Evidence for propagation of cold-adapted yeast in an ice core from a Siberian Altai glacier

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[1] Cold environments, including glacier ice and snow, are known habitats for cold-adapted microorganisms. We investigated the potential for cold-adapted yeast to have propagated in the snow of the high-altitude Belukha glacier. We detected the presence of highly concentrated yeast (over 10^4 cells mL^{-1}) in samples of both an ice core and firn snow. Increasing yeast cell concentrations in the same snow layer from July 2002 to July 2003 suggests that the yeast cells propagated in the glacier snow. A cold-adapted *Rhodotorula* sp. was isolated from the snow layer and found to be related to psychrophilic yeast previously found in other glacial environments (based on the D1/D2 26S rRNA domains). 26S rRNA clonal analysis directly amplified from meltwater within the ice core also revealed the presence of genus *Rhodotorula*. Analyses of the ice core showed that all peaks in yeast concentration corresponded to the peaks in indices of surface melting. These results support the hypothesis that occasional surface melting in an accumulation area is one of the major factors influencing cold-adapted yeast propagation.

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1. Introduction

[2] Ice cores from polar regions provide information about atmospheric composition and climate changes of the past glacial-interglacial cycles [*Petit et al.*, 1999; *Watanabe et al.*, 2003; *Augustin et al.*, 2004]. Similarly, ice core studies of samples obtained from high-altitude glaciers in mountains located at low latitudes and midlatitudes are important in order to understand natural changes in climate and environment [*Thompson et al.*, 1997, 2000]. Furthermore, ice cores are also important for interpreting past microbiological diversity. Bacteria, fungi, and algae are present in ice cores from polar ice sheets and mountain glaciers [*Abyzov et al.*, 1998; *Ma et al.*, 1999, 2000; *Yoshimura et al.*, 2000; *Christner et al.*, 2003; *Uetake et al.*, 2006; *Zhang et al.*, 2006, 2009; *Miteva et al.*, 2009]. In temperate regions, snow algae can accumulate and multiply on glacier surfaces in accumulation areas because of the availability of meltwater, which is essential for their growth. Annually, these cells are presumably buried by snow accumulation. Therefore, the depth distribution of snow algae in an ice core show the location of summer layers and these act as annual markers for ice core dating [*Yoshimura et al.*, 2000; *Shiraiwa et al.*, 2001; *Uetake et al.*, 2006; *Kohshima et al.*, 2007; *Santibañez et al.*, 2008].

[3] Recently, studies of cold-adapted (psychrophilic, psychrotolerant) yeasts in glaciers have become frequent. Psychrophilic yeasts have been isolated from supraglacial and subglacial ice in Svalbard [*Butinar et al.*, 2007], Austrian glacier ice [*Margesin et al.*, 2007, 2009], Italian subglacial meltwater [*Buzzini et al.*, 2005], supraglacial and subglacial ice and meltwater from the Italian Alps [*Turchetti et al.*, 2008], glacial and subglacial waters from northwest Patagonia [*Brizzio et al.*, 2007; *De García et al.*, 2007], and an Antarctic deep ice core [*Amato et al.*, 2009]. At low temperatures (1–4°C), these isolates are able to grow and have ability for phenol degradation [*Margesin et al.*, 2007], undertake extracellular enzymatic activity [*Brizzio et al.*, 2007; *De García et al.*, 2007; *Turchetti et al.*, 2008], and degrade organic macromolecules [*Buzzini et al.*, 2005]. Isolates from deep ice cores have been shown to be metabolically active due to H^3-leucine incorporation under frozen conditions (−5°C) [*Amato et al.*, 2009].

[4] However, these cold-adapted (psychrophilic, psychrotolerant) yeasts had not been reported from accumulation snow on glacier, perhaps due to difficulty to access there. Therefore, their propagation, ecology and seasonal change are still unknown. This study focused on determining possible yeast propagation and their genetic information in the higher accumulation areas of a glacier to clear hypothesis

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yeast also propagate in specific season like a snow algae and to become some markers for ice core study.

2. Methods

2.1. Ice Core and Pit Sampling

[5] In July 2002 and July 2003, we excavated 3 and 4 m deep pits, respectively, on the western plateau of the Belukha glacier (49°49′ N, 86°34′ E; 4100 m above sea level) located on the west side of the summit of the Belukha mountain in the Russian Altai (Figure 1). Samples were obtained at 4–10 cm intervals using ceramic knives and were packed into sterilized plastic bags. The location of 2003 pit is just next of previous year. In July 2003, a 171 m long ice core was excavated at the same site. The details of the drilling of the ice core and the glaciological and meteorological observations at the drill site were described by *Takeuchi et al.* [2004] and *Fujita et al.* [2004]. Surface 48.1 m of 171 m long ice core contains 88 annual layers from 1914 to 2003 and average annual accumulation is 464 mm w.e. from 1915 to 2000 (Okamoto et al., manuscript in preparation, 2011). Both the pit and ice core samples were transported in a frozen state from the glacier to the laboratory of the Research Institute of Humanity and Nature in Kyoto, Japan, and thereafter stored in a freezer. The pit samples were melted in the laboratory and dispensed into clean plastic bottles in a class 100 clean bench. The ice core samples were cut with a band saw at approximately 9–34.5 cm intervals, and 1 cm of the core surface was scraped off with precleaned ceramic knives to remove contamination, using the same process as was used for dust samples from the Dome Fuji ice core [*Fujii et al.*, 2003]. The samples were then packed into sterilized plastic bags (Whirl-Pak, Nasco, United States). After melting, the samples were dispensed into 15 mL centrifuge tubes for isolation and genetic analysis (Iwaki, Japan) and into clean plastic bottles for cell and pollen counts using a clean bench.

2.2. Microscopic Observation of Microorganisms and Pollen Grains

[6] Melted snow and ice samples (10–15 mL) containing pollen and microorganisms were filtered through a hydrophilic polytetrafluoroethylene membrane (Omnipore pore size 0.22 μm; Millipore, United States). We used a glass vacuum filter holder (KGS-04; Advantec, Tokyo, Japan) that can filter a concentrated area (ϕ = 4 mm) of membrane for measuring cells at low concentrations. We counted the number of pollen grains and microorganisms accumulated on the surface of the filters under a fluorescent microscope (E600; Nikon). All pollen grains in the filter area were counted; microorganism cells were counted in 20 fields of view.

2.3. Isolation and Phylogenetic Analysis of Yeasts

[7] To isolate the yeasts, we selected 5 samples at different depths from layers where yeasts were abundant from direct cell counts. A total of 200 μL of the melted ice core samples was inoculated on yeast extract-peptone-dextrose (YEPD) agar medium and incubated at 4°C for 3 months. The 26s rRNA D1/D2 domains of the resulting isolated strain and extracted/present in cells from the melted ice core samples were then amplified. All the manipulations for phylogenetic analysis before PCR were conducted within a class 100 laminar flow

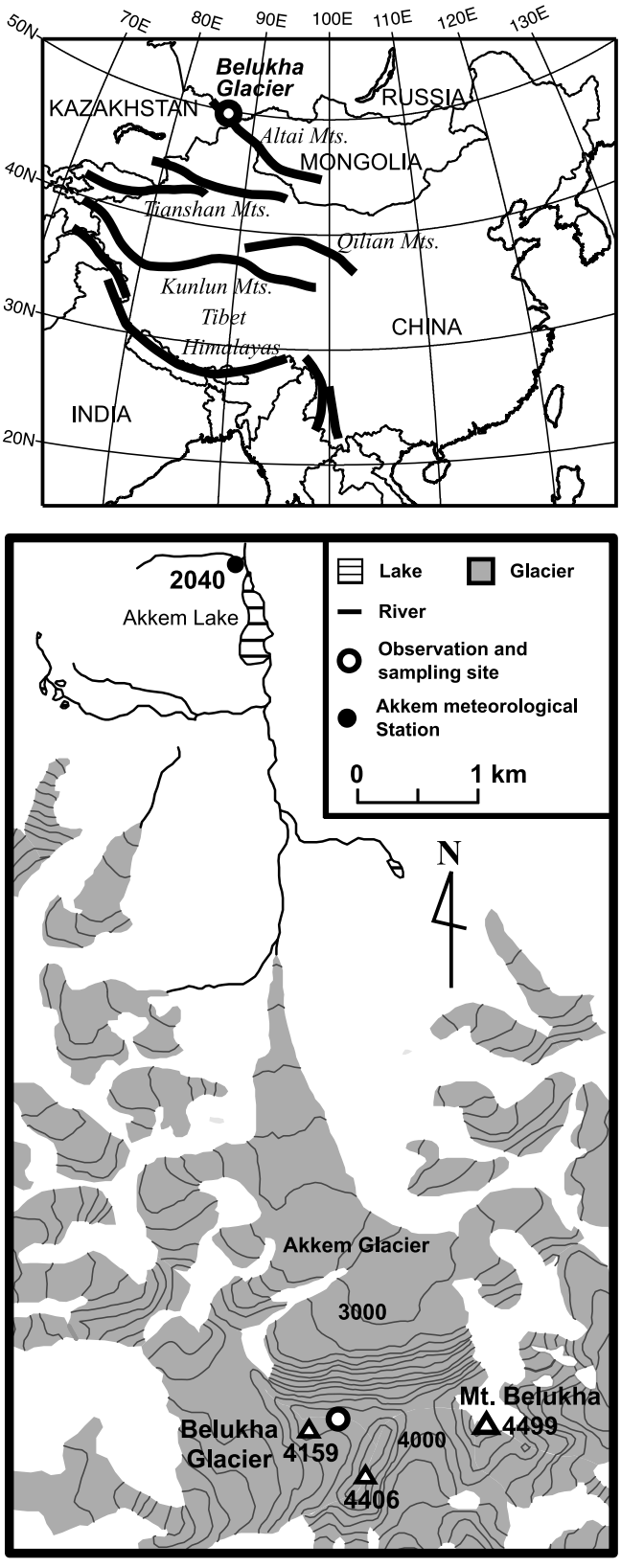

Figure 1. Location of Mt. Belukha and the Akkem meteorological station in Siberian Altai and sampling sites on the glacier.

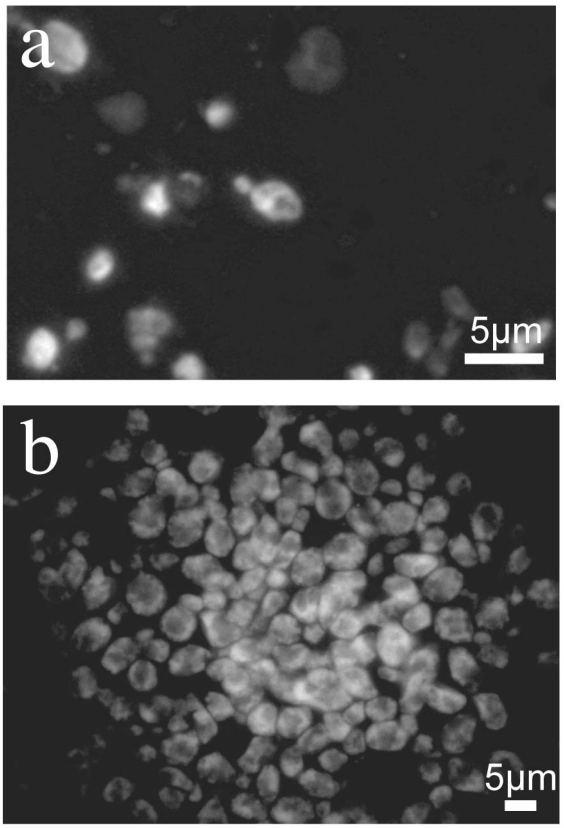

Figure 2. Yeast cells observed in the ice core. (a) Small from depth 5.85–6.07 m and (b) medium-sized from depth 7.15–7.31 m.

clean bench to avoid contamination. For the isolated strain, a colony was removed from the agar medium with a sterile pick and directly added to the PCR mixture. PCR amplification was performed with Ex Taq DNA polymerase (Takara, Shiga, Japan) using the primer pair NL1 (5′-GCA TAT CAA TAA GCG GAG GAA AAG-3′) and NL4 (5′-GGT CCG TGT TTC AAG ACG G-3′), following which the PCR products were sequenced with a 3130xl Genetic Analyzer (Applied Biosystems, California) at the National Institute of Polar Research (NIPR). For molecular cloning, we selected 11 samples from the layers containing high concentrations of yeast cells and added 38.75 μL of the melted ice core samples to PCR mixtures containing 5 pmol of the primer pair [*Christner et al.*, 2001]. PCR amplification was performed in the same way as for the isolated strain. The PCR-amplified DNA fragments from the melted ice core samples were then cloned into the pCR4 vector of the TOPO TA cloning kit (Invitrogen, Carlsbad, California) according to the manufacturer's instructions. The clones obtained from the libraries were sequenced using the same genetic analyzer. Alignment of DNA sequences was performed with Clustal W in Geneious 4.5.2 software. A neighbor-joining analysis was performed and bootstrap consensus trees (1000 pseudoreplicates) were generated using Mega 4.0.2.

2.4. Stratigraphic Observation

[8] The ice core was cut in half lengthwise using a band saw in a low-temperature laboratory, and the melt layers in the horizontal cut section were observed on a light table. The thickness of the ice, infiltration ice and infiltration-recrystalization ice [*Shumskii*, 1964] layers was selected as an index of surface melting. The ratio of the ice and infiltration ice layers to the snow or compressed snow layers in each 30 cm section was calculated as the melt feature percentage (MFP) [*Kameda et al.*, 2004], and the ratio of the infiltration-recrystalization ice layers was calculated as infiltration-recrystalization ice percentage (IRP).

2.5. Measurement of Dissolved Organic Carbon

[9] Each 30 cm of pit samples from 2003 (above 3 m in depth) were measured for organic carbon by TOC analyzer (TOC-V, Shimazu, Kyoto, Japan) in Sumika Chemical Analysis Service, Ltd. Dissolved organic carbon was estimated the subtraction inorganic carbon from total carbon. The samples of dissolved organic carbon (DOC) may contain some of contamination from the sampling process, because all sample snow are once packed and melted in plastic bag (Whirl-Pak, Nasco, United States) and dispensed to preclean glass vials. Therefore, we had measured the control sample which 200mL of ultrapure water in same plastic bag. Control water were strongly shaken by hands for one minutes and dispensed into preclean glass vials.

2.6. Measurement of Particles

[10] Particle concentration was measured in NIPR by laser particle counter (Met One Model-211 with high concentration sensor) as following the method of *Fujii et al.* [2003].

3. Results

3.1. Yeast Concentrations in the Pit and Ice Core Samples

[11] Yeasts were observed in both the pit and ice core samples excavated in 2003. Yeast cells included two morphotypes with cell width by length measurements of 1–2.5 μm × 1–5 μm (small) and 3–5 μm × 3–8 μm (medium) (Figure 2). In the 2003 pit, the yeasts were highly concentrated in layers at depths of 1.9–2.5 m (maximum 1.6×10^4 cells mL^{-1}). The layer in which the cell concentration was highest in the 2003 pit was 40 times higher than the same stratigraphic layer excavated in 2002 (correspond to about surface −80 cm: maximum 4.2×10^2 cells mL^{-1}) (Figure 3). In the 2003 ice core, small yeasts were the primary morphotype observed and these were highly concentrated (over 2 × standard deviation) in layers at 1.64–1.86, 4.4–5.13, 5.41–6.73, 7.46–7.72, 18.59–18.75, 24.52–24.81 m depth (Figure 4).

3.2. Isolation

[12] We selected 5 samples from layers where yeasts were abundant in the ice core. However, we could successfully isolate only 1 species of yeast (*Rhodotorula* sp. BL58 [AB474390]), which was taken from a depth of 6.29–6.408 m. The colonies of the isolated yeast were round, convex, and pinkish. The cells were ellipsoidal (2–4 μm and 3–8 μm) and displayed a polar budding pattern. These cells were similar to those of the yeasts observed in the ice core sample.

3.3. Molecular Analysis

[13] The isolate was closely related (97.2%) to the *Rhodotorula psychrophila* strain A12 (EF151256), which is a psy-

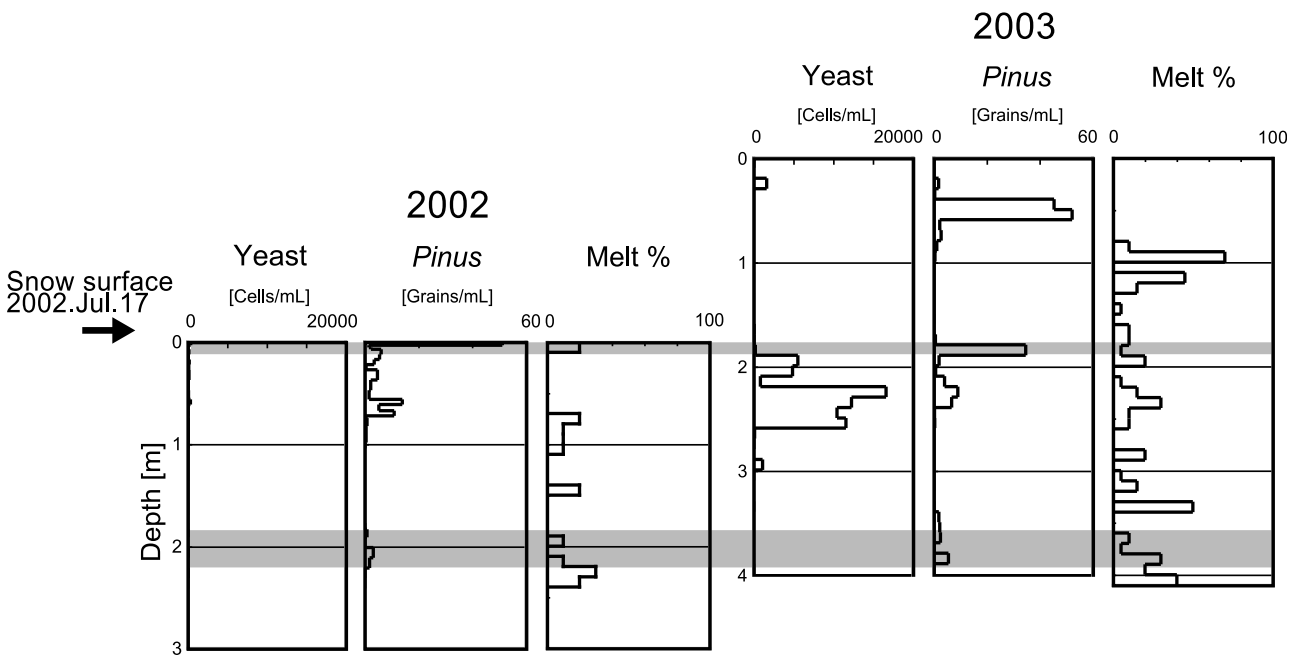

Figure 3. Vertical profiles of yeast cell concentration, *Pinus* pollen, and MFP in the 3 m and 4 m pits on the Belukha glacier excavated in (left) July 2002 and (right) July 2003, respectively. The gray areas are drawn on the basis of the *Pinus* pollen profile.

chrophilic species isolated from an Austrian glacier [*Margesin et al.*, 2007] (Figure 5). For molecular cloning, we selected 11 samples from the layers containing the highest concentrations of yeast cells and 5 of the 11 samples were successfully amplified. Finally, 3 yeast sequences (BL58–2 and BL54, BL58–1) were obtained from 2 of the 11 samples (BL54: 6.012–6.08 m and BL58: 6.29–6.408 m). The BL58–2 clone was closely related (97%) to the *Rhodotorula psychrophenolica* strain DBVPG 4792 (EF643736), which was isolated from an Italian glacier [*Turchetti et al.*, 2008]. This clone shared 96.4% similarity (541/561 bp) with the strain isolated in this study (*Rhodotorula* sp. BL58 [AB474390]). On the other hand, clones BL54 and 58–1 were isolated phylogenetically and closest species is Agaricomycotina, *Exidiopsis grisea* [AY885167] with 88% similarity (580/654 bp).

3.4. Dissolved Organic Carbon

[14] Concentrations of two control samples are 0.5 and 0.9 mg L^{-1}. We had define the lowest limit of this analysis is 1.9 mg L^{-1} (twofold of mean control value). Concentration of DOC from pit sample above 3m depth is higher than 1.9 mg L^{-1} in the layer from 30 −150 cm depth, and highest (8.3 mg L^{-1}) in the layer from 30–60 cm depth (Figure 6).

4. Discussion

4.1. Propagation of Yeasts in Glacier Snow

[15] Both the isolate and clones of the yeast cells taken from the layers of the ice core that had high concentrations of yeast were closely related to psychrophilic yeasts reported previously based on 26S rRNA molecular analysis (Figure 5). Psychrophilic yeasts have been isolated from many low-temperature environments (1–8°C), such as glaciers and soils, in cold regions from around the world [*Ma et al.*, 1999; *Buzzini et al.*, 2005; *Margesin et al.*, 2005; *Starmer et al.*, 2005; *Brizzio et al.*, 2007; *Butinar et al.*, 2007; *Margesin et al.*, 2007; *Turchetti et al.*, 2008; *Amato et al.*, 2009; *Thomas-Hall et al.*, 2010]. *Rhodotorula psychrophila*, which is closely related to the isolate found in this study, has been confirmed to grow at 1°C in a laboratory [*Margesin et al.*, 2007]. Our observations revealed that the snow temperature at our drill site rose to 0°C during summer [*Fujita et al.*, 2004], allowing meltwater to percolate into the substrate.

[16] The observations in 2002 and 2003 showed that the yeast cell concentration at a depth of 1.9–2.5 m in 2003 was 40 times higher than that at the same stratigraphical layer in 2002 (4.2×10^2 versus 1.6×10^4 cells mL^{-1}, Figure 3). This increase in yeast cell concentration is most likely due to in situ propagation of the yeasts because the difference is significantly larger than that observed for other pit layers between 2002 and 2003. This fact would supports the hypothesis that these yeasts actually grew in the glacier snow.

[17] The yeast distribution can be influenced by the DOC concentration in sample. Take into account the high DOC concentration of control samples. DOC is high in the layers from 30 cm to 150 cm. These layers correspond to the layers had contain almost no yeast cells. Otherwise, high yeast concentration layers from 1.9m to 2.7 m correspond to low DOC concentration as low as control samples. If heterotrophic microorganisms had propagated, DOC may decrease by uptake of microorganisms. The low concentration of DOC in high yeast layers may also be a one of the evidence of yeast propagation in the snow.

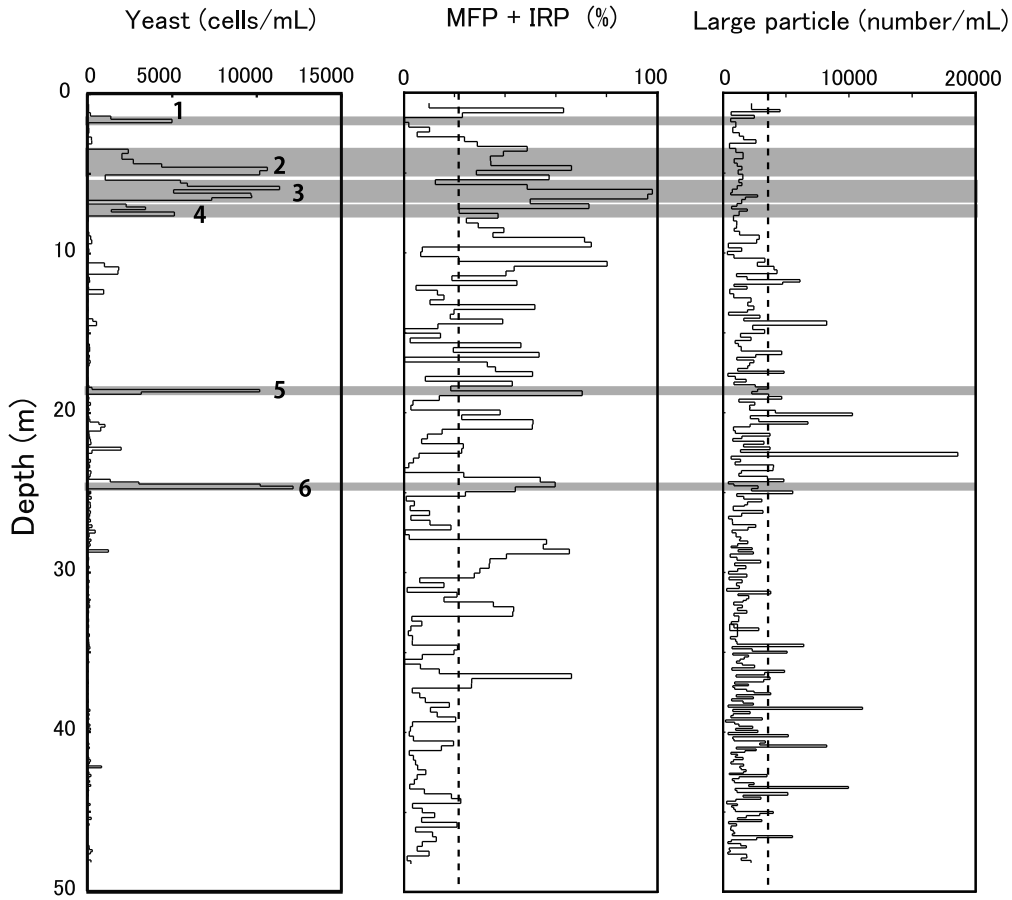

Figure 4. Vertical profiles of yeast cell concentration and MFP and large particle (diameter from 5.7 to 16 μm) in a 48.1 m long ice core. The gray area show that the significantly high concentration peak (over 2 × standard deviation) of yeast. Vertical dot lines in MFP and large particle show that the value of 2 × standard deviation of each.

4.2. Factors Affecting the Annual Variation in Yeast Cell Concentration

[18] All layers showing high concentrations of yeast cells in the ice core corresponded to layers with high MFP + IRP anomalies. There were 6 prominent peaks (over 2 × standard deviation) of yeast cell concentration in the ice core. All 6 yeast peaks were located in layers close to the high MFP + IRP layers (over 2 × standard deviation). High MFP + IRP anomalies show that location of surface melting, because melt formation must be formed by not the compression of snow (depth of firn-ice transition, which the density is close to the 0.9 kg m^{-2}, is around 50 m below the surface) but the melting. In the lower 18.1 m section (30–48.1 m), there is no yeast peak in spite of 6 peaks upper 30 m (0–30 m). These are clearly related to the frequency of surface melting and the age correspond to the lower section is colder than that in upper. The correspondence between the yeast and MFP + IRP peaks suggests that melting of surface snow is one of the major factors influencing cold-adapted yeast propagation, and their growth season is summer.

[19] The high concentration of yeasts in the snow layers may also be due to a large supply of airborne yeast cells from an environment other than the glacier. However, if yeast cells were transported by wind, airborne dust particles as large as yeast would also be highly concentrated in yeast high layers. Yet the depths of the peaks of yeast cell concentrations did not agree with those of the peaks of airborne dust particles which diameter from 5.7 to 16 μm in the ice core (Figure 4). Therefore, airborne yeast cells are not likely to have been responsible for the high yeast cell concentrations observed in the ice core.

[20] This study shows that even in a high-altitude accumulation area, cold-adapted yeast can propagate when there is surface snowmelting. However, yeast had not propagated in every high MFP + IRP anomalies, many other factors could relate to the life cycle of cold-adapted microorganisms in snow. Therefore, we could not utilize yeast growth layer for the seasonal marker of every summer layers as previous studies [*Yoshimura et al.*, 2000; *Shiraiwa et al.*, 2001; *Uetake et al.*, 2006; *Kohshima et al.*, 2007; *Santibañez et al.*, 2008]. Most of these previous studies were of ice cores that experience more frequent melt conditions than our site, because they are lower in altitude and latitude. In contrast, our site has less frequent melt conditions and is, therefore, a more extreme environment. Do to these differences, we are unable to discuss annual variation of these yeast cells or recent climate change, and feel that further study is necessary to understand factors that affect the propagation of cold-adapted yeast and their ecology.

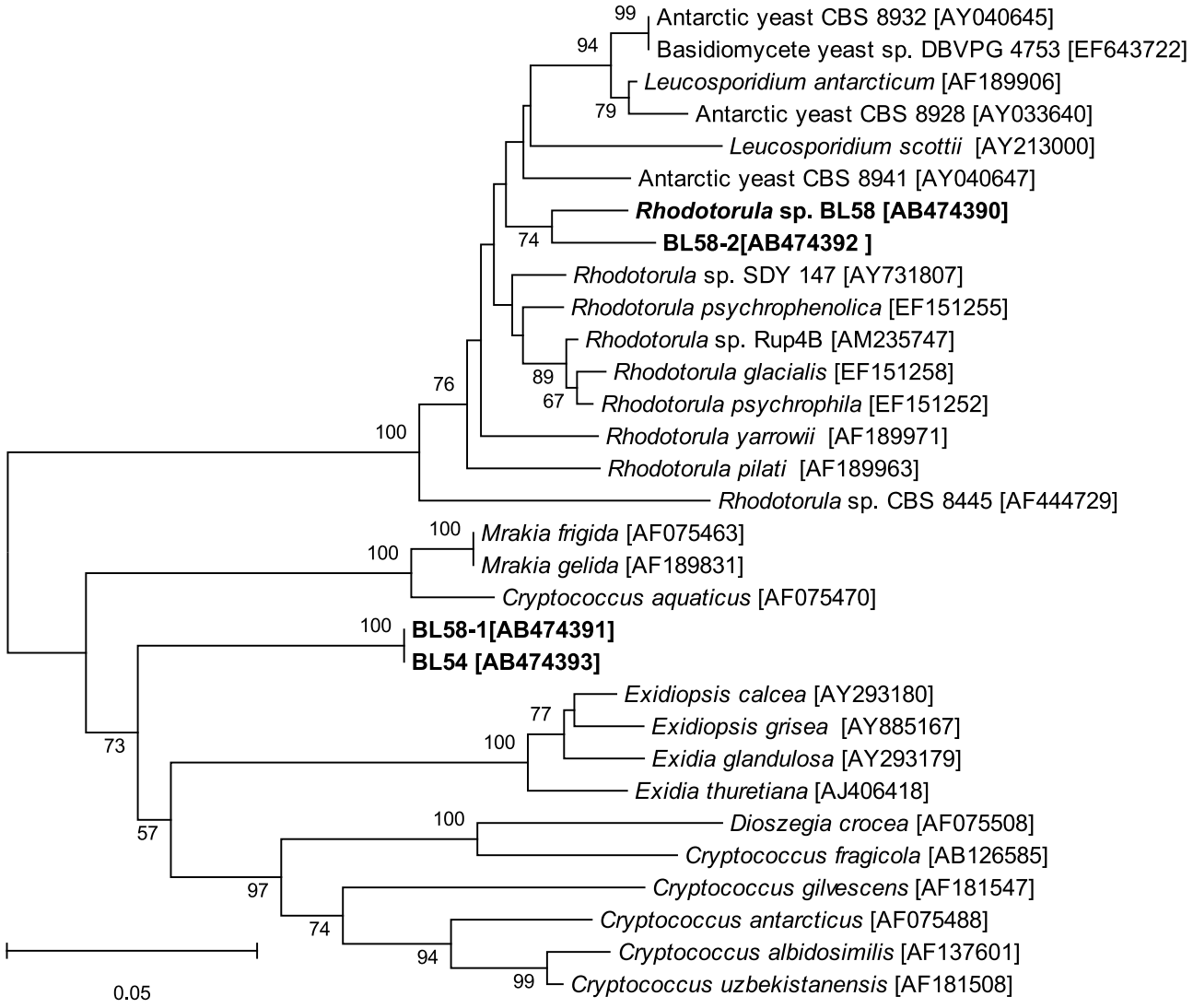

Figure 5. Phylogenetic placement of yeast isolates and clones obtained by neighbor joining of the D1/D2 domains of the 26S rRNA gene. The scale indicates the number of substitutions accumulated every 100 nucleotides. Bootstrap values higher than 60% are shown (1000 replicates).

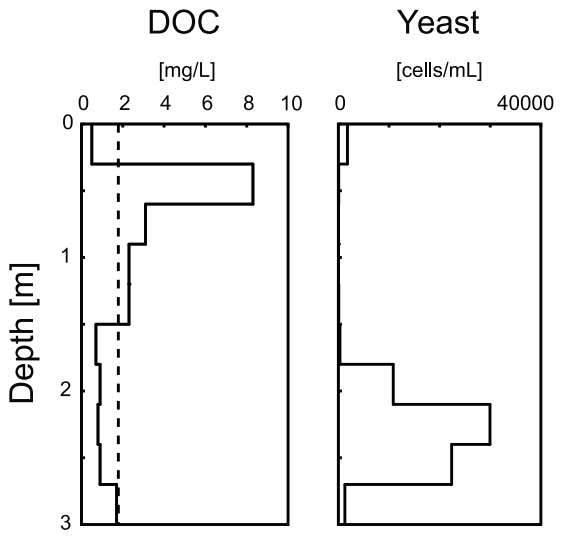

Figure 6. Vertical profiles of dissolved organic carbon (DOC) and yeast concentration in a pit from 2003. The dashed line shows the level of possible contamination from sampling processes.

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