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ICE-BINDING PROTEINS FROM SEA ICE DIATOMS (BACILLARIOPHYCEAE)[1]

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Sea ice diatoms thrive under conditions of low temperature and high salinity, and as a result are responsible for a significant fraction of polar photosynthesis. Their success may be owing in part to secretion of macromolecules that have previously been shown to interfere with the growth of ice and to have the ability to act as cryoprotectants. Here we show that one of these molecules, produced by the sea ice diatom *Navicula glaciei* Vanheurk, is a ~25 kDa ice-binding protein (IBP). A cDNA obtained from another sea ice diatom, *Fragilariopsis cylindrus* Grunow, was found to encode a protein that closely matched the partially sequenced *N. glaciei* IBP, and enabled the amplification and sequencing of an *N. glaciei* IBP cDNA. Similar proteins are not present in the genome of the mesophilic diatom *Thalassiosira pseudonana*. Both proteins closely resemble antifreeze proteins from psychrophilic snow molds, and as a group represent a new class of IBPs that is distinct from other IBPs found in fish, insects and plants, and bacteria. The diatom IBPs also have striking similarities to three prokaryotic hypothetical proteins. Relatives of both snow molds and two of the prokaryotes have been found in sea ice, raising the possibility of a fungal or bacterial origin of diatom IBPs.

Key index words: **cryoprotection; diatoms; *Fragilariopsis cylindrus*; ice-binding proteins; *Navicula glaciei*; sea ice**

Abbreviations: **AB, ammonium bicarbonate; CFB, Cytophaga–Flavibacterium–Bacterioides; DTT, dithiothreitol; EST, expressed sequence tag; IBP, ice-binding protein; UNLV, University of Nevada, Las Vegas**

Sea ice is a habitat of extremophiles, organisms that not only survive but thrive under extremes of temperature, salinity, and light (Thomas and Dieckmann 2002). Prominent among these organisms are diatoms, which during periods of bloom form a dense brown layer at the bottom of sea ice that is responsible for a significant fraction of polar photosynthesis (Thomas and Dieckmann 2002). The diatoms appear to grow best during periods of ice growth (Garrison et al. 1983, Smetacek et al. 1992), and grow well even in brine pockets at temperatures as low as $-6°$ C (Aletsee and Jahnke 1992). One factor that may contribute to survival is the release by the diatoms of exopolymeric substances that can help to preserve a liquid environment as sea ice freezes (Krembs et al. 2002). We have been investigating another potential survival mechanism that involves the production of proteins that have an affinity for ice crystals. Associated with the diatom community are extracellular proteins (formerly called ice-active substances) that strongly interact with ice (Raymond et al. 1994, Raymond 2000). The most conspicuous effect of these proteins is to cause pitting and other deformities on the surface of growing ice crystals, which is a sign of adsorption of an impurity to a crystal surface (Buckley 1951). Accordingly, we call these proteins ice-binding proteins (IBPs). Ice-binding activity has been found in all unialgal populations of Antarctic and Arctic ice diatoms that have been examined so far, and it has not been found in mesophilic diatoms. Thus, it appears that the molecules have a function related to cold or icy environments.

IBPs have been found in many organisms that are exposed to cold, including fish, insects, plants, fungi, and bacteria, in which they act as antifreezes, ice recrystallization inhibitors, and ice nucleators (for reviews of plant antifreezes, see Smallwood and Bowles 2002, Griffith and Yaish 2004). Diatom IBPs resemble plant antifreezes in that they do not appreciably lower the freezing point, which suggests that they mainly

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function as cryoprotectants. Many IBPs, including over a dozen from plants, have been sequenced, and it is now evident that they have little in common beyond an ability to bind to ice. Little is known of the nature of diatom IBPs other than their having the characteristics of proteins. Here we report the cDNA sequences of two diatom IBPs and show that they are more closely related to fungal antifreezes than to any known plant antifreezes.

MATERIALS AND METHODS

Diatoms. Cells of the sea ice diatom *Navicula glaciei* (identified by J. S. K.) were obtained from a dense colony in a crack in the sea ice at Cape Evans, Antarctica. The cells were almost exclusively *N. glaciei*. Additional cells of *N. glaciei*, *Fragilariopsis pseudonana*, *Chaetoceros neogracile*, and *Stellarima microtrias* were obtained near Maxwell Bay, King George Island, Antarctica and were axenically cultured at approximately 3° C under 25 μmol photons $\cdot$ m^{-2} $\cdot$ s^{-1} (24 h light) in modified f/2 medium (Guillard and Ryther 1962) at the Korea Polar Research Institute or at UNLV. Cells of the Arctic sea ice diatoms *Synedra* sp., *Amphora* sp. and *Attheya* sp. were cultured at approximately 2° C at the Provasoli–Guillard National Center for Culture of Marine Phytoplankton, West Boothbay Harbor, Maine. Cells of *F. cylindrus* were isolated from Antarctic sea ice during a "Polarstern" expedition (ANTXVI/3) in the eastern Weddell Sea. Nonaxenic cultures were grown at 0° C under 20 μmol photons $\cdot$ m^{-2} $\cdot$ s^{-1} (24 h light) in double f/2 medium at the Alfred Wegener Institute. Bubbling with air (approximately 150 mL/min) ensured sufficient CO_2 supply and continuous mixing.

Sequencing of F. cylindrus *IBP.* A cDNA library from *F. cylindrus* was constructed from nonaxenic cultures subjected to salt stress conditions. The salinity of double ANT f/2 medium cells was increased by a concentrated brine solution (Instant Ocean, Aquarium Systems, Mentor, OH, USA) from approximately 34 to 60 psu in the middle of the exponential growth phase to induce expression of genes related to osmotic stress. Samples for RNA isolation were taken at increasing intervals within 4 days after brine addition. All samples were pooled for total RNA extraction and mRNA isolation (Ambion Inc., Austin, TX, USA). A cDNA library was synthesized with the CloneMiner cDNA Library Construction Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The 5′ ends of over 1000 clones were sequenced (unpublished data). The IBP sequence from *F. cylindrus* was obtained by annotation of this expressed sequence tag (EST) library using the Genpept data bank (translated NCBI) and the tBlastX algorithm with default options.

Purification of N. glaciei *IBP.* IBP was purified from the Cape Evans ice as described previously (Raymond 2000). In this method, cell-free meltwater was adjusted to an osmolality of approximately 300 mOsm/kg and frozen in centrifuge bottles at −15° C overnight. The bottles were centrifuged upside down at approximately −4° C to expel brine and impurity proteins, leaving IBPs relatively enriched in the ice. The ice fraction was melted and the freeze–thaw cycle was repeated another six times. The final solution was dialyzed, freeze-dried, and subjected to two-dimensional (2-D) (SDS-PAGE/*p*I) electrophoresis at Kendrick Laboratories (Madison, WI, USA) using a 10% acrylamide gel and *p*I range of 3.5–10. Other electrophoresis conditions were as described previously (Raymond and Fritsen 2001). The ice-binding activity of a solution was qualitatively assayed by observing the growth of ice single crystals submerged in the solution at a temperature of approximately 0.1° C below the freezing point. A rough, pitted surface is an indication of the presence of IBPs.

De novo *sequencing of peptides by tandem mass spectrometry (MS/MS).* Part of a protein spot of interest was cut from the gel and subjected to mass spectroscopy analysis at the Molecular Structure Facility, University of California, Davis, CA, USA. The spot was washed with 100 mM ammonium bicarbonate (AB), diced, dried in a SpeedVac (Savant, Instruments, Farmingdale, NY, USA), reduced with 10 mM dithiothreitol (DTT) in AB, pH 8 for 1 h at 55° C, washed with AB to removed excess DTT, alkylated with 55 mM iodoacetamide in 100 mM AB for 45 min in the dark at room temperature, separated from excess reagent, washed with AB, partially dehydrated with acetonitrile, completely dehydrated in a SpeedVac, and digested in 50% AB containing sequence grade, modified trypsin (Promega, Madison, WI, USA) at a final concentration in the range of 10–25 ng/μL at 37° C for 17 h. Peptides were extracted once each with 0.1% TFA in water and 5% formic acid in 50% acetonitrile. The extraction volume was carefully controlled, not exceeding 50 μL, and reduced to 15 μL as the final volume for mass spectrometry analysis. Aliquots of tryptic peptides were cleaned and concentrated using POROS R2 resin (Perceptive Biosystems, Framingham, MA, USA) in a microcolumn following the method described in the Protana manual (Protana, Odense, Denmark). In brief, the peptide mixture (dissolved in 5% formic acid) was loaded onto the microcolumn. The column was then washed with 5% formic acid and a gold-coated nanospray ES capillary obtained from Protana was aligned in continuation of the microcolumn. Peptides were eluted into the nanospray ES capillary using 50% methanol–5% formic acid. Tryptic peptides were analyzed by a hybrid nanospray/ESI-Quadrupole-TOF-MS and MS/MS in a QSTAR mass spectrometer (Applied Biosystems Inc, Foster City, CA, USA). Peptides in 50% methanol–5% formic acid were sprayed from the gold-coated capillary. The QSTAR instrument was calibrated with a standard peptide mixture that gives mass accuracies of 5 ppm or better. Argon gas was used as the collision gas. *De novo* sequencing of peptides was carried out using the QSTAR software (Analyst QS) and double-checked via a manual interpretation of MS/MS spectra.

Initial PCR amplification of N. glaciei *IBP cDNA.* Total RNA was isolated from axenically grown cells using TRIZOL reagent (Invitrogen) and reverse transcribed with a SuperScript first-strand synthesis kit (Invitrogen) using the polydT oligo included in the kit. PCR was performed using a forward primer 5′-CTG CTG TCA ACC TTG GAA CTG C-3′, based on sequences of the IBPs of *F. cylindrus* and *T. ishikariensis*, and a degenerate reverse primer 5′-TAT GCT GCN GAC TAC ACN GC-3′, based on one of the peptide fragments of *N. glaciei* IBP and the corresponding nucleotide sequence of *F. cylindrus* IBP. First-strand-synthesized cDNA was amplified by Taq polymerase (1 u; Promega) in a 50 μL reaction volume containing the following reagents (expressed as final concentration): 5 μL of 10× Taq buffer B, 1.5 mM $MgCl_2$, 0.2 mM dNTP mix, 0.2 μM forward primer, and 0.2 μM reverse primer. Products were amplified using a Eppendorf Mastercycler with initial denaturing for 3 min at 95° C and then 33 cycles as follows: 30 s at 95° C, 30 s at 53° C, and 40 s at 72° C, followed by a final extension for 7 min at 72° C. The PCR products were separated on a 2% TAE agarose gel and a single spot of 210 bp was excised, gel purified (Wizard gel prep, Promega) and cloned into a sequencing vector (pCR2.1, Invitrogen) and transformed into TOP10 competent *E. coli* (Invitrogen) for sequencing. Plasmids were isolated using a Wizard Plus Miniprep kit (Promega) and a subset was subjected to restriction enzyme digestion (*Eco*RI) for positive identification of the insert. The PCR inserts were sequenced with the M13F primer at the Nevada Genomics Center, University of Nevada, Reno, NV, USA.

5′/3′ Rapid amplification of cDNA ends (5′/3′ RACE). SMART RACE (Clontech, Mountain View, CA, USA)

technology was utilized to identify the entire open-reading frame of the *N. glaciei* IBP cDNA as well as identify the full 5′ and 3′ untranslated regions (UTRs). Total RNA was isolated from axenically grown *N. glaciei* cells and was reverse transcribed with Powerscript reverse transcriptase (Clontech) using either the 5′ RACE cDNA synthesis primer or 3′ RACE cDNA synthesis primer. 5′ RACE was performed using a gene-specific reverse primer 5′-GAC GAT GTT GAG AAT GTG TTG CTG GAA TCC-3′ and 3′ RACE was performed using a gene-specific forward primer 5′-CTC ACT ACT GGT CCT ACT GAA GTG ACC GG-3′). The PCR was performed in a 50 μL reaction volume using the 10× universal primer mix (5 μL), the gene-specific primer (0.2 μM final concentration), and reagents, and protocols in the AdvanTAge PCR cloning kit (Clontech). The 5′ RACE amplification was carried out as follows: 2 min at 95° C followed by 30 cycles of 30 s at 95° C, 30 s at 64° C, 2 min at 72° C. The 3′ RACE amplification was carried out as follows: 2 min at 95° C followed by 30 cycles of 30 s at 95° C, 30 s at 68° C, 2 min at 72° C. All amplification reactions were allowed to extend at 72° C for 10 min. The PCR products were gel purified and cloned into the pGEM-T sequencing vector (Promega) and transformed into TOP10 competent *E. coli* (Invitrogen) for sequencing. Plasmids were isolated using a Wizard Plus Miniprep kit (Promega) and a subset was subjected to restriction enzyme digestion (EcoRI) for positive identification of the insert. Twelve plasmids, six containing the 5′ RACE and six containing the 3′ RACE inserts, were sequenced in both directions as described above. Overlapping 5′ and 3′ RACE products allowed the reconstruction of the *N. glaciei* IBP cDNA.

To confirm the continuous open-reading frame of *N. glaciei* IBP cDNA as predicted by RACE and to investigate possible polymorphic variations in *N. glaciei* IBPs, we amplified first strand *N. glaciei* cDNA (described above) with primers designed within the 5′ UTR and 3′ UTR: 5′ UTR: 5′-CCCAA CAATTCAATCAACTCAATTTCC-3′; 3′ UTR: 5′-GGAGATC AACCCGGAACGATGAG-3′. First-strand synthesized cDNA was amplified with Pfx polymerase (1 u, Invitrogen) in a 50 μL reaction volume containing the following reagents (expressed as final concentration): 5 μL of 10× Pfx buffer, 1 mM $MgSO_4$, 0.6 mM dNTP mix, 0.4 μM 5′ UTR primer, and 0.4 μM 3′ UTR primer. Products were amplified using a DNA thermal cycler (9700, Perkin Elmer, Norwalk, CT, USA) with initial denaturing for 2 min at 95° C and then 30 cycles as follows: 30 s at 95° C, 30 s at 55° C, and 2 min at 72° C, followed by a final extension for 10 min at 72° C. The PCR product was separated on a 1% TAE agarose gel and a single band of approximately 850 bp was excised, gel purified and cloned into a Blunt-end sequencing vector (pCR4, Invitrogen) for sequencing. Six positive clones were isolated by miniprep and sent for sequencing in both directions (described above). Sequences were aligned with ClustalW.

Other. Predicted molecular mass and predicted *p*I of the open reading frame (ORF) were obtained with Protein Calculator (http://www.scripps.edu/cgi-bin/cdputnam/protcalc). The N-terminal sequences were examined for signal peptides with the neural network algorithm of SignalP (http://www.cbs.dtu.dk/services/SignalP/) and for mitochondrial and chloroplast targeting signals with TargetP (http://www.cbs.dtu.dk/services/TargetP). Full sequences were examined for *N*-glycosylation sites using NetNGlyc 1.0 Server (http://www.cbs.dtu.dk/services/NetNGlyc/). Putative homologs of the diatom IBPs were found using the BLAST search algorithm against the protein data base hosted by the National Center for Biotechnology Information (NCBI).

RESULTS

Origin of ice-binding activity. Sea ice diatoms have been suspected as being the source of ice-pitting activity in diatom-rich sea ice, but other members of the sea ice community such as bacteria and fungi could not be ruled out. Axenic cultures of seven sea ice diatoms, *N. glaciei*, *C. neogracile*, *F. pseudonana*, and *S. microtrias* from the Antarctic and *Synedra* sp., *Amphora* sp. and *Attheya* sp. from the Arctic, were tested and all were found to release ice-pitting activity into the culture medium. These results demonstrate that sea ice diatoms can produce IBPs and suggest that production of such proteins is a common trait of sea ice diatoms.

N. glaciei *IBP.* An example of the ice-pitting activity of supernatant from melted sea ice in which *N. glaciei* accounted for >99% of the cells is shown in Fig. 1A. The activity was retained through the purification process. 2-D polyacrylamide gel electrophoresis of nearly pure material yielded a spot of approximately 25 kDa with a *p*I of about 5.0 (Fig. 1B). Tandem mass spectrometry sequencing of the band yielded four peptide sequences, NTFSTSSLVTGK, LYAADYTAPTPSK, SDFSTAFTDAAGR, and VGDQ QFYLTGTAK. These sequences were found to be similar to several antifreeze isoforms of the snow mold *Typhula ishikariensis* (NCBI GenBank Acc. Nos. AB109742-AB109748) submitted by T. Hoshino et al. The *T. ishikariensis* protein was identified as an antifreeze because of its ability to depress the freezing point of water by approximately 0.1° C (Hoshino et al. 2003a). A second IBP cDNA from the sea ice diatom *F. cylindrus* Grunow (Acc. No. DR026070) was discovered from an *F. cylindrus* salt stress EST library. Primers based on the nucleotide sequences of the

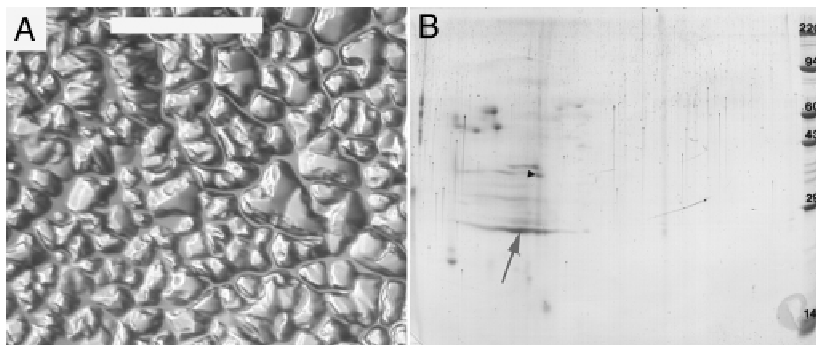

Fig. 1. Ice-binding proteins of *Navicula glaciei*. (A) Surface (basal plane) of an ice crystal grown in the presence of melted sea ice rich in *N. glaciei* cells. Adsorbed proteins inhibit growth on the pit faces, allowing vertical growth on the basal plane surrounding the pits. Scale bar, 500 μm. (B) Polyacrylamide gel of nearly pure ice-binding protein (IBP) of *N. glaciei*. Arrow indicates IBP. The *p*I range of the gel is from 3.5 to 10. Black triangle indicates a protein standard (tropomyosin) with *p*I of 5.2.

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Navicula         ----MMFLAKTVTLLVALVASSVAAEQ-SAVDLGTAGDFAVISKAGVSTT   45
Fragilariopsis   MMNLNLFLISAAAMVSVASASTALPPSPPAVNLGTAEDFVILAKAGVINV   50
Typhula          -----MFSASSLLAVIALAVSSVSAAGPSAVPLGTAGNYVILASTGVSTV   45
Cytophaga        TGIKDAAGAALPSNVIWSFTTGANASVLAVVNLRTAVNYVILAKTAINNN  146
Ferroplasma      VSPASPVTVNGAAITVNVSFTKLAPVSISPVNLGTAGNYAILAKTGISNT  129

Navicula         GPTEVTGDIGTSPIASTALTGFALIKDSSNTFSTSSIVTGKIYAADYTAP   95
Fragilariopsis   PGGAITGDIGVSPIAASAMTGFDIVMDSSNEFSTSTEITGKAYAPDYMSP  100
Typhula          PQSVITGAVGVSPGTAASLTGFSLILSGTGTFSTSSQVTGQLTGADYGIP   95
Cytophaga        PTSAVTGAIGLSPAATSYITGFSIT-NATG-YATSSQVTGHIFAADMVSP  194
Ferroplasma      GTTSIVGNIGVSPASSTYITGISITMNSSGQFSTSSMVTGNVYAATYASP  179

Navicula         TPSKMTTAISDMSTAFTDAAGR-----------SDPDFLELGAGSIEGET  134
Fragilariopsis   TGTKLTTAVSDMITAYNDAAARPVTGGPFGNSISGETYTNLGAGEIGGIT  150
Typhula          TPSILTTAIGDMGTAYINAATR-----------SGPDFLEIYTGAIGGTT  134
Cytophaga        TSSNLTTAINDMQTAYTDAAGR-----------KTPDYVELGTGNIGGKT  233
Ferroplasma      TPSILTTAVGDMQTAYINAAGR-----------TNPNYVNLGAGDINGMI  218

Navicula         IVAGLYKWGTDVSFTS-SLVFDGSATDVWILQVAKDFTVGNGAQMYITGT  183
Fragilariopsis   ITRGVYTYDINVSITSGKVIFHGGADDVFIIKISKSVLQAANTEVVITGG  200
Typhula          ILPGLYKWTSSVGASA-DFTISGTSTDTWIFQIDGTIDVATGKQITIVGG  183
Cytophaga        IQPGLYKWTSSVSVPS-DVIISGGANDVWIFQISGNISLSAGAKITISGG  282
Ferroplasma      IVPGLYKWGTGVSIST-SIILTGNSSSVWIFQISGGITFGNGAHIIISGG  267

Navicula         AKAENIFIQVSGAVNIGTTAHVEGNILSATAIALQTGSSINGKALSQTAI  233
Fragilariopsis   AQAKNIFWSVAQEVNVGAGAHMEGILIVKTAVKFITGSSFVGRVLSATAV  250
Typhula          AQAKNIIWVVAGAVNIEVGAKFEGTIIAKTAVTEKTGSSINGRILAQTAV  233
Cytophaga        AQAKNIFWQVAGTVTAGITSHIEGVILSKIGITENTGASIKGRALAQTAI  332
Ferroplasma      AQPQNIFWQVASGATIGTGATFYGTILSQTAITIATGSSMIGIALAQTAV  317

Navicula         TLDSVTIVS-----------------------------------------  242
Fragilariopsis   TLQSAAITAPATSAPTTRRGPRGLQVA-----------------------  277
Typhula          ALQSATIVEK----------------------------------------  243
Cytophaga        IIDGNIVIQP----------------------------------------  342
Ferroplasma      TLQSDTITAPLEPQSITAAMYGVTFTEAGLPSGTQWNVTLNGVLLSSTVP  367
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FIG. 2. ClustalW alignment of ice-binding proteins from *Navicula glaciei* (Acc. No. DQ062566), *Fragilariopsis cylindrus* (CN212299) and *Typhula ishikariensis* (AB109745), and hypothetical proteins from *Cytophaga hutchinsonii* (ZP_00309837) and *Ferroplasma acidarmanus* (ZP_00608957). Predicted signal peptides are underlined. Gaps have been inserted to improve alignment. Conserved residues are shaded. The N-terminal sequence of the *Cytophaga* protein and the N-and C-terminal sequences of the *Ferroplasma* protein are truncated. Residue numbers are shown at right.

T. ishikariensis and *F. cylindrus* antifreeze genes and the *N. glaciei* peptide sequences resulted in the amplification and cloning of a 210 bp *N. glaciei* cDNA whose predicted amino-acid sequence closely matched the MS-derived *N. glaciei* peptides. Using gene-specific primers designed from the *N. glaciei* 210 bp product and 5′/3′ RACE, nine 5′ and seven 3′ RACE products were sequenced and found to be similar to both the *T. ishikariensis* and *F. cylindrus* IBP sequences. The RACE products overlapped for 63 nts between nucleotides 220 and 282 of the reconstructed IBP shown in Fig. 2. Although 5′ RACE products were not equal in length, all sequences at a common position within the 5′ UTR did not show any sequence variation and were considered identical. Similar to the 5′ UTR, the 3′ RACE products did not show sequence variation in the 3′ UTR until nucleotide 914, which is just proximal to the polyadenylation site where the sequencing results were less reliable. A consensus polyadenylation site (ATAAA) was found at nucleotides 901–905, which is 14 nucleotides upstream of a polyadenylated region. Six PCR products obtained with flanking UTR primers were cloned and sequenced, and none were redundant. They confirmed the 5′/3′ RACE results and revealed a fairly wide range of single nucleotide polymorphisms within the open-reading frame. One of the clones was identical to a corresponding set of 5′ and 3′ RACE products and so it and the 5′ and 3′ RACE products were used to reconstruct a full-length *N. glaciei* IBP (Acc. No. DQ062566, Fig. 2) (Accession numbers of the other five clones are DQ062561–DQ062565). The confirmed full-length *N. glaciei* cDNA contains a 75 nt 5′ UTR, a 726 nt ORF encoding 242 amino acids, and an approximately 121 nt 3′ UTR. The predicted molecular mass and predicted *p*I of the ORF 24,461 Da and 4.45, agree well with the values estimated by 2-D electrophoresis. The closeness of the two molecular mass estimates suggests that the protein has little or no carbohydrate moiety. The molecular mass is consistent with a value of 30 kDa estimated for an IBP associated with the sea ice diatom *Nitzschia stellata* (Raymond et al. 1994). Together, these data cast doubt on estimates of larger sizes of IBPs based on retention by dialysis membranes from two other species of ice diatom (Raymond 2000).

F. cylindrus *IBP.* The above-mentioned cDNA from the *F. cylindrus* salt stress library was found to have high similarity (e-value 5e-43) to isoforms of the *T. ishikariensis* antifreeze, which identified its putative function. The full-length *F. cylindrus* cDNA contains a 63 nt 5′ UTR, an 831 nt ORF encoding 277 amino-acids, and an approximately 49 nt 3′ UTR. The predicted molecular mass and predicted *p*I of the ORF are 27,961 Da and 5.55. A putative polyadenylation signal is located at nucleotides 921–926 (ATTAAA), which is approximately 20 nucleotides upstream of the poly(A+) tail. The predicted amino-acid sequence (Fig. 2) shows 48% identity to that of *N. glaciei* IBP. Although *F. cylindrus* was not tested to see whether it expresses an IBP, axenically grown *F. pseudonana* was confirmed to release ice binding activity into the culture medium and to express an IBP mRNA, whose partial sequence (~530 bp, unpublished data) is very similar to the sequence of *F. cylindrus* IBP.

IBP properties. The N-terminal sequences of the *N. glaciei* and *F. cylindrus* IBPs and each of the *T. ishikariensis* antifreeze isoforms have high probabilities of being signal peptides (with lengths of 21, 20, and 21 residues, respectively; Fig. 2) and low probabilities of being mitochondrial or chloroplast targeting peptides. *F. cylindrus* IBP has one potential *N*-glycosylation site, whereas *N. glaciei* IBP has none, in agreement with its predicted low degree of glycosylation.

Other IBP-like proteins. Other proteins similar to the diatom IBPs were found in the NCBI data base. H. S. Kwan et al. (The Chinese University of Hong Kong) submitted 18 ESTs from the commercially grown shiitake mushroom, *Lentinula enodes* (e.g. Acc. No. CO501811) that closely resembled (~70% amino-acid identity) the *T. ishikariensis* antifreeze sequence (43% amino-acid identity to *N. glaciei* IBP). Shiitake mushrooms are typically exposed to freezing temperatures during their growing period. The supernatant of a cell homogenate of this species was found to have modest ice-pitting activity (our unpublished data), presumably owing to expression of the IBP-like genes. However, the function of these genes remains unclear.

The IBPs also show considerable similarity to hypothetical proteins from a Gram-negative soil bacterium, *Cytophaga hutchinsonii*, (Acc. no. ZP_00309837; 48% amino-acid identity to the *N. glaciei* IBP), an acidophilic archaeon, *Ferroplasma acidarmanus* (Acc. no. ZP_00608957; 51% identity), a denitrifying estuarine bacteria, *Shewanella denitrificans* (Acc. no. ZP_00636736; 43% identity), and a psychrotolerant sediment bacteria, *Rhodoferax ferrireducens* (Acc. no. ZP_00693230; 58% identity) in the regions of overlap. Portions of the *C. hutchinsonii* and *F. acidarmanus* proteins are aligned with the diatom and *T. ishikariensis* IBPs in Fig. 2. Several bacterial strains related to *Cytophaga* and several species of *Shewanella* have been isolated from polar ice. A few other bacterial proteins that are similar to the *Cytophaga* and *Ferroplasma* proteins show lower sequence identities (~30%) to the IBPs. It is interesting that the *Cytophaga* and *Ferroplasma* proteins are also weakly similar (21%–25% identities) to some bacterial ice-nucleating proteins (e.g. Acc. Nos. P09815, P20469, and P06620), although the resemblance may be owing to chance. The phylogenetic relationships of these proteins will be described separately.

Sequence information from the nearly full genome of *Thalassiosira pseudonana* (Armbrust et al. 2004) and from EST data for *Phaeodactylum tricornutum* (Scala et al. 2002), both mesophilic diatoms, have recently become available. No predicted translation products similar to IBPs were found in either species, in agreement with a previous finding that ice-pitting activity in diatoms is limited to polar regions (Raymond et al. 1994).

DISCUSSION

Previous studies have described "ice-active substances" that are "associated" with sea ice diatoms (Raymond et al. 1994, Raymond 2000) because the nature of the substances and their origin were not definitely known. The present results obtained from axenic cultures of diatoms confirm that diatoms produce proteins with ice-binding activity. It seems likely that the activity that we observe in natural communities largely originates from diatoms, although we cannot rule out the possibility that some of the natural activity originates from bacteria. Our results identify the source of the ice-binding activity in *N. glaciei* as a 25 kDa protein and strongly suggest that the IBP gene of *F. cylindrus* expresses a similar protein. Over 200 species of sea ice diatom are found in both the Arctic and Antarctic, representing a total of 58 genera (Horner 1985). Because each of the axenic species of sea ice diatom that we of examined so far have exhibited ice-pitting activity, we expect that each of these genera will have one or more IBP homologs.

IBP homologs. Our results show that the diatom IBPs are likely homologs of "antifreeze" proteins from the snow mold *T. ishikariensis*. Other snow molds appear to have similar proteins (Hoshino et al. 2003a, b). The diatom and snow mold IBPs do not resemble any of the numerous antifreeze proteins that have been identified so far in fish, insects, plants, and bacteria, and thus represent a new class of IBP.

The finding of IBP-like hypothetical proteins in *Cytophaga hutchinsonii* and *Shewanella denitrificans* is interesting because both species are related to well-known sea ice bacteria. One of the most prominent groups of sea ice bacteria are the so-called Cytophaga–Flavibacterium–Bacterioides (CFB) phylum group (Brown and Bowman 2001). Bacteria from this group have been isolated from sea ice in the Arctic (Junge et al. 2002, Groudieva et al. 2004) and Antarctic (Brown and Bowman 2001), and in ice overlaying Lake Vostok in Antarctica (Christner et al. 2001). Furthermore, several lines of evidence suggest that CFB

or CFB-like bacteria contribute strongly to bacterial populations in well-established sea-ice algal assemblages (Brown and Bowman 2001). Several psychrophilic species of *Shewanella* have been isolated from sea ice, often in association with algal assemblages (Bowman et al. 1997a, b). It will thus be interesting to see whether these polar bacteria express IBPs.

Function and mechanism of IBPs. Because the diatom IBPs appear to be limited to icy environments, it is reasonable to believe that they have a role in cold hardiness. It seems most likely that their role is to protect cells in the frozen state because they have little effect on the freezing point. In fact, IBPs have been shown to increase the survival of cells subjected to a freeze–thaw cycle: semi-pure *Fragilariopsis kerguelensis* IBP increased the survival of both polar and nonpolar diatoms (Raymond and Knight 2003, Raymond and Janech 2003) and semipure *N. glaciei* IBP was found to reduce the hemolysis of frozen human red blood cells (Kang and Raymond 2004). The latter results indicate that the IBP protected the cell membrane.

A possible mechanism by which IBPs could prevent freezing injury to membranes is inhibition of the recrystallization of ice, a phenomenon that has been implicated in plant freezing tolerance (Knight et al. 1995, Thomashow 1998, Griffith and Yaish 2004). Recrystallization is a process in which large grains of ice grow at the expense of small grains, which are thermodynamically unstable. The growth of the larger grains is thought to be physically disrupting to cell membranes. Protein recrystallization inhibitors have been found in several cold-hardy plants, including ryegrass (Pudney et al. 2003), Antarctic hair grass (John and Spangenberg 2005), carrot (Worrall et al. 1998, Smallwood et al. 1999), lichens and mosses (Doucet et al. 2000), and winter rye (Griffith et al. 2005). Diatom IBPs are also strong recrystallization inhibitors (Raymond and Janech 2003, Raymond and Knight 2003, Kang and Raymond 2004), as are fish antifreezes (Knight et al. 1988). In hair grass (John and Spangenberg 2005) and winter rye (Griffith et al. 2005), recrystallization inhibitors have been found to be expressed or to accumulate in the extracellular region where ice forms, which supports the idea that they serve to prevent freezing injury. Because diatoms secrete IBPs, the IBPs may also act extracellularly, where they may protect the cells by preventing the recrystallization of external ice. However, other functions cannot be ruled out. For example, diatom IBPs may have a role in preserving brine pockets in sea ice, which appear to be essential for the survival of diatoms in sea ice at low temperatures (Krembs et al. 2002).

Origin of IBPs. Although the diatom IBPs and snow mold antifreezes almost certainly have a role in cold hardiness, their original function may have been different. In other organisms, antifreezes appear to have arisen from a variety of proteins with other functions (Cheng 1998, Griffith and Yaish 2004), although some may retain the original functions (Griffith and Yaish 2004). These proteins fortuitously shared an ability to bind to ice that appears to have been exploited to allow survival in freezing environments. The function of the IBP-like transcripts of the mushroom *Lentinula enodes* is unclear. This species is typically grown over a period greater than a year, during which time temperatures can fall below freezing, so they may act as cryoprotectants, but other roles cannot be ruled out. The route by which the diatoms acquired IBP genes is unknown. Horizontal transfer from fungi is a possibility, as basidiomycotic fungi (which include *Lentinula* and *Typhula*), are known to inhabit sea ice (Melnikov 1997, Gunde-Cimerman et al. 2003), and are believed to have arisen hundreds of millions of years before the diatoms (Heckman et al. 2001, Falkowski et al. 2004). Horizontal transfer from a *Cytophaga*-like bacteria or a species of *Shewanella* is another possibility because of their association with algal assemblages in sea ice and relation to species with IBP-like genes. However, it is too early to rule out the possibility of convergent evolution from different ancestral genes. We are presently attempting to isolate IBPs from other genera of sea ice diatoms, which we hope will shed light on the evolution of these proteins in polar diatoms and possibly on the evolution and radiation of sea ice diatoms themselves.

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