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Isolation of fungi from Lake Vostok accretion ice

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Abstract: Here we report the characterization of fungi from 10 accretion ice sections (3300–5100 y old) as well as two deep glacial ice sections that are close to the bottom of the glacier (1 000 000–2 000 000 y old) from the Vostok, Antarctica, 5G ice core. Fungi were characterized by fluorescence microscopy culturing and sequence analyses of ribosomal DNA internal transcribed spacers. A total of 270 fungal colonies were cultured from the accretion ice of subglacial Lake Vostok and an additional 14 from the glacial ice immediately above the accretion ice. Mean concentrations were 0–4.42 cells mL⁻¹ ice meltwater of which 0–100% exhibited viability (as determined by fluorescence microscopy). Thirty-one unique fungal ribosomal DNA sequences (28 from accretion ice and three from glacial ice) were determined and compared to recent taxa. The results, plus tests for growth at low temperatures, indicated that Lake Vostok contains a mixture of heterotrophic psychrotolerant fungal species. This indicates that the lake is not sterile but contains a unique ecosystem.

Key words: Antarctica, heterotrophs, ITS, subglacial lake

INTRODUCTION

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

exploring evolutionary processes related to extended isolation and extreme conditions.

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

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

the distinct regions of the lake from different times and determine whether there is a relationship between the diversity and concentration of microorganisms based on the origin of the accretion ice.

Microbiological investigations of Lake Vostok have provided results on the biological conditions that might be present in the lake. Freshwater is supplied to Lake Vostok by melting glacial ice near the shoreline and at the ice-water interface in the north (Siegert et al 2000, Studinger et al 2004). In addition, subglacial streams and rivers might add water (Wingham et al 2006). Glacial melting is a source of oxygen and nitrogen, as well as nutrients and organic matter. For example, concentrations of dissolved oxygen in the lake water column are estimated to be as high as 50 times greater than air-equilibrated water (Christner et al 2006, McKay et al 2003). However, gases supplied from the meltwater are excluded from the accretion process, as is evident in the fact that accretion ice is essentially gas free compared to glacial ice (Jouzel et al 1999). Reported dissolved organic carbon (DOC) levels range from adequate for growth of microbial heterotrophs (Christner et al 2006, Priscu et al 1999) to insufficient to support heterotrophic life (Bulat et al 2004). Initial estimates of microbial concentrations in Lake Vostok accretion ice samples have varied from zero to nearly 10^6 cells mL^{-1} (Bulat et al 2004, Priscu et al 1999). Another early study of an ice core section from 3603 m indicated $2\text{--}3 \times 10^2$ cells mL^{-1} (Karl et al 1999). Current estimates for the surface of Lake Vostok, using a partitioning coefficient to account for cells excluded during the refreezing process, are 140–770 cells mL^{-1} (Christner et al 2006). However our previous results from the accretion and glacial ice sections indicate that the total cell concentrations range from 2.33 (sd \pm 0.29) to 12.33 (sd \pm 9.58) cells mL^{-1} and the mean viable cell concentrations range from 0 to 6.56 (sd \pm 3.36) cells mL^{-1} (D'Elia et al 2008). The discrepancies among biological assays are most likely due to natural heterogeneity in accretion ice core sections, as well as the methodologies used in analysis of microbes in the ice.

Fungi have been isolated from a variety of cold environments and have developed several mechanisms to withstand cold temperatures and low nutrient availability. The vast majority are psychrotolerant, able to survive below freezing temperatures but with optimal growth temperatures greater than 15 C. Few are true psychrophiles, which die if exposed to temperatures above 20 C, with optimal growth temperatures below 15 C. Subglacial ice from arctic glaciers provided the first reports of the isolation of yeasts (Butinar et al 2007) and a new species of *Penicillium* (Sonjak et al 2007) from these unique environments. Viable fungi have been recov-

ered after 140 000 y in Greenland glacial ice, which represents a significant amount of time separated from contemporary populations (Ma et al 2000, 2005; Starmer et al 2005). Mycelial fungi have been isolated from Siberian cryopegs, despite subzero temperatures and high salinities (Ozerskaya et al 2004). The fungal component of antarctic environments also has been widely studied. These investigations have identified fungi in glacial ice (Abyzov et al 2004, Poglazova et al 2001), soil (Arenz et al 2006), airborne spore traps (Marshall 1997) and mosses sampled in Antarctica (Tosi et al 2002). Most are reported to be psychrotolerant. Few are true psychrophiles. Several general physiological adaptations are used by fungi to survive in extremely cold environments. Membrane fluidity is maintained by increasing the unsaturated lipid content (Weinstein et al 2000). Additional physiological mechanisms that enhance cold adaptation include an increase in intracellular trehalose and polyol concentrations, secretion of antifreeze proteins (Snider et al 2000) and the use of cold adaptive enzymes (Robinson 2001).

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

MATERIALS AND METHODS

Description of ice core sections.—Ten accretion ice core sections were examined for biological contents (as described below). Five (at 3540, 3563, 3582, 3585 and 3591 m) represented ice that had accreted over a shallow embayment and were approximately 3800–5100 y old (Bell et al 2005, Christner et al 2006). Five (at 3606, 3610, 3613, 3619, and 3621 m) accreted over the main lake basin and were approximately 3300–3500 y old. Two additional cores were examined from deep glacial ice (3501 and 3520 m), which were approximately 1 000 000–2 000 000 y old (Salamatin et al 2003, 2004). All were examined for the presence of living and dead fungi. Sections from 3613, 3619 and 3621 m were from the Russian allocation and were kindly shared by Prof Sergey Bulat and colleagues. All other ice core sections were obtained from the National Ice Core Laboratory (NICL) in Denver, Colorado.

Decontamination.—The outer ice core surfaces were decontaminated with 5.25% sodium hypochlorite with a protocol shown to be effective at removal of all external microbial and nucleic acid contamination (Rogers et al 2004, 2005). Quartered ice core sections (6 cm long) were warmed at 4 C at least 30 min before surface decontamination. Work surfaces in a room isolated from the working laboratory were sterilized with 5.25% sodium hypochlorite (undiluted Clorox), 70% ethanol and UV irradiation 1 h before handling of the ice core sections. Inside a sterile Class II cabinet (biosafety laminar flow hood) ice core sections were surface decontaminated by immersion in 500 mL 5.25% sodium hypochlorite solution (prechilled to 4 C) 10 s followed by two rinses with 200 mL sterile water (4 C, 18.2 MΩ, < 1 ppb total organic carbon). The core section was transferred into a sterile funnel and was melted at room temperature by collection of 30–50 mL aliquots. This process allows for collection of “shells” of meltwater corresponding to initially the outer portion and sequentially more interior portion of the ice core section. Up to five shells were collected for each ice core section (based on size of core section). A portion of the meltwater was used immediately for culturing, and the remaining water was frozen for subsequent molecular and microscopic investigation. During the handling process four agar plates (nutrient agar and malt extract agar, see below) were placed in the laminar hood. The lids were removed to allow continuous exposure to the working environment. These controls allowed for monitoring air quality within the laminar flow hood.

Fluorescence microscopy.—Ice core meltwater samples were viewed by fluorescence microscopy with the LIVE/DEAD BacLight™ Viability Kit (Molecular Probes, Eugene, Oregon). Using aseptic techniques in a laminar flow hood, meltwater samples were concentrated 10-fold by preparing 1 mL aliquots meltwater that were centrifuged in sterile 1.5 mL microfuge tubes for 10 min at 10 000 g. The supernatant was removed and saved. The remaining pellet was resuspended in 100 µL supernatant. A total of 10 mL meltwater for each core was used for cell counts and viability with this procedure. The 100 µL concentrated meltwater aliquots were stained with 3 µL 1:1 mixture of both stains (3.34 mM SYTO 9 and 20 mM propidium iodide in DMSO). Slides were prepared with 10 µL stained meltwater and examined by epifluorescence microscopy (Zeiss Axiophot Epifluorescence Microscope, FITC long pass filter set) at 1000× final magnification. The samples were not filtered for examination to avoid false negatives (filtering might kill viable cells or introduce contaminating cells), accumulation of debris and multiple manipulations that could introduce contaminants. Sterilized water preparations also were examined under the same condition (negative controls).

Scanning electron microscopy (SEM).—Meltwater samples were prepared for SEM by filtering 5 mL water through a sterile 0.2 µm polycarbonate filter. The filter was fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) 1 h and rinsed three times (10 min each) in 0.1 M phosphate buffer followed by dehydration 10 min each in 40%, 60%, 80%, 95% and 100% (three times) ethanol. All

solutions used for processing meltwater samples were filter sterilized by passage through a 0.2 µm filter before use. A 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 with a Polaron E500 SEM coating unit. Samples were observed under SEM (Hitachi S-2700) for the presence of microorganisms. Control filters were processed in identical manners with sterilized water and were examined for the presence of any microorganisms that indicated contamination.

Culturing.—Meltwater collected from the surface-decontaminated ice core sections was used to assay for viable microorganisms by distribution and incubation on a variety of agar media to encourage the growth of fungi. Culturing was performed by spreading 200 µL meltwater from each shell onto duplicates of each media. The media included malt extract agar (1.28% maltose, 0.27% dextrin, 0.24% glycerol, 0.08% peptone, 1.5% agar [pH 4.7]), potato dextrose agar (0.4% potato starch, 2% dextrose, 1.5% agar [pH 5.6]), rose bengal agar (0.5% soytone, 1% dextrose, 0.1% monopotassium phosphate, 0.005% rose bengal, 1.5% agar [pH 7.2]), nutrient agar (0.3% beef extract, 0.5% 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]) and water agar (sterile distilled water with 2% agar). The inoculated agar plates were incubated at 8 C at least 2 wk, followed by 15 C at least 2 wk and then maintained at 22 C. Plates were examined for fungal growth weekly. Any isolates obtained from meltwater culturing were immediately subcultured to obtain pure cultures for molecular identification. Subcultures were tested for growth at 4, 8, 22 and 37 C. Triplicates of each isolate were cultured on MEA plates and incubated at the respective temperature. Mean growth rates were calculated based on colony diameter measurements after 2 wk incubation.

PCR amplification of isolates.—Nuclear ribosomal DNA (rDNA) internal transcribed spacers (ITS1 and ITS2) and 5.8S gene were amplified with primers ITS4 and ITS5 (White et al 1990). DNA was amplified with a GeneAmp PCR Reagent Kit (Applied Biosystems, Branchburg, New Jersey). Each reaction consisted of 5 µL cell suspension, 50 pmol each primer, 10 pmol each dNTP, 2U *Taq* DNA polymerase, 50 mM KCl, 1.5 mM MgCl₂, in 50 µL total volume. In some cases native *Taq* DNA polymerase (Fermentas Inc., Hanover, Maryland) was used. The thermal cycler (Mastercycler gradient, Eppendorf, Westbury, New York) program used was 95 C for 8 min, 40 cycles of 1 min at 94 C, 1 min 30 s at 54 C and 2 min at 72 C, followed by an incubation for 8 min at 72 C. PCR reactions

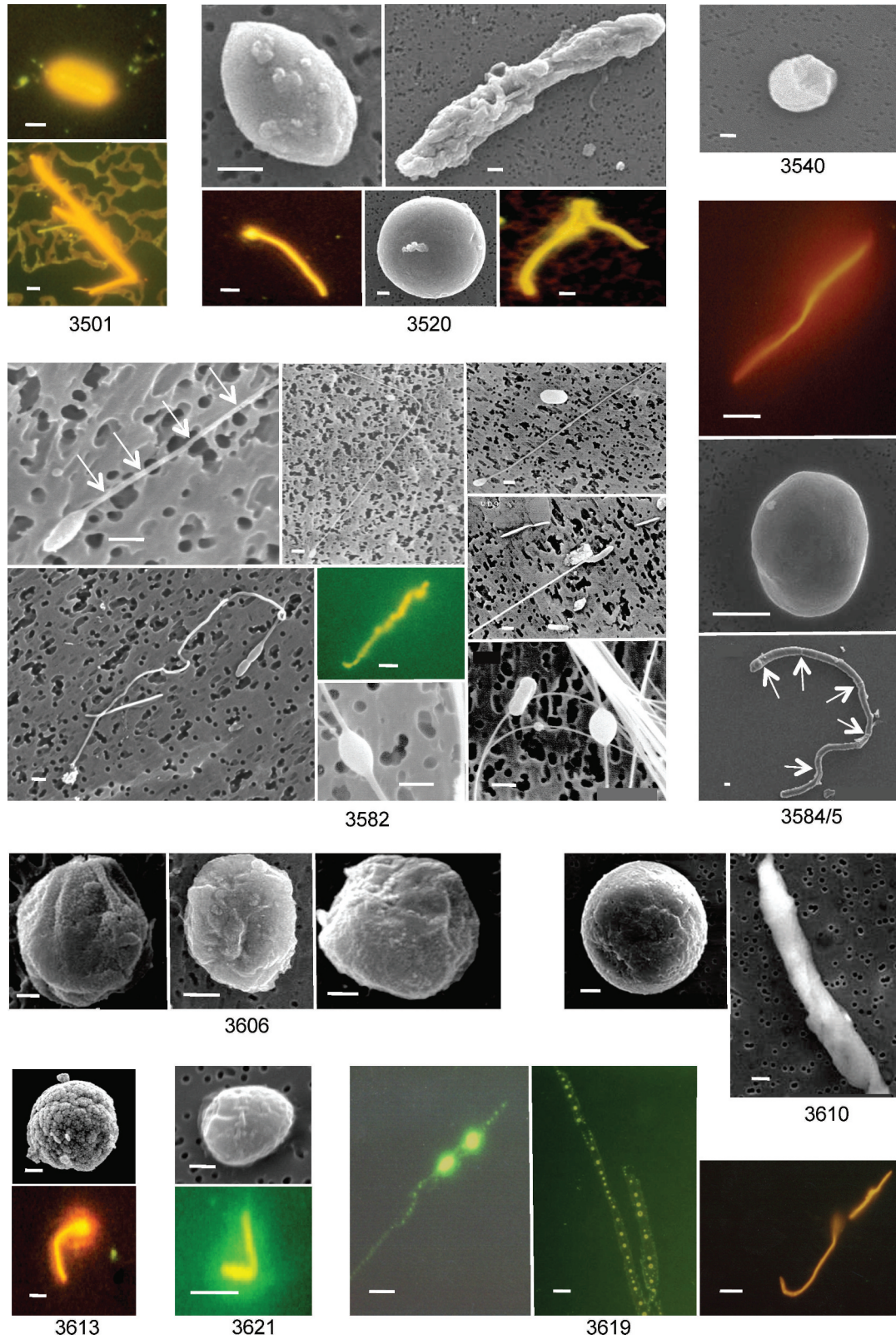


FIG. 1. Micrographs of cells found in the glacial (3501 and 3520 m) and accretion (3540, 3582, 3584, 3606, 3610, 3613, 3619 and 3621) ice core sections. No cells consistent with fungi were found in sections 3563 or 3591. Cells in the fluorographs were stained with a LIVE/DEAD BacLight Viability Kit (Molecular Probes, Eugene, Oregon). Green fluorescence indicates possible viable cells, based on membrane integrity. Red fluorescence indicates possible dead cells based on intracellular staining.

were viewed on 1.0% agarose gels, with TBE (90 mM Tris-borate, 2 mM EDTA, pH 8.0), and 0.5 $\mu\text{g mL}^{-1}$ ethidium bromide, illuminated with UV and photographed with a digital camera.

Cloning and sequencing.—PCR amplified ITS rDNA from the fungal isolates were inserted into a pCR 4-TOPO vector (TOPO TA Cloning Kit for Sequencing, Invitrogen, California) following the manufacturer's instructions. The reaction was set up as follows: 2.5 μL PCR product, 1.0 μL salt solution (200 mM NaCl, 10 mM MgCl_2) and 1.5 μL vector (10 ng μL^{-1}). The reaction was used to transform One Shot[®] TOP10 Competent *E. coli* cells as described by the manufacturer. Plasmid DNA was isolated from transformed cells with the Cyclo-Prep Plasmid DNA isolation kit (Amresco, Solon, Ohio) and analyzed for inserts by restriction digestion with *EcoRI*. Digested plasmids were analyzed by electrophoresis through a 1% agarose gel and viewed by ethidium bromide (0.5 $\mu\text{g mL}^{-1}$) staining and UV irradiation and fluorescence. Plasmids containing positive inserts were diluted to 50 ng μL^{-1} and sequenced commercially (Gene Gateway LLC, Hayward, California).

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

RESULTS

Meltwater analysis by microscopy.—Fluorescence microscopy revealed a wide variety of morphologically

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

Cultured isolates and DNA sequence analysis.—Culturing of meltwater from the two glacial ice and 10 accretion ice samples resulted in 284 colonies of fungi (TABLE I). No growth was observed on any control plates (sterile water or culture plates opened in the hood) that were incubated 12–24 mo. Only 14 colonies in total were isolated from deep glacial ice

←

Yellow is from the fluorescence of both dyes and thus is ambiguous. Bars represent 5 μm on fluorographs and 1 μm on electron micrographs. 3501: A spore and a possible branched hypha. 3520: Spores and germinating spores. The organism in the upper right 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 fact that we recovered no fungal isolates from this core section (FIG. 2). 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 their termini (as in upper left micrograph). Some had enlarged portions in the middle of the filaments (as in the lower center 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 (center). 3584/5: Filamentous structure and spherical cells were found. One of the filamentous structures was large (approximately 10 μm wide and 500 μm long) and appeared to have septa (lower, arrows). 3606: Only distorted spores were observed. This is consistent with partial grounding of the glacier in this region, causing increased and uneven pressures. Low cell viabilities were measured in this core section (FIG. 2). 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 organisms were filamentous. The two on the left exhibited what appear to be multiple nuclei as well as heavier fluorescence at two foci (leftmost micrograph). The filaments in the center micrograph also appear to be multinucleate. This core section exhibited a spike in the concentration of total cells and viable cells, although sequence diversity was low (FIG. 2). 3621: One spore and a germinating spore.

core sections, 3501 m and 3520 m. For eight of the accretion ice cores the number of total colonies was 0–11. The remaining two accretion ice sections from 3610 m and 3582 m respectively had the highest number of colonies, 72 and 169. The high number of colonies from core section 3582 was mainly distributed among three culture plates with the remaining plates having fewer colonies. This indicates the heterogeneity in these samples and might reflect a high concentration of microbes adhering to mineral inclusions entrapped in the ice. One of these accretion ice cores (3610 m) is from open water, while the other (3582 m) is in a transition zone that contains both type I and type II ice and is associated with the shallow embayment.

A total of 38 fungal cultures were selected for sequence analysis of ribosomal ITS regions. All sequences were obtained directly from cultured isolates (all attempts to PCR amplifying fungal ITS DNA directly from ice core meltwater were unsuccessful). Of these 30 were from at least four genera of basidiomycetes (27 from accretion ice and three from glacial ice), while eight were from at least five genera of ascomycetes (all from accretion ice). Sequences closest to *Rhodotorula mucilaginosa* were the most frequently observed. A total of 26 *Rhodotorula* ITS sequences were obtained for isolates from every accretion ice core section except 3540 m and 3585 m (isolates GI822, GI902-904, GI908-911, GI914, GI926-929, GI931, GI933, GI939, GI945-949, GI951, GI952, GI959, GI962 and GI966). Three additional isolates were collected from the deep glacial ice core, section 3501 m (GI966, GI962 and GI959). Isolates related to *Rhodotorula* were most abundant in the 3582 m core section, which formed over the embayment. Percent similarity scores for the *Rhodotorula* isolates were 93–100% compared to taxa in NCBI. Isolates of *Rhodotorula* were the only fungi found in the glacial ice as well as the accretion ice. All other isolates were found only in the accretion ice. Isolates initially were collected from a variety of media, including minimal nutrient media, after incubation of 15 d to more than 9 mo. *Rhodotorula* isolates showed optimal growth at 22 C but also grew well at 15 C and 4 C (TABLE II), indicative of psychrotolerance. Other basidiomycetes included a single isolate (GI913) belonging to genus *Cystofilobasidium* (96% similarity to GenBank) and two isolates (GI817 and GI944) that are most closely related to members of genus *Cryptococcus*. A final basidiomycete was isolate GI895 that was closest to species of *Pseudozyma* and *Ustilago* and was psychrotolerant (TABLE II).

A total of seven ascomycete isolates were identified in six (3563, 3582, 3585, 3610, 3613 and 3619 m) of

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

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

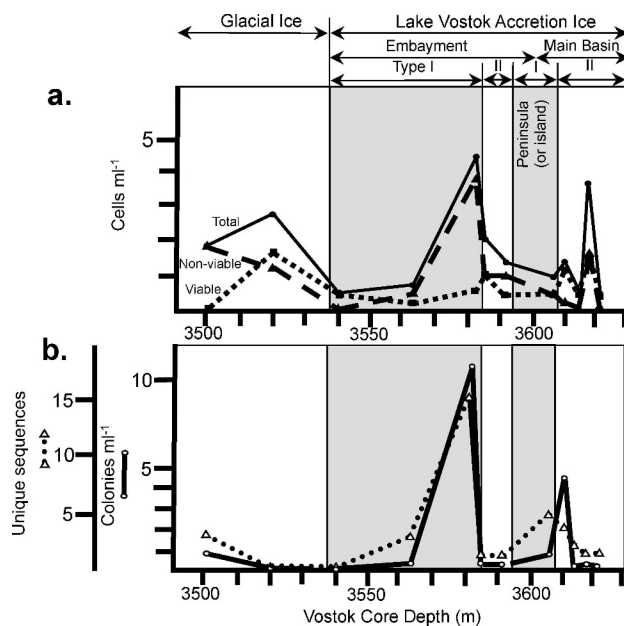


FIG. 2. a. Cell concentrations in the ice core sections that were studied. Calculations were based on counts of fluorescently stained cells viewed by microscopy from 10 mL meltwater from the two glacial and 10 accretion ice core sections. Cells were counted as viable if they fluoresced bright green to yellow. Cells were considered nonviable if they fluoresced orange or red. The regions of the lake are represented in each ice core section and the ice characteristics (type I and type II) are indicated at the top. The highest concentrations of cells and viable cells are in the areas near the transitions from type I to type II, as well as in type II from the main basin. b. Number of fungal colonies from ice core section meltwater. Also shown is the number of unique sequences determined from each ice core section. The peaks are near the type I to type II transition zones.

accretion ice core section 3582 m grouped closely together, while isolates from core sections 3606 m and 3501 m were more widely distributed throughout the *Rhodotorula* subclade. Another basidiomycete, isolate GI913 (3606 m), was closest to genus *Cystofilobasidium*, grouping with the cold-adapted yeast *Cystofilobasidium capitatum*. Isolates GI817 and GI944 grouped with antarctic isolates of *Cryptococcus* and GI895 was closest to antarctic isolates of *Pseudozyma*.

The ascomycetes in the phylogram are fewer in total number (FIG. 3). Isolate GI920 displayed strong affinity to species of *Penicillium* from Antarctica, glacial and deep sea environments and was in the same clade as a Greenland glacial isolate (*P. griseoroseum*). Isolates GI898 and GI932 grouped respectively with marine members of *Aspergillus* and *Aureobasidium*. 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 y old Greenland glacial ice (Ma et al 2000) as well as other *Cladosporium* isolates from Antarctica.

DISCUSSION

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

Seasonal extremes in Antarctica, along with the variety of habitats on the continent, leads to the selection of microorganisms that use a generalist survival strategy that includes psychrotolerance (Vincent 2000). The majority of the isolates obtained from all ice core sections were isolated originally at 15 C, and all isolates tested were determined to be psychrotolerant (TABLE II). Psychrotrophic fungi capable of growth at 0–25 C have been isolated from a variety of polar habitats (Bergero et al 1999, Tosi et al 2002, Ozerskaya et al 2004, Ma et al 2000). Studies of prokaryotic isolates from Antarctica have found that the optimal growth temperature is often higher than expected for the environment but is typically lower compared to similar taxa from nonpolar environments (Franzmann et al 1996). This is consistent with fungi isolated from Vostok accretion ice. The fungal component from the accretion ice is composed of psychrotolerant species (TABLE II), based on the examination of the isolates obtained from accretion

TABLE I. Summary of accretion ice data for fungal isolates

Region	Depth (m)	Percent viable cells ^a	Number of colonies ^b	Unique sequences	Closest taxon in BLAST analyses ^c [% similarity]	Characteristics of closest taxon ^d
Glacial ice	3501	0	14	3	<i>Rhodotorula mucilaginosa</i> [97–99]	atm, ant, aqu, gla, soi
	3520	59	0	0	None	—
Accretion Embayment Grounded	3540	100	0	0	None	—
	3563	33	3	3	<i>Rhodotorula mucilaginosa</i> [99] Unclassified soil fungus [69]	ant, aqu, atm, gla soi asc, unk
Open water	3582	85	169	9	<i>Aureobasidium pullulans</i> [95] <i>Rhodotorula</i> [94–95] <i>Rhodotorula mucilaginosa</i> [95–99] Unknown fungus [92] Unclassified soil fungi [97–99]	frw, gla, sal, per, psy ant, aqu, atm, gla, soi ant, aqu, atm, gla, soi ant, asc, gal, psy, lak, soi ant, asc, atm, gla, sno, soi, tol ant, gla, lks, pnd, soi, tol
	3585	50	6	1	<i>Penicillium</i> [87–88]	
Peninsula Main basin Grounded	3591	71	2	1	<i>Rhodotorula mucilaginosa</i> [98]	ant, aqu, atm, gla, soi
	3606	50	11	5	<i>Cystoflabasidium informiniatum</i> [96] <i>Rhodotorula mucilaginosa</i> [95–100] <i>Sporidiobolales</i> [90–98] Unclassified soil fungus [95] <i>Rhodotorula mucilaginosa</i> [95] <i>Aspergillus</i> [84] Unclassified soil fungi [92–94] <i>Cryptococcus</i> [89] <i>Penicillium</i> [85] ^e <i>Rhodotorula</i> [93] ^e <i>Pseudozyma</i> [94]	ant, gla, oce, sno, soi ant, aqu, atm, gla, soi ant, aqu, frw, oce ant, asc, atm, sno, soi, tol ant, aqu, atm, gla, soi ant, atm, gla, soi ant, asc ant, sno, soi ant, gla, lks, pnd, soi ant, aqu, per, pol, tol, sno, soi ant, lks
Open water	3610	14	72	4		
	3613	0	3	2		
	3619	51	3	3		
	3621	0	1	1		

^aBased on cell counts from 10 mL of concentrated ice meltwater with LIVE/DEAD staining.

^bCultures of fungi on all media.

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

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

^ePartial sequence, not included in phylogenetic analysis.

TABLE II. Mean growth (mm per day \pm sd) of fungal isolates collected from glacial and accretion ice core sections. Each isolate was tested at four temperatures. Rates are given as the average of triplicate testing. Maximum rates are indicated by bold type, NG indicates no growth observed. All were grown on MEA medium

Closest taxon in BLAST analyses Fungi (GI number)	Core depth (m)	Temperature			
		37 C	22 C	15 C	4 C
<i>Aspergillus niger</i>	Control	4.2 \pm 0.00	4.3 \pm 0.00	2.3 \pm 0.06	NG
<i>Pseudozyma</i> (GI895)	3621	1.21 \pm 0.06	3.00 \pm 0.06	2.81 \pm 0.06	0.28 \pm 0.12
<i>Penicillium chrysogenum</i> (GI920)	3585	1.29 \pm 0.06	3.83 \pm 0.17	2.24 \pm 0.46	0.34 \pm 0.11
<i>Penicillium chrysogenum</i>	Control	0.6 \pm 0.03	1.4 \pm 0.00	0.9 \pm 0.00	NG
<i>Rhodotorula mucilaginosa</i> (GI911)	3606	0.64 \pm 0.07	1.25 \pm 0.00	1.02 \pm 0.00	0.57 \pm 0.00
<i>Rhodotorula mucilaginosa</i> (GI914)	3591	0.51 \pm 0.00	1.25 \pm 0.00	1.10 \pm 0.03	0.51 \pm 0.00
<i>Rhodotorula mucilaginosa</i> (GI928)	3582	0.55 \pm 0.07	1.04 \pm 0.09	0.97 \pm 0.06	0.55 \pm 0.03
<i>Rhodotorula mucilaginosa</i> (GI952)	3563	0.76 \pm 0.03	1.37 \pm 0.00	1.14 \pm 0.11	0.59 \pm 0.03
<i>Rhodotorula mucilaginosa</i> (GI959)	3501	0.40 \pm 0.00	1.04 \pm 0.03	0.95 \pm 0.09	0.59 \pm 0.03
<i>Saccharomyces cerevisiae</i>	Control	0.99 \pm 0.03	1.00 \pm 0.07	0.68 \pm 0.00	NG
Uncultured soil fungus (GI944)	3582	0.21 \pm 0.07	1.14 \pm 0.00	0.95 \pm 0.03	0.61 \pm 0.03

ice. Even though accretion ice isolates were found related to ubiquitous genera, such as *Penicillium* and *Rhodotorula*, a distinction is observed in the phylogenetic separation and BLAST percent similarities compared to other taxa (TABLE I, FIG. 3).

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

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

(Butinar et al 2007), which might explain the frequency of *Rhodotorula* in the accretion ice from each region of Lake Vostok.

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

Ascomycetes isolated from the accretion ice also were found to be similar to taxa from other polar environments. Two isolates that grouped phylogenetically with *Cladosporium* were obtained from 3610 m and 3582 m. *Cladosporium* is a prevalent genus of fungus worldwide. Analysis of 140 000 y old GISP-2D glacial ice recovered viable *Cladosporium* isolates (Ma et al 2000). *Cladosporium* is the second most frequently trapped airborne fungal spore in Antarctica (Marshall 1997). It is possible therefore that *Cladosporium* spores were aeri ally deposited in the overlying glacier and have been able to remain viable over the long period of delivery to the lake. Isolate

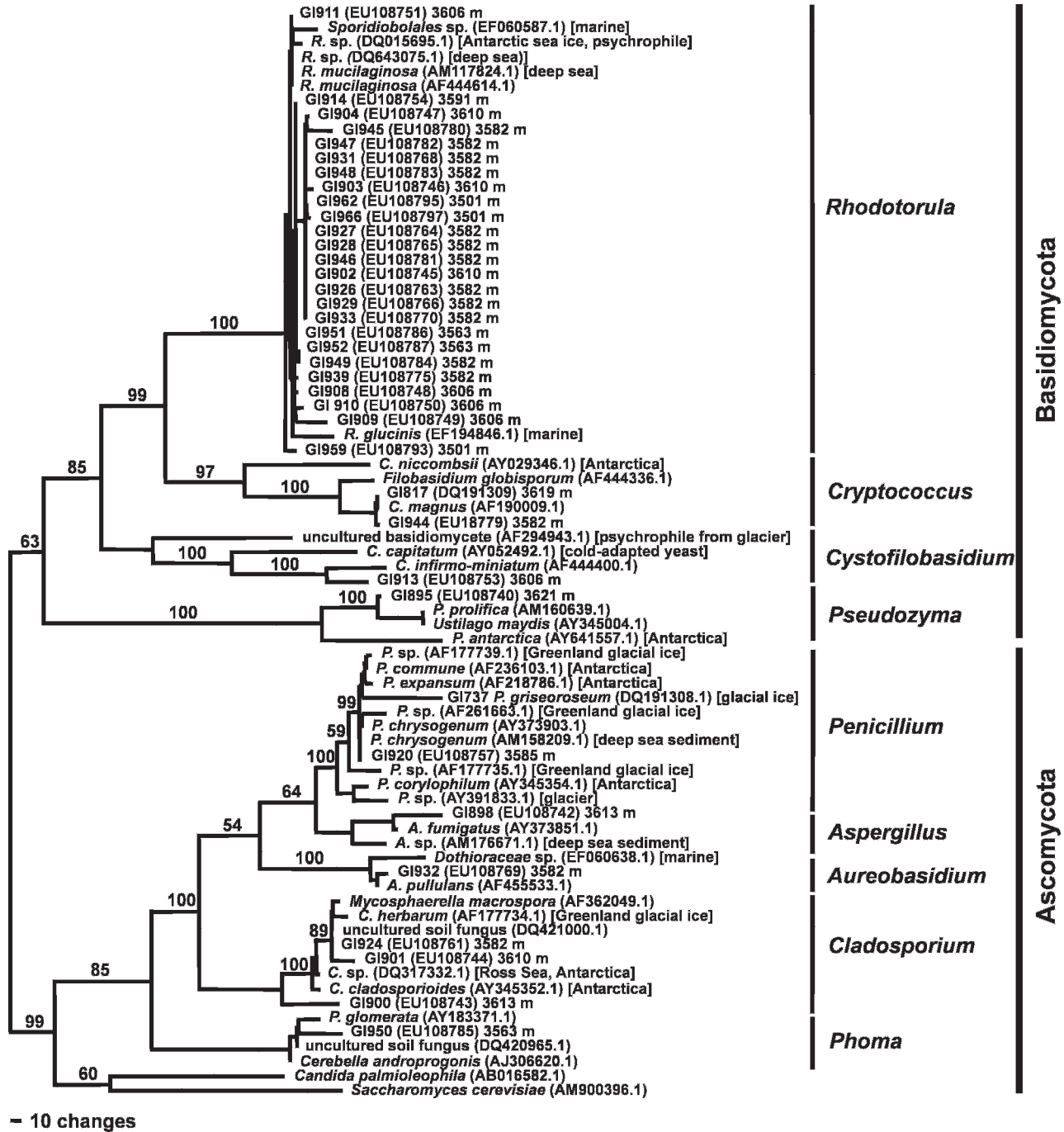


FIG. 3. Phylogram of rRNA ITS sequences (with maximum parsimony on PAUP, Swofford 1999) from glacial and accretion ice fungal isolates (alignment and tree on TreeBase, accession numbers S2238, M4443). Taxonomic groupings are indicated on the right. The primary basidiomycetes are species of *Rhodotorula*. This is the only genus that was isolated from both glacial and accretion ice sections. All sequences that have the prefix GI are isolates from the ice core sections. NCBI accession numbers (in parentheses) and depths (in meters below the glacial surface) are provided for each. The sequences closest to the unknown isolates in this study were chosen for inclusion in the phylogenetic analyses. GI737 was isolated in our lab from a Greenland ice core (Dye 3) at a depth corresponding to ice that was 5500 y old and is included for comparison. Sources and environments are included in square brackets (when available). Bootstrap values (1000 replications) are provided for branches with greater than 50% support.

GI920 was closest to *Penicillium* spp. (88% ITS similarity) and was from 3585 m meltwater. Species belonging to *Penicillium* have been isolated from polar glacial ice (Ma et al 1999, 2000), and many species have been found in arctic glacial and subglacial environments (Sonjak et al 2006, 2007). Considering the long period of time that Lake Vostok has been isolated, the finding of a viable *Penicillium* isolate with low percent sequence similarity indicates that the lake potentially is harboring a set of *Penicillium* species that may be uniquely adapted to this extreme subglacial environment. Accretion ice isolate GI950 grouped within the subclade containing *Phoma* (FIG. 3). *Phoma* species have been identified in glacial ice (Ma et al 1999) and isolates from arctic soils have been characterized as psychro-oligotrophic (Bergero et al 1999). The ability to tolerate both the cold and oligotrophic conditions in Lake Vostok make species of *Phoma* potentially compatible with conditions in the lake. Another ascomycete isolated from 3582 m (GI932) was identified as *Aureobasidium pullulans*. This species has been isolated from arctic and antarctic environments (Starmer et al 2005, Tosi et al 2002).

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

high diversity of microorganisms, including fungi. This indicates that Lake Vostok might be a complex ecosystem instead of a sterile body of water.

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