RESEARCH ARTICLE

Isolation of oligotrophic yeasts from supraglacial environments of different altitude on the Gulkana Glacier (Alaska)

Jun Uetake[1,2], Yoshitaka Yoshimura[3], Naoko Nagatsuka[4] & Hiroshi Kanda[2]

[1]Transdisciplinary Research Integration Center, Tokyo, Japan; [2]National Institute of Polar Research, Tokyo, Japan; [3]Graduate School of Agriculture, Tamagawa University, Tokyo, Japan; and [4]Graduate School of Science, Chiba University, Chiba, Japan

Correspondence: Dr Jun Uetake, National Institute of Polar Research, C306 10-3 Midoricho, Tachikawa, Tokyo 190-8518, Japan. Tel.: +81 42 512 0768; fax: +81 42 528 3492; e-mail: juetake@nipr.ac.jp

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Abstract

Psychrophilic yeasts have been isolated from supra- and subglacial ice at many sites worldwide. To understand the ecology of psychrophilic yeasts on glaciers, we focused on their adaptation to wide range of nutrient concentrations and their distribution with altitude on the Gulkana Glacier in Alaska. We found various culturable psychrophilic yeasts on the ice surfaces of the glacier, and 11 species were isolated with incubation at 4 °C in four different dilutions of agar medium. Some of our isolated species (*Rhodotorula psychrophenolica*, *Rhodotorula* aff. *psychrophenolica*, *Rhodotorula glacialis*, and *Basidiomycota* sp. 1) can grow on the low dissolved organic matter (DOC) concentrations medium (7.6 mg L^{-1}) which is close to the typical level of supraglacial melt water, suggesting that these species can inhabit in any supraglacial meltwater. Otherwise, most of other species were isolated only from higher DOC concentration medium (183 mg L^{-1}–18.3 g L^{-1}), suggesting that these are inhabitant around the cryoconite, because DOC concentrations in melted surface-ice contained cryoconite is much higher than in melted water. Similarity of altitudinal distribution between culturable yeast and algal biomass suggests that the ecological role played by the cold-adapted yeasts is as organic matter decomposers and nutrient cyclers in glacier ecosystem.

Introduction

For many years, glacial environments were believed to be virtually abiotic. However, recent studies have shown that glaciers are biotic environments containing many psychrophilic organisms (Hodson *et al.*, 2008), including snow algae (Kol, 1969; Hoham & Duval, 2001), cyanobacteria (Takeuchi, 2001), yeasts (Turchetti *et al.*, 2008), bacteria (Segawa *et al.*, 2005; Simon *et al.*, 2009), invertebrates (Kohshima, 1984), metazoans (DeSmet & Van Rompu, 1994), and protozoa (Säwström *et al.*, 2002). A large number of studies of psychrophilic yeasts on glaciers are currently being conducted. Psychrophilic yeasts have been isolated from supra- and subglacial ice in Svalbard (Butinar *et al.*, 2007), from Austrian glacial ice (Margesin *et al.*, 2007, 2009); Italian subglacial meltwater (Buzzini *et al.*, 2005); supra- and subglacial ice and meltwater of the Italian Alps (Turchetti *et al.*, 2008; Thomas-Hall *et al.*, 2009); glacial and subglacial waters from northwest Patagonia (Brizzio *et al.*, 2007; De García *et al.*, 2007), Antarctica (Thomas-Hall *et al.*, 2009), and an Antarctic deep ice core (Amato *et al.*, 2009). These isolates can grow and degrade phenol (Margesin *et al.*, 2007) and organic macromolecules (Buzzini *et al.*, 2005) at low temperatures (1–4 °C), and also exhibit extracellular enzymatic activity (Brizzio *et al.*, 2007; De García *et al.*, 2007; Turchetti *et al.*, 2008). Isolates from deep ice cores have been shown to be metabolically active, displaying H^3-leucine incorporation under frozen conditions (−5 °C) (Amato *et al.*, 2009). Although various studies have been carried out on isolated fungi from glacial environments, there is little information on the ecology of these organisms, such as their adaptation to low-nutrient and cold supraglacial environments.

To understand the ecology of psychrophilic yeasts on glaciers, we focused on their adaptation to a wide range of nutrient concentrations and altitudes on the Gulkana Glacier in Alaska. The Gulkana Glacier is a small

mountain glacier with a length of approximately 4 km and an area of around 21.8 km^2. The glacier has been monitored since 1960s by the University of Alaska and the United States Geological Survey (http://ak.water.usgs.gov/glaciology/gulkana/), and is easily accessible by vehicle from the Richardson Highway. It flows west to south from Icefall Peak (~2440 m asl) to its termination at ~1220 m asl. The snow line on 29 August 2008 was around 1750 m asl by visual observation. The altitudinal distributions of snow algae and bacteria and the spectral reflectivity of biogenic materials (red-colored snow-algal cells and cryoconite) on this glacier have been reported previously (Takeuchi, 2001, 2009; Segawa *et al.*, 2010).

Materials and methods

Sampling

Snow and ice were sampled at five sites on the Gulkana Glacier in Alaska (S1, 1270 m asl; S2, 1385 m asl; S3, 1470 m asl; S4, 1585 m asl; and S5, 1680 m asl) between 29 August and 2 September 2008 (Fig. 1). Five samples were collected from randomly selected points at each sampling site. Snow and ice from the surface to a depth of 1 cm were directly collected into 50-mL sterile centrifuge tubes. The centrifuge tubes were transported to the National Institute of Polar Research (NIPR), Japan. The samples for yeast isolation were transported on ice (0 °C) during the full transportation process, whereas the samples for molecular cloning and chemical analysis were transferred to a freezer (−20 °C) on 3 September for transport in a freezer cargo plane.

Isolation of yeasts and yeast cell concentrations

Aliquots of 20–50 μL of the melted samples were inoculated onto yeast extract peptone dextrose (YEPD) agar medium, YEPD agar medium diluted 10-fold (1/10 YEPD), YEPD agar medium diluted 100-fold (1/100 YEPD), and ultrapure water agar (UWA) medium within a class 100 laminar-flow clean bench. For all media, the agar was mixed with ultrapure water. The cultures were incubated at 4 °C for 2–5 weeks (Butinar *et al.*, 2007; De García *et al.*, 2007; Turchetti *et al.*, 2008; Branda *et al.*, 2010). All media contained 2% agar (010-08725; Wako Pure Chemical Industries, Osaka, Japan) and chloramphenicol (100 mg L^{-1}) to prevent the growth of prokaryotes. Colonies were selected for isolation based on macromorphology (small, 1–2 mm; medium, 3–4 mm; or large, 5–6 mm), taking care to isolate all morphotypes represented on each medium at different incubation

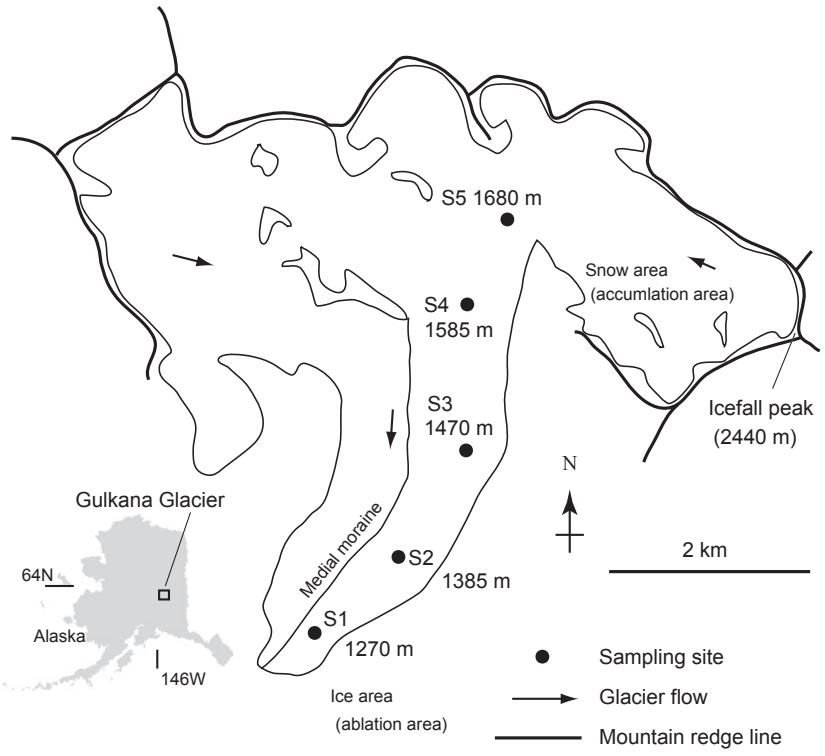

Fig. 1. Map of the Gulkana Glacier, Alaska. Solid circles show the locations of the sampling sites.

mediums. After first incubation, the colonies on each medium were subcultured onto new medium, to purify the isolates. Isolate had not been deposited in culture collection and keep frozen with glycerol in laboratory. After incubation for 2–5 weeks, all the colonies were counted, and the total numbers of colonies were expressed as colony-forming units (CFU) mL^{-1}. Multiple comparisons were made between the sampling sites, using the Tukey–Kramer test.

Genetic analysis of isolated yeasts and environmental samples

The 26S ribosomal RNA (rRNA) D1/D2 domains of the isolated strains were amplified. All manipulations before PCR analysis were made within a class 100 laminar-flow clean bench, to avoid contamination. A colony was selected from each agar medium with a sterile pipette tip and added directly to the PCR mixture or extracted with a Dr GenTLE® (from Yeast) High Recovery Kit (Takara Bio, Shiga, Japan). PCR amplification was performed with Ex Taq DNA polymerase (Takara Bio) for 35 cycles, using the primer pair NL1 (5′-GCATATCAATAAGCGGAG-GAAAAG-3′) and NL4 (5′-GGTCCGTGTTTCAAGACGG-3′). The PCR products from the isolates were sequenced with the 3130xl Genetic Analyzer (Applied Biosystems, Carlsbad, CA) at NIPR. The DNA sequences were aligned with ClustalW in the Geneious 4.5.2 software. Neighbor-joining analysis was performed, and bootstrap consensus trees (1000 pseudo-replicates) were generated with Mega 4.0.2. Strains were identified by comparing the sequences obtained with the GenBank database. Strains that are differed from the closest related type strain by two or fewer nucleotides in the D1/D2 region were considered to be the same species (Fell *et al.*, 2000).

Chemical analysis

The concentration of dissolved organic carbon (DOC) was measured in YEPD agar medium diluted 500-fold (1/500 YEPD) and in the nutrient eluted supernatant of the 10% UWA medium using a Spectroquant test kit (Merck, Darmstadt, Germany) and a spectral photometer (photLab S12; Wissenschaftlich-Technische Werkstätten, Weilheim, Germany). All samples and media were filtered through a polycarbonate membrane (K040A047A; Advantec, CA). The nutrient eluted supernatant of the 10% UWA agar medium was collected after shaking (110 r.p.m.) for 24 h, according to Uzuka (1992). The melted surface-ice samples were mixed from two aliquots of samples from four sites (S1, S2, S4, and S5) and from three aliquots of samples from one site (S3) to achieve the volumes required for analysis.

Results and discussion

Isolation of cold-adapted yeast

Fifty-nine yeast strains were isolated from the ice and snow samples collected from the glacier surface and incubated at 4 °C in four different agar media (Table 1). The number of isolates obtained from each agar medium was as follows: 10 strains from YEPD, 26 strains from 1/10 YEPD, 17 strains from 1/100 YEPD, and six strains from UWA. The D1/D2 domain of the 26S rRNA gene sequence analysis of 59 yeast strains allowed them to be assigned to 11 species (Fig. 2, Table 1): *Rhodotorula psychrophenolica* (five strains), *Rhodotorula* aff. *psychrophenolica* (10 strains), *Rhodotorula glacialis* (three strains), *Dioszegia hungarica* (one strain), *Mrakia* sp., *Basidiomycota* sp. 1 (five strains), *Basidiomycota* sp. 2 (10 strains), *Basidiomycota* sp. 3 (10 strains), *Basidiomycota* sp. 4 (five strains), *Basidiomycota* sp. 5 (one strain), and *Basidiomycota* sp. 6 (one strain), and their nucleotide sequences have been deposited in the GenBank database under accession numbers AB558439–AB558455 and AB671320–AB671328.

Rhodotorula psychrophenolica and *Rhodotorula glacialis* are closely related to the strain from glacier cryoconite in Austrian glacier (Margesin *et al.*, 2007) and *Rhodotorula* aff. *psychrophenolica* is 3–5 nucleotides different from *Rhodotorula psychrophenolica* strain A12 (EF151256). In Gulkana Glacier, glacier surface in research area was covered by cryoconite and supposed to be similar environment to Austrian glacier. *Mrakia* sp. has 100% similarity with many species of genus *Mrakia* (*Mrakia frigida*, *Mrakia robertii*, and *Mrakia* sp.) and not possible to determine the species by analysis of 26S rRNA D1/D2 domains. *Dioszegia hungari* is closely related to the strain from glacial outflow meltwater river (De García *et al.*, 2007), cloud water, soil, and malt with 99% similarity.

Basidiomycete sp 1. and sp. 2 are closely related to uncultured clone (AB474392) from ice core in Russian glacier with 96–99% similarity, respectively. This unclutured clone was retrieved from snow layer which is affected by meltwater percolation during summer time, and Uetake *et al.* (2011) showed evidence for the propagation of these yeast from a low-DOC (maximum of 8.3 mg L^{-1}) contained snow in ablation area. *Basidiomycota* sp. 3 are closely related to Antarctic yeast CBS 8941 (AY040647), which is isolated from Antarctica with 99–100% similarity. *Basidiomycota* sp. 4 are closely related to *Basidiomycota* sp. CRUB 1733 (FJ841888), which are isolated from Patagonian glacier with 100% similarity. *Basidiomycota* sp. 5 and 6 are undescribed species, because closest relatives of these species are far from GenBank (*Basidiomycota* sp. CRUB 1733: FJ841888 with

Table 1. Physical appearance of the colonies (color and diameter) of 11 species isolated from this study in each growth mediums at five sites (S1–S5) on the Gulkana Glacier. The physical appearances of the colonies are defined as follows: W, white; C, cream; R, red; O, orange; S, small; M, medium; L, large

		Sample site				
Species	Medium	S1	S2	S3	S4	S5
Rhodotorula psychrophenolica	YEPD					
	YEPD1/10					WM
	YEPD1/100					WM
	UWA					CM
Rhodotorula aff. *psychrophenolica*	YEPD	WM	WM			
	YEPD1/10		WM	WM		
	YEPD1/100	WM, WL				WM, CM
	UWA	WS	WS	WS		
Rhodotorula glacialis	YEPD					
	YEPD1/10				WM	
	YEPD1/100			WL		
	UWA				WS	
Dioszegia hungarica	YEPD	OM				
	YEPD1/10					
	YEPD1/100					
	UWA					
Mrakia sp.	YEPD			WL	WL	
	YEPD1/10	WL	WL	WL	WL	
	YEPD1/100		WL, CL			
	UWA					
Basidiomycota sp. 1	YEPD					
	YEPD1/10			CM	CM	
	YEPD1/100			WM, CM		
	UWA					WS
Basidiomycota sp. 2	YEPD					WS, RM
	YEPD1/10	RM	RM	WS, RM		WS, RM
	YEPD1/100			WM		WS
	UWA					
Basidiomycota sp. 3	YEPD				CM	CM
	YEPD1/10		CM, CL, RM			CM, RM
	YEPD1/100		WM			CM
	UWA					
Basidiomycota sp. 4	YEPD				WS	
	YEPD1/10	WS	WS			
	YEPD1/100	WS			WS	
	UWA					
Basidiomycota sp. 5	YEPD					
	YEPD1/10					
	YEPD1/100			WS		
	UWA					
Basidiomycota sp. 6	YEPD					
	YEPD1/10					
	YEPD1/100			WS		
	UWA					

96% similarity and *Zymoxenogloea eriophori* strain CBS8387: AF189905 with 95% similarity, respectively).

Clearly, the colony color of both *Basidiomycota* sp. 2 and *Basidiomycota* sp. 3 includes three types of color (red, cream, and white) (Table 1). Pigmentation may be for resistance from strong UV on surpraglacial environment; however, the reason why different color mixed in same specie is unknown. Pigmentation is one of the common physiological features of the isolate (Margesin & Miteva, 2011), and difference of color can divide more strains. But further genetic analysis using ITS region may be helpful for the identification of strain.

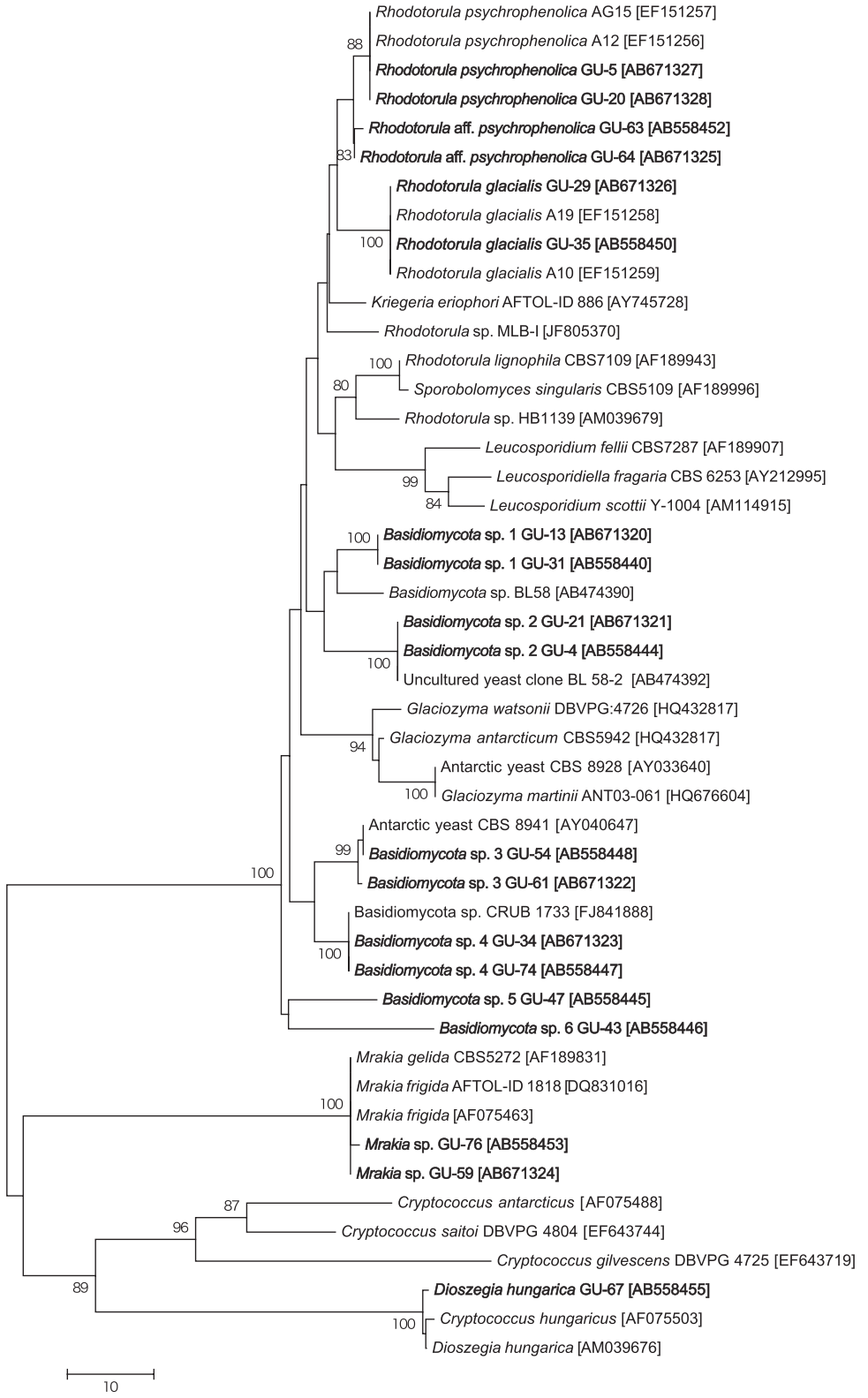

Fig. 2. Phylogenetic placement of the isolates and clones calculated with the neighbor-joining method applied to the 26S rRNA D1/D2 domain sequences. The scale indicates the number of substitutions accumulated every 100 nucleotides. Bootstrap values higher than 80% are shown (1000 replicates).

Growth in wide range of DOC concentrations

All species which can be retrieved more than three strains are able to grow on wide range of nutrient condition (Table 1). For example, *Rhodotorula psychrophenolica* was isolated form three different mediums (YEPD1/10, YEPD1/100, and UWA) in S5. The estimated concentrations of DOC in 2% UWA and 1/100 YEPD were 7.6 and 183 mg L^{-1}, respectively. (These values were estimated from measured concentration of 10% UWA (37.8 mg L^{-1}) and 1/500 YEPD (36.6 mg L^{-1}).) Therefore, DOC concentration of mediums which we used for this study covered from 7.6 to 18300 mg L^{-1}.

The DOC concentration of the supraglacial environment is reported to be 0.252 mg L^{-1} on the John Evans Glacier (Barker *et al.*, 2006), 1.3 ± 0.52 mg L^{-1} in Werenskioldbreen (Stibal *et al.*, 2008) in the Arctic, 1.9–3.9 mg L^{-1} in cryoconite hole water in the glaciers around Dry Valley (Foreman *et al.*, 2007), 5.897 mg L^{-1} in the Victoria Upper Glacier in Antarctica (Barker *et al.*, 2006), 0.188–0.303 mg L^{-1} in the Outre Glacier in Canada (Barker *et al.*, 2006), and 0.275–1.052 mg L^{-1} on Chinese high-altitude glaciers (Liu *et al.*, 2009).

These DOC concentrations are usually recognized as representing oligotrophic environments; however, Priscu *et al.* (1999) estimated from a study of subglacial lake ice that a DOC level of 1.2 mg L^{-1} is adequate to support heterotrophic growth. Therefore, lowest-DOC-contained UWA is most closed to supraglacial condition (cryoconite hole water, meltwater, and melted ice) and sustainable for yeast growth. Some of our isolate adapt to this oligotrophic condition, because four of 11 species (*Rhodotorula psychrophenolica*, *Rhodotorula* aff. *psychrophenolica, Rhodotorula glacialis*, and *Basidiomycota* sp. 1) can grow on the UWA. Therefore, species retrieved from UWA are able to grow in any water environment on/in glacier.

Otherwise, most of other species were isolated from higher DOC concentration medium (YEPD, YEPD1/10, YEPD1/100). YEPD1/100 is estimated 183 mg L^{-1} and much higher than meltwater and melted ice. We had analyzed the DOC concentrations in melted surface-ice contained much of cryoconite. DOC concentrations were much higher (5.5–770 mg L^{-1}) than typical supragracial meltwater described earlier, probably attributable to lysis from cryoconite. Because the samples that we analyzed had experienced many freeze–thaw cycles before analysis, the cytoplasm of the microorganisms and other organic contents in cryoconite would be lysed by this process. Although we had not measured the DOC from cryoconite contained water, in the near surface or inside of cryoconite, DOC concentration may be locally higher than other supraglacial melted water environment; therefore, species adapted to high DOC concentration could inhabit around the cryoconite.

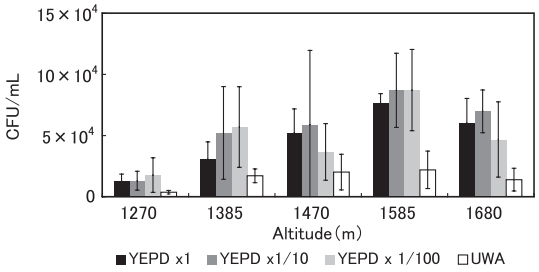

Fig. 3. Altitudinal distributions of yeasts (CFU mL^{-1}) in four different dilutions of YEPD medium: YEPD, 1/10 YEPD, 1/100 YEPD, and UWA 2%. The agar medium contained chloramphenicol (100 mg L^{-1}) to prevent the growth of prokaryotes.

Altitudinal distributions of the yeasts

The altitudinal distributions of yeasts, in terms of CFU mL^{-1}, on the ice of the glacier surface ranged from 1.3×10^4 (S1) to 8.7×10^4 (S4) in YEPD medium, from 1.3×10^4 (S1) to 8.7×10^4 (S4) in 1/10 YEPD medium, from 1.7×10^4 (S1) to 8.7×10^4 (S4) in 1/100 YEPD medium, and from 3.5×10^3 (S1) to 2.1×10^4 (S4) in UWA medium (Fig. 3). The yeast CFU in YEPD medium at S1 were significantly lower ($P < 0.01$) than those at S3, S4, and S5. And CFU in both 1/10 YEPD and 1/100 YEPD media were lower ($P < 0.05$) than those at S4.

The altitudinal distributions of yeast CFU differed from those calculated with direct bacterial counts on the same glacier (Segawa *et al.*, 2010). The altitudinal distributions of bacterial cell concentrations were highest at the lowest altitude (S1). In contrast, yeast cell concentration at the same site was not significantly higher than at the other sites. Segawa *et al.* (2010) showed that most bacteria at S1 were of a peri-glacial environmental origin and had been transported onto the glacier by the wind, because the number of singletons (sequences unique among the entire 16S rRNA gene clone library constructed from a glacial sample) at S1 was much higher than at the other altitudes.

On the other hand, the similar altitudinal distribution of snow-algal biomass has been reported for the Gulkana Glacier and other Alaskan glaciers (Takeuchi, 2001; Takeuchi *et al.*, 2003). Both yeast CFU and algal biomass are higher in the higher altitude. Takeuchi (2001) reported that the amount of meltwater could wash out the snow algae near the glacier termination; therefore, biomass increased with increasing altitude on the ice area. While we were at the Gulkana Glacier between 29 August and 2 September 2008, we saw many surface meltwater streams around S1 and S2. Therefore, wash-out by meltwater

would also affect the populations of yeasts that inhabit the glacier.

Similarity of altitudinal distribution between culturable yeast and algal biomass may show that ecological relationship on the glacial ecosystem. Ecological role played by psychrophilic yeasts is as organic matter decomposers and nutrient cyclers, because many studies showed the ability of extracellular enzymatic activity of psychrophilic yeast (Margesin *et al.*, 2003, 2007; Brizzio *et al.*, 2007; De García *et al.*, 2007; Turchetti *et al.*, 2008). Therefore, psychrophilic yeast species on glacier are distributed with snow algae as a primary producer in the supraglacial ecosystem. However, our altitudinal data refer only to the short period of the melting season. To better understand the ecology of cold-adapted yeasts, we must analyze the DOC concentrations in fresh samples with long-term observation and sampling of the glacier.

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