Extremophiles
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A bacterial ice-binding protein from the Vostok ice core

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Abstract Bacterial and yeast isolates recovered from a deep Antarctic ice core were screened for proteins with ice-binding activity, an indicator of adaptation to icy environments. A bacterial strain recovered from glacial ice at a depth of 3,519 m, just above the accreted ice from Subglacial Lake Vostok, was found to produce a 54 kDa ice-binding protein (GenBank EU694412) that is similar to ice-binding proteins previously found in sea ice diatoms, a snow mold, and a sea ice bacterium. The protein has the ability to inhibit the recrystallization of ice, a phenotype that has clear advantages for survival in ice.

Keywords Subglacial Lake Vostok · Flavobacteriaceae · Ice-binding protein · Recrystallization inhibition · Ice grain boundaries

Introduction

The Vostok Ice Core represents a cross-section of the East Antarctic Ice Sheet to a depth of over 3,600 m, which is <100 m above Subglacial Lake Vostok (Petit et al. 1999), the largest subglacial lake in Antarctica. Using a quantitative decontamination protocol, cells were detected in melted ice samples from all ice core depths analyzed and microbes in the deepest sections were shown to be metabolically active when provided with organic substrates (Christner et al. 2006). Subsequently, a number of bacterial and yeast species were cultured and isolated from these samples. We examined these species to see if any were specifically adapted to surviving in ice.

Some cold-adapted microorganisms increase their survival at sub-zero temperatures by producing proteins that bind to and inhibit the growth of ice crystals. Examples include a bacterial antifreeze protein (Garnham et al. 2008), and proteins from a sea ice bacterium (Raymond et al. 2007), snow molds (Hoshino et al. 2003) and sea ice diatoms (Janech et al. 2006) that inhibit the recrystallization of ice. We hypothesized that such proteins could increase the survival of microorganisms that become entrapped in glacier ice. Furthermore, as ice-active proteins have been described only in species inhabiting frozen environments, production of such proteins by a microbe would provide additional evidence that it is not a contaminant, a question that often arises in reference to isolations of microbes from ancient ice (Inman 2007). Here we describe such a protein from one of the Vostok ice core isolates and show how it might enhance survival under frozen conditions by affecting the physical structure of the ice.

Materials and methods

Sampling and culturing procedures

Contaminating cells on the ice core exterior were removed using a quantitative decontamination protocol (Christner

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et al. 2005). The cleaned ice samples were melted and incubated at 4°C as described previously (Christner et al. 2001). The isolates were subsequently grown in R2 liquid or agar-solidified media (Difco, Inc.). Fourteen isolates were examined. These included two yeast species (*Cryptococcus* sp. and *Rhodotorula* sp.) and 12 bacteria (*Subtercola* sp., *Sphingomonas* sp., a Flavobacteriaceae and nine unidentified bacteria).

Activity measurements

Ice-binding activity was assayed by measuring the ability of a solution to cause pitting and other distortions in a growing ice crystal (Raymond and Fritsen 2000). Recrystallization inhibition (RI) activity was measured as described previously (Raymond and Fritsen 2001). Briefly, 3-µl drops of solutions with and without IBP were frozen side-by-side with a liquid nitrogen-cooled glass plate, immersed in hexane, stored in a −4°C bath and photographed through crossed polarizers. The experiment was done in triplicate with similar results.

IBP purification and characterization

Gel permeation chromatography was used to estimate the molecular size of the active fraction in the culture medium. Culture medium was concentrated by vacuum centrifugation and applied to a G-50 Sephadex column (2.5 cm × 50 cm) and 1.7-ml fractions were collected and assayed for ice-binding activity. IBP was semipurified by exploiting its affinity for ice (Raymond and Fritsen 2000). The semipurified material was subjected to 2D electrophoresis by K. Schegg of the University of Nevada Proteomics Center, Reno. Spots on the gel were cut out and subjected to a mass fingerprinting analysis by A. Chien of the Stanford University Mass Spectrometry Laboratory.

Genome sequencing

High-molecular weight genomic DNA was isolated using a conventional phenol/chloroform-based extraction protocol (Murray and Thompson 1980). A draft version of the genome was sequenced by the 454 method (Margulies et al. 2005). A sequencing library was constructed from sheared genomic DNA and loaded onto a GS 20 Sequencing System (454 Life Sciences, Branford, CT, USA) according to the manufacturer's protocol.

Protein prediction

p*I* and molecular mass were predicted with the ExPASy p*I* tool (http://ca.expasy.org/tools/pi_tool.html). The presence of a signal peptide was predicted with SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP/).

Recombinant IBP

The IBP gene was amplified from lysed cells by PCR with with forward and reverse primers 5′-ATGAATAAAT TTTTACTACTGGCTGCTTCGGTTG-3′ and 5′-TTATG GCTTAGCAATCAGTTTACCTGCCTG-3′. The resulting product was inserted in the pEXP-NT/TOPO vector (Invitrogen), which was then transformed into One-Shot competent *Escherichia coli* cells (Invitrogen). Plasmids were purified from several clones with a Wizard Plus SV miniprep kit (Promega) and sequenced. A plasmid in which the IBP insert had the correct orientation and reading frame with respect to an N-terminal His tag was then incubated with the reaction mixture of the Expressway cell-free expression system (Invitrogen) for 4.5 h at 30°C. The supernatant from the reaction mixture (100 µl) was mixed with 100 µl water, resulting in a final osmolality of about 460 mOsm kg^{-1}, and then assayed for ice-binding activity as described above. A reaction mixture in which the DNA was replaced with an equal volume of water served as a negative control.

Partitioning of cells in ice

3519-10 cells were cultured in R2 at 4°C. A dilute suspension (10^7 cells ml^{-1}) was stained with SYBR Gold™ (Molecular Probes, Inc., cat. no. S-11494) for 15 min and frozen in a test tube at −80°C. The ice suspension formed was thin sectioned in a −5°C environmental room with a microtome. A thin ice section was viewed on a microscope (Olympus BX51-TRF epifluorescence microscope) equipped with a Linkam large area thermal stage.

Results and discussion

Fourteen of the bacterial and yeast isolates from depths of 171–3,622 m were cultured at 4°C. Cell-free media were recovered from the stationary phase of growth and examined for ice-binding activity using an ice-pitting assay. One of the bacterial isolates from a depth of 3,519 m (designated 3519-10) was clearly active, causing hexagonal pits to form on the ice (Fig. 1a), while the others showed no activity under the conditions tested. The pits were formed by vertical growth of the ice around the pits, indicating that growth on the pit faces was inhibited. In gel permeation chromatography, the active fraction eluted early, indicating that the activity was associated with a macromolecule. Conditioned growth media from 3519-10 also contained a substance with ice RI activity (see below). The ice at a

depth of 3,519 m is near the bottom of a layer of deformed glacial ice and has an age in excess of 420,000 years (Petit et al. 1999). This depth is ~20 m above the layer of accretion ice originating from Subglacial Lake Vostok and has a temperature of ~−8°C (Salamatin et al. 2004).

A draft version of the 3519-10 genome (manuscript in preparation) yielded a full-length 16S rRNA gene sequence (deposited in GenBank as EU694411). The sequence was most similar (97% identity) to 16S sequences of two genera in the Flavobacteriaceae family (*Haloanella* and *Chryseobacterium*) (GenBank acc. nos. AB035150 and AM423083, respectively), and 96 and 97% identical to partial sequences (~1.4 kb) from Antarctic and Himalayan glacier ice isolates, respectively (GenBank acc. nos. AF479331 and DQ223664, respectively).

Similarity searches of the genome for open reading frames resembling all other known ice-binding genes yielded a gene encoding a protein of 533 amino acids that significantly matched (~26% identities, ~45% similarities) ice-binding proteins (IBPs) from a snow mold, sea ice diatoms and a sea ice bacterium (Raymond et al. 2007). The protein was designated IBPv, and its gene was deposited in GenBank as EU694412. To identify the molecular component in the conditioned medium responsible for ice-binding activity, extracellular proteins in the 3519-10 culture medium were selected for their affinity for ice. The "ice-loving" fraction was confirmed to have ice-binding activity and examined by 2D electrophoresis. A spot on the gel matched the predicted mass (54.1 kDa) and p*I* (6.1) of IBPv. In addition, the mass fingerprint of the spot was found to perfectly match IBPv, thus confirming that IBPv encodes an IBP that is expressed under cold conditions. The IBPv gene was cloned and expressed in a cell-free expression system and the product showed very strong ice-binding activity (Fig. 1c), confirming its identity.

IBPv has a predicted N-terminal secretory peptide and two nearly complete IBP domains (Fig. 1b). In a

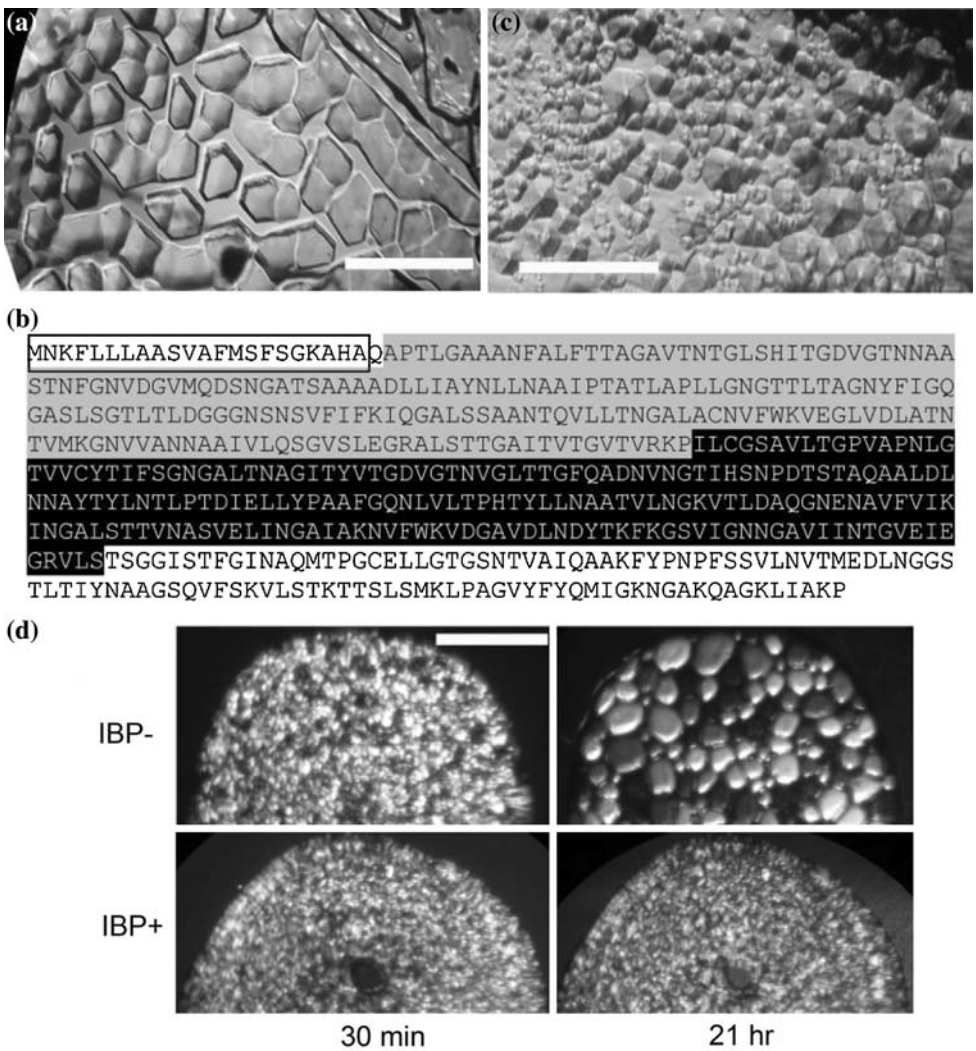

Fig. 1 An ice-binding protein (*IBP*) from the Vostok ice core bacterium 3519-10. **a** Pitting of an ice crystal grown in culture medium, indicating the presence of an IBP. **b** Predicted amino acid sequence of the IBP. *Boxed* sequence is a predicted secretory peptide; *gray* and *black* portions indicate two IBP domains, each of which is 28% identical and about 45% similar to that of a sea ice bacterium (Raymond et al. 2007). **c** Dense pitting of an ice crystal grown in a solution of recombinant IBP. **d** Recrystallization of frozen drops of solutions with and without recombinant IBP held at −4°C for 21 h. *Scale bars* 1 mm

phylogenetic tree of protein sequences with IBP-like domains, the two IBP domains of IBPv clustered with hypothetical proteins from two psychrophilic sea ice bacteria of the Flavobacteriaceae, *Psychroflexus torquis* (Bowman et al. 1998) and *Polaribacter irgensii* (Gosink et al. 1998) (Fig. 2). IBPv was most closely related to one of the *P. irgensii* sequences, which also contains two IBP domains.

The recombinant protein also showed ice RI activity (Fig. 1d), a property that is thought to prevent the mechanical disruption of cells during recrystallization (Urrutia et al. 1992). However, RI activity might have another benefit in deep ice that has not been previously recognized. Recrystallization, a natural process in which larger grains of ice grow at the expense of smaller grains, is common in warm basal ice under large strain (Paterson 1994) and was prevalent in the deformed glacial ice sample from which 3519-10 was isolated. As shown in Fig. 1d, RI activity could conserve the boundaries between ice grains in glacier ice. Such boundaries constitute a network of liquid veins that harbor microbial communities in sea ice (Deming 2002) and have been proposed as a microbial habitat in deep Antarctic ice (Price 2000). Triple junctions, where the boundaries of three ice crystal grains meet, are considered especially important as microbial refugia because of their greater liquid volume and tendency to accumulate ions and nutrients excluded from the ice (Price 2000; Mader et al. 2006). In fact, cultured cell suspensions of 3519-10 were found to accumulate at triple junctions when frozen under laboratory conditions (Fig. 3). In the ice vein environment of glacier ice, microbes that do not produce an IBP could benefit from commensal relationships with species such as 3519-10, as has been observed in cold-adapted soil bacteria (Walker et al. 2006).

We have shown that a bacterium recovered from deep within the East Antarctic Ice Sheet possesses a protein with ice-binding activity that has sequence similarity to IBPs from species inhabiting other ice-bound habitats. These

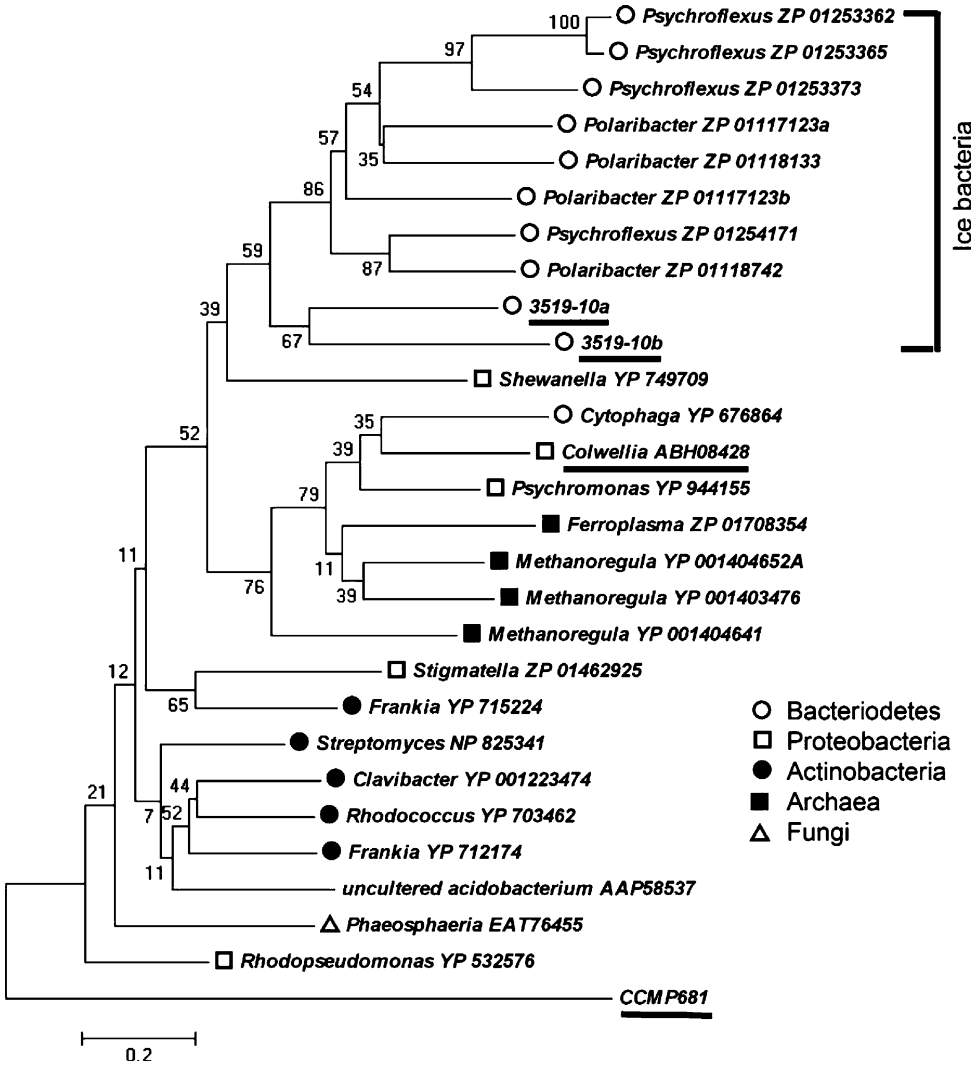

Fig. 2 Neighbor-joining tree showing the phylogenetic relationship among the two IBP domains of IBPv, a known bacterial IBP, and other hypothetical proteins in GenBank with IBP-like domains. The sequences analyzed include all sequences that matched either of the IBPv domains with expect thresholds of 1×10^{-10} or less. *Numbers* at nodes indicate bootstrap values for 1,000 replicate runs. The tree is based on deduced amino acid sequences and was constructed with MEGA version 4 (Tamura et al. 2007). GenBank accession numbers are shown. An unrelated IBP (unpublished) from a *Chlamydomonas*-like species (CCMP681) was used as an outgroup. *Underlines* indicate proteins with known ice-binding activity

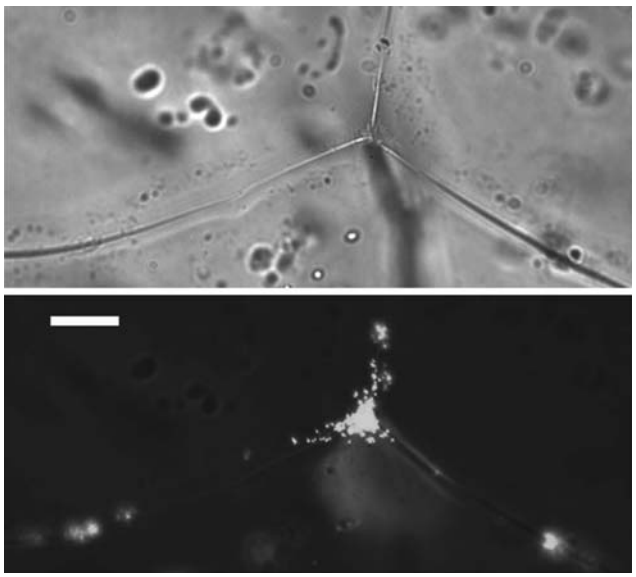

Fig. 3 Cryostage microscopic image at −20°C of 3519-10 cells partitioned within a triple junction. The ice was formed from a solution of dilute mineral media that contained 10^7 cells ml^{-1}. *Upper* image is a bright field image of the ice; the *lower* image is the same area under epifluorescence showing SYBR Gold-stained cells in the interstitial veins between the ice crystals. *Scale bar* 100 μM

findings argue strongly that the strain is not a human- or laboratory-derived contaminant. Furthermore, in view of recent reports demonstrating the viability of bacteria in ancient ice (Miteva et al. 2004; Bidle et al. 2007), our results reveal a molecular adaptation that may be important for the long term survival of microbes in glacier ice.

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