly from ice core meltwater were unsuccessful. Of these, 29 were from at least 4 genera of basidiomycetes (26 from accretion ice and 3 from glacial ice), while 7 were from at least 5 genera of ascomycetes (all from accretion ice). Sequences that were closest to Rhodotorula mucilaginosa were the most frequently observed, although no two
Table 1. Summary of accretion ice biological data.

<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 searches[^cd] [% similarity]</th>
<th>Characteristics of closest taxon</th>
</tr>
</thead>
<tbody>
<tr>
<td>GLACIAL ICE</td>
<td>3501</td>
<td>15</td>
<td>19</td>
<td>7</td>
<td>Bacillus (b) [94]</td>
<td>end, gla, tol, vos</td>
</tr>
<tr>
<td></td>
<td>3520</td>
<td>26</td>
<td>3</td>
<td>3</td>
<td>Bacillus (b) [67-94]</td>
<td>end, gla, tol, vos</td>
</tr>
<tr>
<td>ACCRETION ICE EMBAYMENT</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grounded</td>
<td>3540</td>
<td>14</td>
<td>0</td>
<td>0</td>
<td>none</td>
<td>---</td>
</tr>
<tr>
<td>Open Water</td>
<td>3563</td>
<td>40</td>
<td>3</td>
<td>2</td>
<td>Rhodotorula (f) [99]</td>
<td>ant, aqu, atm, gla soi</td>
</tr>
<tr>
<td></td>
<td>3582</td>
<td>84</td>
<td>562</td>
<td>13</td>
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<td></td>
<td>3584</td>
<td>52</td>
<td>14</td>
<td>6</td>
<td>Carnobacterium (b) [69-99]</td>
<td>laks, per, psy, pol, soi, vos</td>
</tr>
<tr>
<td></td>
<td>3584</td>
<td>52</td>
<td>14</td>
<td>6</td>
<td>Penicillium (f) [69]</td>
<td>laks, per, psy, pol, soi, vos</td>
</tr>
<tr>
<td>PENINSULA</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MAIN BASIN</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Grounded</td>
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<td>24</td>
<td>2</td>
<td>1</td>
<td>Rhodotorula mucilaginosa (f) [98]</td>
<td>ant, aqu, atm, gla soi</td>
</tr>
<tr>
<td></td>
<td>3606</td>
<td>19</td>
<td>12</td>
<td>5</td>
<td>Cystofilobasidium informominutum [96]</td>
<td>ant, gla, oce, sno, soi</td>
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<tr>
<td></td>
<td>3613</td>
<td>5</td>
<td>4</td>
<td>4</td>
<td>Bacillus (b) [66-67]</td>
<td>end, gla, tol, vos</td>
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<td>Open Water</td>
<td>3610</td>
<td>16</td>
<td>72</td>
<td>5</td>
<td>Rhodotorula mucilaginosa (f) [95-100]</td>
<td>ant, aqu, atm, gla soi</td>
</tr>
<tr>
<td></td>
<td>3613</td>
<td>5</td>
<td>4</td>
<td>4</td>
<td>Bacillus (b) [66-67]</td>
<td>end, gla, tol, vos</td>
</tr>
</tbody>
</table>
Table 1 (continued). Summary of accretion ice biological data.

<table>
<thead>
<tr>
<th>Region</th>
<th>Depth (m)</th>
<th>Percent Viable Cells&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Number of Colonies&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Unique Sequences</th>
<th>Closest taxon in BLAST searches&lt;sup&gt;c,d&lt;/sup&gt; [% similarity]</th>
<th>Characteristics of closest taxon</th>
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</thead>
<tbody>
<tr>
<td>Open Water</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Aspergillus (f) [84] ant, atm, gla, soi</td>
<td></td>
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<tr>
<td></td>
<td>3619</td>
<td>44</td>
<td>6</td>
<td>6</td>
<td>unclassified soil fungi (f) [92-94] ant</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Cryptococcus (f) [89] ant, sno, soi</td>
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<tr>
<td></td>
<td>3621</td>
<td>10</td>
<td>6</td>
<td>2</td>
<td>Friedmaniella (b) [86] ant, san</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Kocuria (b) [94] ant, lak, oce, pnd, psy</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Paenibacillus (b) [48] end, gla, tol, vos</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Penicillium (f) [85] ant, gla, lks, pnd, soi</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Leucosporidium (f) [93] ant, aqu, per, pol, tol, sno, soi</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3621</td>
<td>10</td>
<td>6</td>
<td>2</td>
<td>Micrococcus (b) [75] ant, lak, pnd, psy</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Pseudozyma (f) [94] ant, lks</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Based on cell counts from 10 ml of concentrated ice meltwater.

<sup>b</sup> Cultures of all bacteria and fungi on all media.

<sup>c</sup> For bacteria and fungi, 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 for in BLASTN searches of GenBank on the NCBI internet site. If the similarity was 95% of 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. Bacteria = b, fungi = f

<sup>d</sup> A list of some characteristics within the taxon represented by the sequences that are consistent with conditions in Lake Vostok.

Abbreviations: atm = common in the atmosphere; ant = Antarctic; aqu = aquatic; dpo = deep ocean; end = forms endospores; frw = fresh water; gla = glaciers ; lak = lakes; lks = lake sediments; nif = nitrogen fixation; oce = oceans; per = permafrost; pol = polar; pnd = ponds; psy = some species are psychrophilic; sal = saline and salt marshes; san = sandstone; sno = snow; soi = soil; tol = some species are psychrotolerant; unk = unknown; vos = reported previously in Vostok accretion ice.
sequences were identical. A total of 22 *Rhodotorula* ITS sequences were obtained for isolates from every accretion ice core section except 3540 m and 3585 m (isolates GI902-904, GI908-911, GI914, GI926-929, GI931, GI933, GI939, GIGI945-949, GI951, and GI952). 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 shallow embayment. Percent similarities scores for the *Rhodotorula* isolates ranged from 94-100% compared to taxa in GenBank. Isolates were initially collected from a variety of media, including minimal nutrient media, after incubation periods of 15 days to over 9 months. *Rhodotorula* isolates showed optimal growth at 22° C, but also grew well at 15° C and 4° C (Table 2), indicative of psychrotolerance. Other basidiomycetes included a single isolate (GI913) belonging to the genus *Cystofilobasidium* (96% similarity to GenBank) and two isolates (GI817 and 944) that are most closely related to members of the genus *Cryptococcus*. A final basidomycete was isolate GI895 that was closest to species of *Pseudozyma* and *Ustilago*, and was psychrotolerant (Table 2).

A total of seven isolates belonging to the ascomycetes were identified in six (3563, 3582, 3585, 3610, 3613, and 3619 m) of the ten accretion ice core sections (Table 1). A single isolate (GI920) from 3585 m was determined to be most closely related to the genus *Penicillium*, and showed 88% ITS sequence similarity to *P. chrysogenum*. Isolate GI737 was from the Greenland Dye-3 ice core section that was approximately 5,500 years old. It was included for comparison, since it exhibits many sequence changes from similar *Penicillium* species from Antarctica. Isolate GI920 also displayed psychrotolerant growth characteristics (Table 2). Accretion ice samples that formed over
Table 2: Mean growth rates of the fungal isolates collected from glacial and accretion ice core sections.

<table>
<thead>
<tr>
<th>Closest Taxon in BLAST Search</th>
<th>Core Depth (m)</th>
<th>Media</th>
<th>Temperature Growth Rate(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fungi (GI Number)</td>
<td></td>
<td></td>
<td>37(^\circ)C</td>
</tr>
<tr>
<td>Pseudozyma (GI895)</td>
<td>3621</td>
<td>MEA</td>
<td>1.21</td>
</tr>
<tr>
<td>Penicillium chrysogenum (GI920)</td>
<td>3585</td>
<td>MEA</td>
<td>1.29</td>
</tr>
<tr>
<td>Penicillium chrysogenum</td>
<td>Control</td>
<td>MEA</td>
<td>0.6</td>
</tr>
<tr>
<td>Aspergillus niger</td>
<td>Control</td>
<td>MEA</td>
<td>4.2</td>
</tr>
<tr>
<td>Rhodotorula mucilaginosa (GI911)</td>
<td>3606</td>
<td>MEA</td>
<td>0.64</td>
</tr>
<tr>
<td>Rhodotorula mucilaginosa (GI914)</td>
<td>3591</td>
<td>MEA</td>
<td>0.51</td>
</tr>
<tr>
<td>Rhodotorula mucilaginosa (GI928)</td>
<td>3582</td>
<td>MEA</td>
<td>0.55</td>
</tr>
<tr>
<td>Rhodotorula mucilaginosa (GI952)</td>
<td>3563</td>
<td>MEA</td>
<td>0.76</td>
</tr>
<tr>
<td>Rhodotorula mucilaginosa (GI959)</td>
<td>3501</td>
<td>MEA</td>
<td>0.40</td>
</tr>
<tr>
<td>Uncultured soil fungus (GI944)</td>
<td>3582</td>
<td>MEA</td>
<td>0.21</td>
</tr>
<tr>
<td>Saccharomyces cerevisiae</td>
<td>Control</td>
<td>MEA</td>
<td>0.99</td>
</tr>
</tbody>
</table>

\(^a\)Mean growth rates (mm/day) of the fungal isolates collected from glacial and accretion ice core sections. Each isolate was tested at four different temperatures. Rates are given as the average of triplicate testing. Maximum rates are indicated by bold type, NG indicates no growth observed.
the shallow embayment (3582 m) and open main basin of the lake (3610 m) each contained single isolates (GI924 from 3582 m; and GI900 and GI901 from 3613 m) with closest sequence similarities to unknown fungi (92 and 95% respectively) that are most likely within the genus *Cladosporium*. Isolate GI932 also originated from ice core section 3582 m, and shares 95% ITS sequence similarity with *Aeurobasidium pullulans*. One isolate (GI898) was closest to *Aspergillus* species, but differed significantly from all sequences in the NCBI database. A single ascomycete was collected from accretion ice representative of the grounding line of the ice sheet in the shallow embayment. This isolate (GI951) from 3563 m showed very low sequence similarity to taxa in GenBank (highest similarity of 69%), although it is closest to *Cerebella androprogonis*. Tolerance to low growth temperatures was observed for all of the isolates classified as ascomycetes. All of the isolates were originally detected after incubation at 15° C. No ascomycetes were isolated from the glacial ice core sections, estimated to be between 1.5 and 2.0 million years old (Salamatin et al 2004).

**Phylogenetic analysis**

Phylogenetic analysis was performed on ITS sequences obtained from the 36 ice core isolates, and an additional 39 fungal sequences obtained from the NCBI database related to contemporary taxa and isolates from polar and frozen environments (Fig. 4). The phylogenetic analysis revealed two major groups comprised of ascomycetes and basidiomycetes. The basidiomycete group contained four major subclades including the genera *Rhodotorula*, *Cryptococcus*, *Cystofilobasidium*, and *Ustilago*. The majority of the sequences isolated from the accretion ice cores grouped within the *Rhodotorula* subclade.
Fig. 4. Maximum parsimony phylogram created from ITS sequences of fungal isolates from accretion and glacial ice. Fungal isolates cultured from melt water (GI, glacial isolate numbers) and sequences collected from BLAST searches were aligned.
using ClustalX. The multiple sequence alignment was used for constructing the phylogenetic tree using PAUP (Swafford 1999). The tree was created using heuristic searches of maximum parsimony and midpoint rooting. Brief descriptors are provided for each of the sequences determined by BLAST searches to be the taxa closest to the sequences from this research. NCBI (GenBank) accession numbers are shown for each sequence. Bootstrap values are shown on branches with >50% support.
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 the 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 section 3606 m and 3501 m were more widely distributed throughout the *Rhodotorula* subclade. Another basidiomycete, isolate GI913 (3606 m), was closest to the genus *Cystofilobasidium*, grouping with the cold adapted yeast *Cystofilobasidium capitatum*.

The ascomycetes in the phylogram are fewer in total number and include more distinct taxa than the basidiomycetes (Fig. 4). Isolate GI920 displayed strong relation to species of *Penicillium* from Antarctica, glacial and deep sea environments. The ITS sequences of three cultured isolates (GI900 from 3613 m, GI901 from 3610 m, and GI924 from 3582 m) from the ascomycete group were 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 year old Greenland glacial ice (Ma et al 2000).
DISCUSSION

Accretion ice from Lake Vostok enables a unique opportunity to investigate the microbiology of the extreme subglacial environment that has been completely ice-covered for millions of years. Glacial and accretion ice core sections have been extensively studied in terms of the bacterial component and chemistry. The findings in this report offer this first time fungi have been specifically targeted for culture and molecular identification. As such, this is the initial detailed study of known heterotrophic microbes in Lake Vostok accretion ice. Polar regions and subglacial environments have been increasingly analyzed in regards to fungi and yeasts. These environments are a potential source of microbes that have been removed from the global gene pool for evolutionarily significant periods of time, and may allow for the study of processes of microbial speciation (Vincent 2000). 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 insulation from UV exposure and temperature fluctuations (Sharp et al 1999). The Lake Vostok ecosystem is of particular interest because of its length of isolation and the extreme conditions present. It is hyperbaric (approximately 350 atmospheres of pressure), cold (average temperature is -3°C), dark, oligotrophic, and possibly hyperoxic. Isolation and identification of fungi from the accretion ice adds new evidence to the range of microbial diversity in the ice, and further supports the likelihood of a complex ecosystem within the lake.

Many microorganisms isolated from Antarctic environments show some degree of relatedness to taxa found in a wide range of habitats indicating that microbial distribution is occurring on a global scale or at least polar scale. Seasonal extremes in Antarctica,
along with the variety of habitats on the continent, leads to the selection of microorganisms that utilize a generalist survival strategy that includes psychrotolerance (Vincent 2000). The majority of the isolates obtained from all ice core sections were originally isolated at 15°C, and all of the isolates tested were determined to be psychrotolerant (Table 2). Psychrotrophic fungi capable of growth at temperatures ranging from 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). Previous 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 non-Antarctic environments (Franzmann et al 1996). Based on the examination of the isolates obtained from the accretion ice, it appears that the fungal component of Lake Vostok also is composed of psychrotolerant species. 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 1, Fig. 4). Lake Vostok and the surrounding subglacial sediment were likely seeded with a wide range of fungi prior to becoming isolated by the Antarctic Ice Sheet. Additionally, biological and non-biological materials constantly are deposited in the lake by melting glacial ice, and possibly by subglacial rivers and streams. However, the microbes isolated from the glacial ice were mostly different from those isolated from the accretion ice. Both the ITS sequence analysis and psychrotolerant characteristics of the isolates, along with the consistently negative controls, supports that the isolates are actual members of the Lake Vostok microbial community and not modern contaminants.
All of the isolates identified are related to polar taxa from a variety of permanently cold environments. 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* has also been shown to maintain a high percentage of viability after freezing (Butinar et al 2007), which may explain the frequency of *Rhodotorula* in the accretion ice from each region of Lake Vostok. *Rhodotorula* may be transported to the lake via freeze-thaw cycles with the accretion ice and lake water, with melting of the deep glacial ice, or by deposition from river systems. *Rhodotorula* also has been 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 and all were pink in pigmentation. Two additional yeasts isolated from the accretion ice core, isolates GI944 and GI913, were related to *Cryptococcus magnus* and *Cystofilobasidium* sp. respectively. Species of *Cryptococcus* have been isolated from Greenland GISP ice (Ma et al 1999), Vostok glacial ice (Abyzov et al 2004) and 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 subglacial ice (Butinar et al 2007).
Ascomycetes isolated from the accretion ice also were found to be similar to taxa from polar environments. Two isolates that grouped phylogenetically with *Cladosporium* were obtained from 3610 m and 3582 m. *Cladosporium* is a very prevalent genus of fungus worldwide. Analysis of 140,000 year old GISP glacial ice has recovered viable *Cladosporium* isolates (Ma et al 2000). *Cladosporium* has been determined to be the second most frequently trapped airborne fungal spore in Antarctica (Marshall 1997). It is therefore likely 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 GI920 was identified as *Penicillium* sp. (88% ITS similarity), and was cultured from meltwater from 3585 m. Species belonging to *Penicillium* have been isolated from polar glacial ice (Ma et al 1999, 2000) and many species have been found in Arctic 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 is potentially harboring a set of *Penicillium* species that may be uniquely adapted to this extreme subglacial environment. Accretion ice isolate GI951 was grouped within the subclade containing *Phoma* by phylogenetic analysis. *Phoma* species have been identified in glacial ice (Ma et al 1999) and isolates from Arctic soils have been characterized as psychrooligotrophic (Bergero et al 1999). The ability to tolerate both the cold and oligotrophic conditions in Lake Vostok makes species of *Phoma* potentially compatible to conditions within the lake. Another ascomycete isolated from 3582 m, was identified as *Aureobasidium pullulans*. This species has been previously 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 number 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 depths between 3572 m and 3612 m have previously indicated the capacity to support heterotrophic metabolisms and contain diverse compositions of microorganisms (Christener et al 2006). This range of ice core depths includes the passage of the ice sheet from the shallow embayment to the main lake basin. The concentration of organic carbon (100 µmol L\(^{-1}\)) and cell density (430 ± 23 cells ml\(^{-1}\)) reaches their highest levels in this transitional region relative to the open lake and deep glacial ice (Christner et al 2006). Total and viable cell counts also were previously shown to be highest for accretion ice from 3582 m (Fig. 3; D’Elia et al 2008). Respiration of \(^{14}\)C-labeled 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, 3610, and 3619 m. Accretion ice from adjacent core sections also has 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 this region are sufficient to support heterotrophic metabolisms and a high diversity of microorganisms, including fungi.

There is an increasing amount of evidence that supports the conclusion that the microorganisms identified in accretion ice originate from the lake. The total number of culturable microorganisms was higher for accretion ice than the glacial ice. Total cell
counts also peak in accretion ice samples relative to deep glacial ice (D’Elia et al 2008, Christner et al 2006). If microorganisms in accretion ice were composed only of cells that were released from glacial melting and then became entrapped by the accretion process, both the concentration and diversity would be expected to be higher in the glacial ice compared to the accretion ice. It is difficult to imagine a non-biological process whereby cell concentrations would be higher in the accretion ice than in the overlying glacial ice. Open water regions (or regions closely bordering the open water areas) of the shallow embayment (3582 m) and main lake basin (3610 m) also are high in total colonies and unique fungal isolates. These regions are more distant from where the ice sheet contacts the sediment and bedrock near the shoreline. There remains a possibility that scouring of subglacial sediments near the shoreline releases microbes into the lake. However, the low cell counts in the glacial ice, the absence of viable microbes in the glacier grounding region (e.g., 3540 m), and the few taxa identified in both glacial and accretion ice indicates that the microorganisms originate in the lake. Additionally, the isolates are consistent with fungi that might be found in Lake Vostok. All of the isolates identified have relatives that are psychrophilic or psychrotolerant, and are also similar to fungi isolated from other permanently cold oligotrophic aquatic environments.

Analysis of accretion ice is the only way currently available to study the microbiological conditions that may be present in Lake Vostok. All of the evidence collected to date indicates that the lake harbors a diverse set of microorganisms. The isolation of fungi from the accretion ice further supports that Lake Vostok is a complex ecosystem. It is becoming increasingly evident that fungi play important roles in microbial communities of subglacial environments (Butinar et al 2007, Sonjak et al 2006,
Future investigations of subglacial environments can only be considered complete if they also include analysis of the mycobiota present. This will be specifically important if or when Lake Vostok water is sampled directly.
LITERATURE CITED


CHAPTER III

ISOLATION OF MICROBES FROM LAKE VOSTOK ACCRETION ICE

ABSTRACT

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.
INTRODUCTION

Subglacial Lake Vostok, the eighth largest lake on Earth (area = 14,000 km², volume = 5,600 km³) (Masalov et al 2001, Siegert et al 2001), 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) (Salamatin et al 2003). 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 (Bulat et al 2004, Salamatin et al 2004). 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 (Christner et al 2006, Priscu et al 1999, Salamatin et al 2004). This ice is very clear ice and has been termed type II accretion ice (Salamatin et al 2003). The top section (from 3538 to 3595 m), which accreted within and near the embayment, primarily consists of type I ice, although there are some regions of type II ice (Bell et al 2005). Bacteria from this ice, including potentially psychrophilic and psychrotolerant species as well as the molecular signature of a thermophilic bacterium, have been reported (Abyzov et al 2005, Bulat et al 2004, Castello et al 2005, Christner et al 2001, Karl et al 1999, Priscu et al 1999). 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.
RESULTS

Descriptions and cell concentrations

Nine Vostok 5G ice core sections were assayed. Five sections represented ice that accreted over the shallow embayment (depths of 3540, 3563, 3582, and 3584 m, all type I ice, and 3591 m, type II ice) (Bell et al 2005) approximately 3,800 to 5,100 years ago (Bell et al 2005, Christner et al 2006, Salamatin et al 2003, 2004), two accreted 3,400 to 3,500 years ago over the main lake basin (3606 m, type I ice, and 3610 m, type II ice), and two were glacial ice cores near the bottom of the glacier (3501 and 3520 m, approximately 1 to 2 million years old) (Salamatin et al 2003, 2004). The surfaces of all of the ice core sections were decontaminated prior to melting, as described previously (Rogers et al 2004). Ice core meltwater initially was analyzed using a live/dead stain (BacLight viability kit; Molecular Probes, Eugene, OR) to count cells from 10 1-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 ± 9.58 cells/ml (Fig. 1). Considering the partition coefficient (0.56) (Christner et al 2006), 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 (Christner et al 2006, Salamatin et al 2003, 2004).
Fig. 1. Cell concentrations (upper panel), colonies of viable cells, and numbers of unique sequences (lower panel) in the ice core sections. Cell concentrations were based on cell counts of fluorescently stained cells visualized via microscopy from 10 ml of meltwater from two glacial and seven accretion ice core sections. The graph also shows the region of the lake that is represented in each ice core section. The highest concentrations of cells, viable cells, cultures, and unique sequences are in the areas near the transitions between type I and type II accretion ice.
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 Rogers et al 2004, with primers described in Rachman et al 2004) and sequenced, followed by BLAST searches, CLUSTAL alignment, and phylogenetic analysis (as in Ma et al 2000). 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 variety 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).
Fig. 2. Fluorescence and scanning electron micrographs of cells found in the glacial (3501 and 3520 m) and accretion ice (3540, 3563, 3582, 3584, 3591, 3606, and 3610 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 [Christner et al 2001]); 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.
Fig. 3. Maximum-parsimony phylogram (determined using PAUP, version 4 [Sinaur Academic Publishers]) of portions of the small subunit genes from bacterial isolates in this study (GI, glacial isolate numbers) as well as comparable sequences from
bacteria that were closest to these sequences in BLAST searches. Brief descriptors are provided for each of the sequences determined by BLAST searches to be the taxa closest to the sequences from this research. NCBI (GenBank) accession numbers are shown for each sequence. Bootstrap values are shown on branches with >50% support.
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 (Karl et al 1999). Some have suggested that Lake Vostok is sterile, since parts of the lake may be extremely oligotrophic (Bulat et al 2004). However, all of our data indicate that the lake supports a diverse microbial assembly, as has been concluded elsewhere (Christner et al 2006, Karl et al 1999, Priscu et al 1999, Sambrotto and Burckle 2005). 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 (Bulat et al 2004), 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.
LITERATURE CITED


APPENDIX A: Comparison of glacial and accretion ice data.

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<sup>a</sup>Based on cell counts from 10 ml of meltwater.
<sup>b</sup>Cultures of all bacteria and fungi from all media.
<sup>c</sup>Frequency of occurrence for select genera of bacteria and fungi in each ice core section. Identification of genus based on BLAST results using DNA sequences obtained from cultured glacial and accretion ice isolates.
APPENDIX B: GenBank accession numbers.

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Isolation of Microbes from Lake Vostok Accretion Ice

Tom D’Elia, Ram Veerapaneni, and Scott O. Rogers*

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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 1-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 ±...
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 variety of Firmicutes, Actinobacteria, and Proteobacteria, as indicated by the phylogenetic analysis (Fig. 3).
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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REFERENCES