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Antifreeze activities of various fungi and Stramenopila isolated from Antarctica

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Abstract: We examined the antifreeze activities of culture filtrates from cold-adapted fungi and Straminopila isolated from terrestrial materials in Antarctica. All of the isolates could grow at -1º C on suitable media and antifreeze activities were detected in various taxa including: an isolate of an unknown species in Oomycota, an isolate of an unknown species in Blastocladiomycota, *Antarctomyces psychrotrophicus*, *Penicillium camemberti* (Ascomycota) and several basidiomycetous yeasts. Unique ice crystal structures in media and depression of the freezing point of water for some are presented as evidence of fungal antifreeze proteins that can protect cells in cold climates.

Key words: Antarctica, *Antarctomyces psychrotrophicus*, antifreeze protein, Blastocladiomycota, Hyphochytriomycota, *Leucosporidium antarcticum*, Oomycota

Introduction: Many living organisms that have adapted to the cold environment of Polar Regions have various biochemical and ecological strategies to protect themselves against intracellular freezing. Antifreeze proteins (AFPs) have the unique ability to attach to hexagonal ice crystals to inhibit their growth, resulting in depression of the freezing point of water and leading to protection of cells from freezing injury as a result of ice crystal formation (Duman et al. 1993). Such depression of the freezing point is 500-times greater than that of colligative salts on a molar basis and occurs noncolligatively because of AFP-induced thermal hysteresis: a disparity between the melting and freezing points of the solution. AFPs also affect the morphology of ice crystals, creating bipyramidal ice crystals (Fig. 1 B) (Duman et al. 1993, Hoshino et al. 2009).

AFPs were first found in the body fluids of various kinds of polar fishes. AFPs have also been found in several cold-adapted microorganisms, including basidiomycetous fungi (Duman and Olsen 1993, Hoshino et al. 2003a, 2003, Newsted et al. 1994, Raymond and Janech 2009, Snider et al. 2000) and lichens (Doucet et al. 2000, Sidebottom et al. 1999). Purified and cloned AFPs from basidiomycetous fungi (Hoshino et al. 2003a, 2003b, Raymond and Janech, 2009) are most similar to ice-binding proteins from polar diatoms (Janech et al. 2006) and an Antarctic sea ice bacterium (Raymond et al. 2007). However, these fungal AFPs are thought to be a new class of AFPs.

Antifreeze proteins have not been found in groups such as snow molds in Oomycota and Ascomycota or psychrophilic fungal phytopathogens under snow cover (Hoshino et al.
2003a, 2003b). However, Kawahara et al. (2006) recently reported antifreeze activities in certain genera of Ascomycota. Their findings suggest that AFPs might also be present in cold-adapted fungi outside the Basidiomycota.

In this study, we screened a wide range of cold-adapted fungi and Oomycota and Hyphochytriomycota from Antarctica, a region with one of most severe cold climates. In such situations the need for antifreeze activities is great. Here we report new fungal and non-fungal groups that possess extracellular antifreeze activities.

Materials and Methods: Various terrestrial materials (soils, mosses, algal mats, etc.) were collected near Great Wall Station in King George Island, South Shetland Islands in 1996 and 2007, Zhongshan Station in Larsemann Hills, Prydz Bay, East Antarctica in 1996 and Soya coast, East Antarctica in 2007. The samples were stored at 4°C and -20°C before inoculation and were placed directly on potato dextrose agar plates (PDA, Difco) and Pythium-selective NARM plates (Morita and Tojo 2007) at -1°C for 2 months. Chytrids, Blastocladiomycota and Oomycota were also collected using the pine pollen baiting method (Gaertner, 1968) at 10°C and 15°C. Isolates of some of the same species previously found in Antarctica were obtained from NITE NBRC (Chiba, Japan).

Morphological characteristics of all isolates were examined on PDA plates, corn meal agar plates (Difco) or PYG agar plates (1.25 g bactopeptone, 1.25 g yeast extract, 3 g glucose, 15 g agar per liter). DNA sequences (ITS region) of tested isolates were also obtained from liquid culture at -1˚C according to Gardes and Bruns (1993).

Isolates were cultured in liquid media (potato dextrose broth, PDB; Difco and PYG broth) at -1˚C for 1-2 months. The antifreeze activities of each culture medium were further examined by observation of ice morphology using a Leica DMLB 100 photomicroscope (Leica Microsystems AG, Wetzlar, Germany) equipped with a Linkam LK600 temperature controller (Linkam, Surrey, UK). The culture medium of our

isolates and other strains from the culture collection was momentarily frozen (at about -25˚C) and warmed to 0˚C on the sample stage of the photomicroscope to create several ice crystal seeds in solution. This solution was then cooled to approximately -1 to -5˚C, and growth of the ice crystal seeds was monitored.

Results: We obtained isolates from a range of taxa on terrestrial materials in Antarctica: 12 of Oomycota, 2 of Hyphochytriomycota, 12 of Chytridiomycota, 2 of Blastocladiomycota, 15 of Zygomycota, more than 50 of Ascomycota, and more than 50 of Basidiomycota.

Major genera of our isolates were *Pythium* for Oomycota, *Hyphochytrium* for Hyphochytriomycota, *Chytriomyces*, *Kappamyces*, *Powellomyces* and *Rhizophydium* for Chytridiomycota, an unknown genus for Blastocladiomycota, *Mortierella* for Zygomycota, *Acremonium*, *Antarctomyces*, *Embellisia*, *Geomyces*, *Leptosphaeria*, *Penicillium*, *Phialocephala*, Helotiales and Thelebolaceae for Ascomycota; and *Cryptococcus*, *Dioszegia*, *Leucosporidium*, *Mrakia* and *Rhodotorula* for Basidiomycota. All our isolates of collected Basidiomycota were yeast forms.

Extracellular antifreeze activity was detected in various taxa (Table 1). Culture media of one isolate of an unknown species in Oomycota (not *Pythium*), one isolate of unknown species in Blastocladiomycota, three isolates of *Antarctomyces psychrotrophicus* Stchigel & Guarro from maritime and continental Antarctica, *Penicillium camemberti* Thom NBRC 32215 strain (Ascomycota), seven isolates of *Leucosporidium antarcticum* Fell, Statzell, I.L. Hunter & Phaff, and two isolates of *Rhodotorula glacialis* Margesin & Sampaio (basidiomycetous yeasts) showed ice modification activity.

Table 1. Extracellular antifreeze activities* of various fungi from terrestrial materials collected in Antarctica.

Taxa	Number of tested isolates	Mycelial growth at -1°C	Isolates with antifreeze activity
Stramenopila			
Oomycota	14	12	1
Hyphochytriomycota	2	2	0
Fungi			
Chytridiomycota	12	12	0
Blastocladiomycota	2	2	1
Zygomycota	15	15	0
Ascomycota	50	50	2
Basidiomycota	50	50	9
Total	**145**	**143**	**13**

*based on ice crystal morphologies and freezing point depression.

Ice morphologies of culture media from these fungi and related species were different. Ice crystals of the isolate of the unknown species in Oomycota and the isolate of unknown species of Blastocladiomycota were similar in shape and consisted of hexagonal plates with rough surfaces (Fig. 1D-E). These morphological characteristics have not been reported for known fungal AFPs from Basidiomycota (Hoshino et al. 2009) or antifreeze substances from Ascomycota (Kawahara et al. 2006). Neither culture media demonstrated any thermal hysteresis. The ice crystals in the culture medium of *A. psychrotrophicus* showed a typical hexagonal bipyramidal shape (Fig. 1F) similar to fish AFP-bound ice crystals (Fig. 1B) and weak thermal hysteresis (ca 0.1º C). Ice crystals in the culture medium of *Leucosporidium antarcticum* (Fig. 1G) had a distorted hexagonal bipyramid shape with rough surfaces like the AFP-bound ice crystals of the basidiomycetous snow mold *Typhula ishikariensis* S. Mai (Fig. 1C) and weak thermal hysteresis (ca.0.22ºC).

Discussion: We found extracellular antifreeze activity in culture media supporting growth of various fungi and pseudofungi in Stramenopila from terrestrial habitats in Antarctica. We also obtained the first evidence of the presence of antifreeze activity in Oomycota, Blastocladiomycota and Basidiomycota outside Agaricomycotina (as defined by Blackwell et al. 2006). These results suggested that AFPs and/or antifreeze substances are widely distributed in fungal and non-fungal kingdoms.

Various amino acid sequences of AFPs have been detected in other living organisms such as fish, insects and plants. On other hand, the known fungal AFPs from only the Agaricomycotina (Basidiomycota) show high similarity among different species based on gene sequences and immuno-cross reaction (Hoshino et al. 2003b, Raymond and Janech 2009). Our isolates of *L. antarcticum* in Pucciniomycotina (Basidiomycota) produced extracellular antifreeze activities, and the protein profile of *L.*

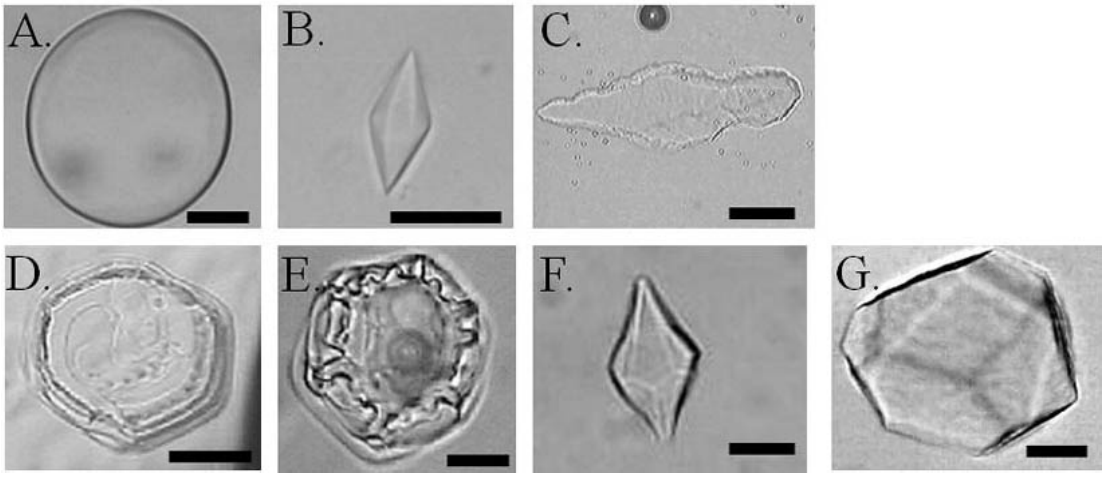

Fig. 1. Ice crystal morphologies in culture media supporting growth of various fungi and Straminopila from Antarctica and controls for comparison. A. control (100 mM ammonium bicarbonate), B. purified fish type III antifreeze protein, C. purified antifreeze protein from the basidiomycetous snow mold *Typhula ishikariensis*, D. culture medium of an unknown species in Oomycota, E. culture medium of unknown species in Blastocladiomycota, F. culture medium of *Antarctomyces psychrotrophicus* (Ascomycota) and G. culture medium of *Leucosporidium antarcticum* (basidiomycetous yeast). Bar is 50 μm.

antarcticum culture medium showed 22 kDa protein of major comportment significance (Fujiu et al. unpublished results). These results suggest that different subphyla in the Basidiomycota probably have the same isoform of AFP.

However, antifreeze substances from Antarctic Ascomycota differ from basidiomycetous AFPs in biochemical characteristics (Kawahara et al. 2006). Ice crystal morphologies of culture media with the isolate of an unknown species in Blastocladiomycota and *Antarctomyces psychrotrophicus* were also different from AFPs produced by Basidiomycota in this study. These findings indicate the possibility of AFP diversity at the phylum level within the fungal kingdom.

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