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Properties and biotechnological applications of ice-binding proteins in bacteria

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One sentence summary: This review analyzes the current information available on bacterial antifreeze and ice-nucleating proteins and their biotechnological applications in diverse fields, including their future perspectives in agriculture.

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ABSTRACT

Ice-binding proteins (IBPs), such as antifreeze proteins (AFPs) and ice-nucleating proteins (INPs), have been described in diverse cold-adapted organisms, and their potential applications in biotechnology have been recognized in various fields. Currently, both IBPs are being applied to biotechnological processes, primarily in medicine and the food industry. However, our knowledge regarding the diversity of bacterial IBPs is limited; few studies have purified and characterized AFPs and INPs from bacteria. Phenotypically verified IBPs have been described in members belonging to Gammaproteobacteria, Actinobacteria and Flavobacteria classes, whereas putative IBPs have been found in Gammaproteobacteria, Alphaproteobacteria and Bacilli classes. Thus, the main goal of this minireview is to summarize the current information on bacterial IBPs and their application in biotechnology, emphasizing the potential application in less explored fields such as agriculture. Investigations have suggested the use of INP-producing bacteria antagonists and AFPs-producing bacteria (or their AFPs) as a very attractive strategy to prevent frost damages in crops. UniProt database analyses of reported IBPs (phenotypically verified) and putative IBPs also show the limited information available on bacterial IBPs and indicate that major studies are required.

Keywords: Antarctic bacteria; antifreeze proteins; ice-nucleating proteins; ice-binding proteins; psychrophilic bacteria

INTRODUCTION

Cold environments have opened a new window in bioprospecting, which is the discovery and commercialization of new products (such as enzymes and bioactive compounds) from biological resources (Vester, Glaring and Stougaard 2015). Two of the most amazing examples are psychrophilic and psychrotolerant bacteria. Psychrophilic bacteria are defined as bacteria with an optimal growth temperature at ≤15 °C, while psychrotolerant...
bacteria are defined as bacteria with optimal growth at >15 °C, but that can tolerate and survive at lower temperatures (Helmkne and Weyland 1995). In nature, finding bacteria that are able to survive and proliferate at low temperatures is not surprising given that 71% of the earth’s surface is covered by oceans (with an average temperature from 2 °C to 4 °C below 1000 m depth), of that 15% is polar regions, glaciers and alpine mountains and permafrost (Feller 2013).

Bacteria from cold environments are characterized by long-term survival at low temperatures with low water and nutrient availability (Vishniavetskaya et al. 2006); these bacteria have been reported in a wide variety of habitats, such as Antarctic subglacial lakes (D’Elia, Veerapaneni and Rogers 2008), Antarctic glacial ices (Bidle et al. 2007) and the deep sea (Xu et al. 2003). However, bacteria that have adapted to cold environments have been scarcely explored, and their roles in nature have primarily been associated with the dissolution and oxidation of minerals in sediments beneath ice masses, and with the degradation of recalcitrant soil organic matter in permafrost (Pautler et al. 2010).

Ice-binding proteins (IBPs), such as antifreeze proteins (AFPs) have been found in organisms that survive and proliferate at cold temperatures. AFPS act by binding to ice crystals causing (i) thermal hysteresis (TH) and (ii) ice recrystallization inhibition (IRI) (Lorv, Rose and Glick 2014). TH is a non-colligative effect defined by a difference between the freezing and melting points of a solution (Raymond and DeVries 1977). In IRI, AFPS prevent the generation of large ice crystals by boundary migration of smaller ice crystals (Yu et al. 2010). Another type of IBPs is the ice-nucleating proteins (INPs), which induces ice formation at high subzero temperatures (Lindow, Arny and Upper 1982). Water freezing is a stochastic process that is determined by the temperature and orientation of water molecules. Spontaneous or homogeneous ice nucleation in pure water occurs at a temperature of −38.5 °C, and bacterial INPs can act as water molecule organizers that promote ice nucleation at higher temperatures (−2 °C to −10 °C) (Lee, Warren and Gusta 1995).

IBPs are highly attractive for biotechnologists as they can be applied in various fields, mainly in food industry (Fletcher et al. 1997; Kontogiorgos et al. 2007; Yeh, Kao and Peng 2009; Zhang, Wang and Chen 2010) and in medicine (Koushafar and Rubinsky 1997; Muldrew et al. 2001; Amir et al. 2003; Hirano et al. 2008; Lee et al. 2012). For example, AFPS are used in ice-cream production to keep their quality (Warren, Mueller and McKown 1992; Feeney and Yeh 1998) and INPs are used for the production of artificial snow (Cochet and Widehem 2000). Nevertheless, few IBPs from bacteria have been described to date, and their applications have only been demonstrated at the laboratory level. In the light of these facts, a better understanding of current and future biotechnological applications of bacterial IBPs is required. Thus, this minireview summarizes the current information on bacterial IBPs (both AFPS and INPs) and their application in biotechnology, with an emphasis on the potential application in less explored fields such as agriculture.

ANTIFREEZE PROTEINS

AFPs were first identified in Antarctic teleost fish (DeVries and Wohlschlag 1969). However, AFPS are not limited to fish and have subsequently been found in a great variety of living organisms from cold environments, such as plants (Bravo and Griffith 2005; Middleton et al. 2009), insects (Graether and Sykes 2004; Kristiansen et al. 2011) and microorganisms such as fungi (Hoshino et al. 2003; Xiao et al. 2010), microalgae (Janech et al. 2006; Raymond, Janerch and Fritsen 2009), yeast (Lee et al. 2010) and archaea (Saunders et al. 2003).

Based on differential ice-binding patterns, AFPS have been classified into two primary categories: moderately active and hyperactive. The difference between moderately active and hyperactive AFPS is still being actively researched. A main driving force for their difference may be in their selectivity for different faces of ice, where the hyperactive AFPS bind to the prism and planes basal of ice and block growth along its c-axis (Pertaya et al. 2008). Indirect observation in a mutational study on an insect protein (Tenebrio molitor AFP, TmAFP) supports this mechanism, where the modification of the residues from threonine to valine resulted in lower activity of AFP, possibly due to a lower binding affinity of the protein for the basal plane of ice (Liou et al. 2000).

Moderately active AFPS bind only to the prism plane, leaving the basal plane uncovered (Scotter et al. 2006). In addition, extensive ice surface coverage by hyperactive AFPS increases TH activity up to 6 °C in the meal worm T. molitor at submillimolar concentrations (Graham et al. 1997). In comparison, moderately active AFPS, commonly found in fish, are characterized by a lower TH of 0.5 °C–1.0 °C at millimolar protein concentrations (Hanada et al. 2014).

BACTERIAL AFPs

Studies describing the isolation, purification and characterization of AFPS produced by environmental bacteria are scarce. As shown in Table 1, the first study that discovered AFPS in bacteria was performed by Duman and Olsen (1993) in Micrococcus cryophilus and Rhodococcus erythropolis. However, these AFPS remain uncharacterized. The second study, by Sun et al. (1995), presented a glycoprotein AFP from Pseudomonas putida isolated from the rhizosphere of plants grown in the Canadian Arctic. These authors reported an apparent molecular weight of ~34 kDa with a low TH of ~0.1°C. Subsequently, Xu et al. (1998) characterized this AFP as an extracellular protein with an apparent molecular weight of 164 kDa. They have also described that aggregates of this AFP display ice nucleation activity, which favors extracellular ice nucleation, followed by the stabilization effect of IRI. Both activities are suggested to maintain ice crystals at a small harmless size.

A hyperactive AFP was reported in the marine bacterium Marinomonas primoryensis (MpAFP) with a molecular weight >1000 kDa, and was shown to be a calcium-dependent protein with a TH of 2 °C at a concentration of 0.5 mg mL−1 (Gilbert, Davies and Laybourn-Parry 2005; Garnham et al. 2008) (Table 1). The X-ray crystallographic structure of MpAFP is shown in Fig. 1 (Garnham, Campbell and Davies 2011). The side chain atoms of threonine and asparagine residues in the AFP contribute to the binding of water molecules to the ice facets. The flatness of the ice-binding face, another likely important property in the interaction between this protein and its ligand, is reminiscent of two insect AFP structures (Graether et al. 2000; Liou et al. 2000). The MpAFP structure also revealed that the internal Ca2+ atoms are coordinated by the side chain carboxyl groups from asparagine residues and the backbone carbonyl groups from glycine residues down one side of the protein. Removal of the Ca2+ results in a loss of antifreeze protein activity, showing that these cations are essential for maintaining the correct AFP structure. Recently, MpAFP was described as an outer membrane anchored protein, serving to bind bacteria to ice (Guo et al. 2012).

Raymond, Fritsen and Shen (2007) isolated from Colwellia sp. (ColAFP), an extracellular protein with a molecular
Table 1. Characteristics of phenotypically verified antifreeze proteins (AFPs) isolated from bacteria.

<table>
<thead>
<tr>
<th>Name</th>
<th>Molecular weight (kDa)</th>
<th>TH (°C)</th>
<th>Reported concentration</th>
<th>Isolated from</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycoprotein</td>
<td>~34</td>
<td>~0.11</td>
<td>n.d.</td>
<td>Pseudomonas putida GR12-2</td>
<td>Sun et al. (1995)</td>
</tr>
<tr>
<td>Lipoprotein</td>
<td>52</td>
<td>~0.171</td>
<td>0.1 mg mL⁻¹</td>
<td>Moraxella sp.</td>
<td>Yamashita et al. (2002)</td>
</tr>
<tr>
<td>Uncharacterized protein</td>
<td>80</td>
<td>0.059</td>
<td>n.d.</td>
<td>Pseudomonas fluorescens</td>
<td>Kawasaki et al. (2004)</td>
</tr>
<tr>
<td>Ca²⁺ dependent protein</td>
<td>n.d.</td>
<td>~0.8</td>
<td>11 mg mL⁻¹</td>
<td>Marinomonas primoryensis</td>
<td>Gilbert, Davies and Laybourn-Parry (2005)</td>
</tr>
<tr>
<td>Recombinant (MpAFP)</td>
<td>&gt;1000</td>
<td>2</td>
<td>0.5 mg mL⁻¹</td>
<td>Marinomonas primoryensis</td>
<td>Garnham et al. (2008)</td>
</tr>
<tr>
<td>Adhesin ice-binding activity (MpAFP)</td>
<td>1500</td>
<td>n.d.</td>
<td>n.d.</td>
<td>Marinomonas primoryensis</td>
<td>Guo et al. (2012)</td>
</tr>
<tr>
<td>β-helix protein (ColAFP)</td>
<td>~25</td>
<td>&lt;0.1</td>
<td>n.d.</td>
<td>Colwellia sp. SLW05</td>
<td>Raymond, Fritsen and Shen (2007)</td>
</tr>
<tr>
<td>Recombinant β-helix protein (ColAFP)</td>
<td>n.d.</td>
<td>~4</td>
<td>0.14 mM</td>
<td>Colwellia sp. SLW05</td>
<td>Hanada et al. (2014)</td>
</tr>
<tr>
<td>Malate-dependent protein</td>
<td>59</td>
<td>0.04</td>
<td>0.7 mg mL⁻¹</td>
<td>Flavobacterium xanthum</td>
<td>Kawahara et al. (2007)</td>
</tr>
<tr>
<td>Ice-binding proteins (FJBP)</td>
<td>~26</td>
<td>2.2</td>
<td>0.005 mM</td>
<td>Flavobacterium frigoris PS1</td>
<td>Do et al. (2014)</td>
</tr>
</tbody>
</table>

TH: thermal hysteresis; n.d: not described.

Figure 1. Structure of MpAFP from Marinomonas primoryensis based on Garnham, Campbell and Davies (2011) model. A cartoon representation of this Antarctic bacterial AFP (PDB id 3P4G) is shown with coil structure colored white, α-helix colored orange and β-strands colored blue. The figure is oriented with the N-terminus at the top of the page and the ice-binding face towards the viewer.

ICE-NUCLEATING PROTEINS

The molecules that act as ice-nucleating agents are diverse in nature (such as dust and pollen) and have temperature ranges for ice nucleus formation between −8°C and −15°C (Margaritis weight of 25 kDa with a TH < 0.1°C. However, studies with recombinant ColAFP characterized this protein as a member of the β-helix hyperactive AFP family, with no repetitive sequence motif and a TH of ~4°C at a concentration of 0.14 mM (Hanada et al. 2014). The differences in TH reported in these studies could be principally attributed to a lower protein concentration that was not given by Raymond, Fritsen and Shen (2007). Recently, ColAFP has also been shown to have a sequence similar to the AFP (54 kDa) that is produced by a Flavobacteriaceae bacterium isolated from subglacial Vostok Lake at a 3519 m depth (Raymond, Christner and Schuster 2008). This finding suggests that AFP genes might be transferred between microorganisms by horizontal gene transfer events (Raymond, Christner and Schuster 2008; Sorhannus 2011; Raymond and Kim 2012). A lipoprotein with a similar molecular weight (52 kDa) was also discovered in Moraxella sp. by Yamashita et al. (2002). Other extracellular and intracellular AFPs that have been reported were produced by P. fluorescens and Flavobacterium xanthum, with TH values of 0.059°C and 0.04°C, respectively (Kawahara et al. 2004, 2007). Recently, a 25.7 kDa AFP produced by F. frigoris PS1 (FJBP), and firstly reported by Raymond and Kim (2012), showed a TH of 2.2°C at a concentration of 0.005 mM (Do et al. 2014). Thus, the TH values given in these investigations are dependent on the concentrations and purity of recombinant protein assays and may have no physiological meaning regarding their activity in nature. At natural concentrations, bacterial IBPs have negligible TH and thus are probably not true AFPs. Their purpose may be to just modify the growth of ice to preserve water channels or liquid pockets to create a habitat for bacterial growth (Raymond, Christner and Schuster 2008; Raymond and Kim 2012; Davies 2016).
and Bassi 1991). Despite that INPs have been found in insects (Duman 2001), fungi (Tsumuki et al. 1992) and plants (Brush, Griffith and Mlynarz 1994) that live in cold environments. Nevertheless, most INP studies have been performed in bacteria which are active ice nuclei at temperature ranges between 0 °C and −10 °C (Cochet and Widehem 2000). In this context, Schmid et al. (1997) provided a classification method used to describe subpopulations of culturable bacteria; this classification is based on the heterogeneous threshold temperatures of INP activity. The most effective bacterial INPs are termed type I, and can initiate ice formation from 0 °C to −4 °C. Type II INPs initiate ice formation from −5 °C to −7 °C, whereas type III INPs initiate ice formation from −8 °C to −10 °C. The basis for these subpopulations of INPs is their monomer, trimer and oligomer aggregation states (Burke and Lindow 1990). This assumption was demonstrated through radiation inactivation of these proteins, which showed that 150 kDa is the minimum molecular mass needed to nucleate ice at temperatures in the range from −12 °C to −13 °C (Govindarajan and Lindow 1988).

**BACTERIAL INPs**

In bacteria, the INP phenotype (known as Ina^{-}) is encoded by a structural gene that expresses a membrane-bound protein. This INP is located at the outer membrane of bacteria and catalyzes ice formation at high subzero temperatures (Lindow 1987). Wölber et al. (1986) cloned the InaZ gene responsible for ice nucleation in *P. syringae* and described its primary structure as an octapeptide repeat sequence; the Ala-Gly-Tyr-Gly-Ser-Thr-Leu-Thr motif is repeated along a 1200 amino acid sequence. Two other orders of periodicity were detected, following a 16 residue repeat and another repeat of 48 residues found in two regions of the sequence. The regular repeating pattern present in the protein sequence is likely responsible for organizing water molecules by influencing their spatial orientation and by favoring molecule alignment for water crystallization (Gurian-Sherman and Lindow 1992; Zachariassen and Kristiansen 2000). Xanthomonas campestris, which causes considerable damage in plants due to its ice nucleation activity (Cambours et al. 2005), secretes an INP encoded by the inaX gene and characterized by 153 octapeptide units repeated in the sequence (Zhao and Orser 1990). *Eruina uredovora* carries the InaU gene in its genome, which encodes a 1034 amino acid protein with a central repetitive domain of 16 amino acids (Michigami et al. 1994). Similarly, *E. herbicola* (now named *Pantoaea agglomerans*) (Lindow, Arny and Upper 1978; Gavini et al. 1989) carries the iceE gene in its genome and secretes a protein with a sequence of 1258 amino acids, with similar codon usage to that found in the InaZ gene from *P. syringae* S203 and the InaW gene from *P. fluorescens* MS1650 (Warren and Corotto 1989).

**BIOTECHNOLOGICAL APPLICATIONS**

Many industrial and biotechnological processes use cold-active biomolecules from organisms adapted to cold environments (Cavicchioli et al. 2011). IBPs, such as AFPs and their recombinants, have been primarily applied in diverse fields such as food industry and medicine (Amir et al. 2003; Kontogiorgos et al. 2007; Hirano et al. 2008) (Table 2). Recombinant AFPs from fish are used in the industry to improve food preservation during freezing (Fletcher et al. 1997; Yeh, Kao and Peng 2009). Recombinant AFPs from the yeast *Leucosporidium* sp. have been used for cryopreservation of red blood cells (Lee et al. 2012). In cryosurgery, the production of needle-like ice crystals at high AFP concentration (≥5 mg ml^{-1}) favors the ablation of cells (Koushafar and Rubinsky 1997). Only fish AFPs have been successfully used in the ablation of subcutaneous rat tumor cell lines; however, despite that those studies have been done at laboratory level, a great application potential for the medical industry is predicted (Koushafar and Rubinsky 1997; Muldrew et al. 2001).

INPs have also been applied in various fields (Table 2), particularly those bacterial INPs with activity at the warmest temperature (type I) (Gurian-Sherman and Lindow 1992). Several food technology studies have reported favorable results when using bacterial INPs to increase the ice nucleation temperature, with consequently reduced freezing times and ice-crystal size and improved frozen solid food quality (Zhang, Wang and Chen 2010). As the consequence of ice formation at higher temperatures, a lower energy cost is required to freeze foods. Freezing food with less cooling has also been shown to be possible when using biological ice nuclei produced by bacteria (i.e. from *P. syringae* and *E. herbicola*). Widehem and Cochet (2003) demonstrated that freezing processes could be improved by the addition of lyophilized cells of *P. syringae* which could act as ice nuclei, leading to a significant decrease in the super-cooling point, forming a greater number of ice crystals. Notably, INP-producing bacteria have been closely related to the water cycle due to the presence of these organisms in samples collected from rainfall and snow (Morris et al. 2008). In fact, the presence of bacteria in cloud water has revealed that this mechanism of transport has made biological ice nucleation a widespread phenomenon in the atmosphere, affecting meteorological processes such as rainfall and hailstorms (Christner et al. 2008). In addition, one of the most important applications in the industry is the current use of Snomax (Telemet, Inc, New York, USA), a freeze-dried bacterial INPs for the production of artificial snow (Cochet and Widehem 2000).

Despite that engineering or genetic modifications for enhancing the function of IBPs are scarce, improvements in recombinant protein production has been achieved by linking a determined protein function to the encoding gene. This technology has increased our ability to modify specific peptides to obtain desired properties in vitro (Daugherty 2007). As an example, protein engineering, by modification of certain residues in isoforms of AFPs, resulted in a gain of antifreeze activity as described by Garnham et al. (2012).

**PERSPECTIVES IN AGRICULTURE**

Frost damage is responsible for more economic losses in agriculture than any other weather-related phenomenon in the United States, and in many other regions across the globe (Pearce 2001; Chevalier et al. 2012). Sessile organisms such as plants have primarily adopted IRI mechanisms to tolerate cold temperatures (Thomashow 1998; Lory, Rose and Glick 2014). Studies have revealed that freeze-tolerant plants such as winter rye (Secale cereale) and ryegrass (*Lolium perenne*) secrete AFPs into intercellular spaces and the apoplast, affecting the freezing point and ice-crystal growth (Griffith et al. 1992; Middleton et al. 2012). Freeze-tolerant plants (*L. perenne* and others) accumulate fructans as a carbohydrate source that are also considered to play a relevant role in freezing-tolerance (Varuluru and Van Den Ende 2008; Varuluru et al. 2008). In contrast, other plant species regulate freezing point depression by the presence of cryoprotectants (trehalose and sucrose) that allow the plants super-cooling, reaching lower temperatures in tissues, without ice formation (Kawahara 2008). Studies in blueberry (*Vaccinium* spp.) also have shown that...
### Table 2. Summary of bacterial ice-binding protein applications.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Application field</th>
<th>Organism source (protein type)</th>
<th>Main effect</th>
<th>Level of application</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>AFP</td>
<td>Food industry</td>
<td>Fish (type III)</td>
<td>Applied as recombinant protein to improve milk fermentation to store frozen yogurt and ice-cream production</td>
<td>Industrial</td>
<td>Fletcher et al. (1997); Feeney and Yeh (1998)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Screened for proteolytic and lipolytic activity to obtain a heat-stable IRI protein</td>
<td>Laboratory</td>
<td>Kontogiorgos et al. (2007)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fish (type I)</td>
<td>Applied as recombinant protein to improve frozen meat and dough quality</td>
<td>Laboratory</td>
<td>Yeh, Kao and Peng (2009)</td>
</tr>
<tr>
<td>Medicine</td>
<td></td>
<td>Fish (type I and III)</td>
<td>Applied to improve the cryopreservation of rat hearts</td>
<td>Laboratory</td>
<td>Amir et al. (2003)</td>
</tr>
<tr>
<td>Medicine</td>
<td></td>
<td>Fish (type III)</td>
<td>Applied as natural and recombinant protein to improve cryopreservation of mammalian cells</td>
<td>Laboratory</td>
<td>Hirano et al. (2008)</td>
</tr>
<tr>
<td>Medicine</td>
<td></td>
<td>Yeast</td>
<td>Applied as a recombinant protein to improve the cryopreservation of red blood cells</td>
<td>Laboratory</td>
<td>Lee et al. (2012)</td>
</tr>
<tr>
<td>Medicine</td>
<td></td>
<td>Fish (type I)</td>
<td>Applied in cryosurgery of subcutaneous rat tumor cell lines</td>
<td>Laboratory</td>
<td>Koushafar and Rubinsky (1997); Muldrew et al. (2001)</td>
</tr>
<tr>
<td>Agriculture</td>
<td></td>
<td>Fish (AFGP)</td>
<td>Inhibition of ice nucleating activity of membrane vesicles of Erwinia herbicola bacterium</td>
<td>Laboratory</td>
<td>Parody-Morreale and Murphy (1998)</td>
</tr>
<tr>
<td>Agriculture</td>
<td></td>
<td>Bacteria</td>
<td>Applied as biofertilizer to increase plant growth at cold temperature</td>
<td>Laboratory</td>
<td>Sun et al. (1995)</td>
</tr>
<tr>
<td>Agriculture</td>
<td></td>
<td>Fish (type III)</td>
<td>Inhibition of ice nucleation process by adsorbing to ice nuclei and dust particles.</td>
<td>Laboratory</td>
<td>Du, Liu and Hew (2003)</td>
</tr>
<tr>
<td>Agriculture</td>
<td></td>
<td>Plant</td>
<td>Recombinant Lolium perenne (Lp (AFP)) reduced ice nucleation temperature of bacterial ice nucleating proteins (INP)</td>
<td>Laboratory</td>
<td>Tomalty and Walker (2014)</td>
</tr>
<tr>
<td>INP</td>
<td>Food industry</td>
<td>Bacteria</td>
<td>Applied as active bacterial cells to improve frozen food quality by reducing ice-crystal size.</td>
<td>Laboratory</td>
<td>Zhang, Wang and Chen (2010)</td>
</tr>
<tr>
<td>Food industry and wastewater treatment</td>
<td></td>
<td>Bacteria</td>
<td>Freezing processes improved by adding lyophilized Pseudomonas syringae cells, decreasing super-cooling point and higher ice-crystals size</td>
<td>Laboratory</td>
<td>Widehem and Cochet (2003)</td>
</tr>
<tr>
<td>Agriculture</td>
<td></td>
<td>Bacteria</td>
<td>Inoculation of non-INP bacteria will reduce the frequency of biological ice nucleus and damages in plants</td>
<td>Laboratory</td>
<td>Lindow (1984, 1987)</td>
</tr>
<tr>
<td>Agriculture</td>
<td></td>
<td>Bacteria</td>
<td>Applied as active bacterial cells to control insect pest in plants</td>
<td>Laboratory</td>
<td>Castrillo et al. (2001); Lee et al. (2001)</td>
</tr>
<tr>
<td>Environmental science</td>
<td></td>
<td>Bacteria</td>
<td>Applied as active bacterial cells to promote artificial rainfall</td>
<td>Theoretical</td>
<td>Levin et al. (1986); Joly et al. (2013)</td>
</tr>
<tr>
<td>Sport/entertainment</td>
<td></td>
<td>Bacteria</td>
<td>Applied as active bacterial cells for the production of artificial snow (Snowmax: Genecor, Rochester, USA)</td>
<td>Industrial</td>
<td>Cochet and Widehem (2000)</td>
</tr>
</tbody>
</table>

AFP: antifreeze protein; INP: ice-nucleating protein; IRI: ice recrystallization inhibition; AFGP: antifreeze glycoprotein.

controlled ice nucleation is a beneficial mechanism for avoiding supercooling in the protoplasm, inducing extracellular freezing and/or accommodating ice crystal in specific tissues (stem bark) (Kishimoto et al. 2014). However, frost sensitive plants can be affected by the occurrence of INP-producing bacteria, which induce ice formation, causing damages and dehydration in plant cells (Lindow, Arny and Upper 1982; Cambours et al. 2005).

Current methods to prevent frost damage in agro-ecosystems are primarily physical, i.e. heaters, wind machines, artificially generated fog and water sprinkling directly on
plants. However, these methods are costly. A different method to mitigate frost damage effects is the control of INP-producing bacteria populations to enhance the natural ability of plants to super-cool (Lindow 1983). In this context, bactericides (such as oxytetracycline and streptomycin) have been applied as protectants to prevent the establishment of INP-producing bacteria in plants (Lindow 1984). Nevertheless, the use of antibiotics is undesirable in foodstuffs given that the remaining antibiotics could affect intestinal flora, generate allergic reactions, and also facilitate bacterial resistance to antibiotics (Jing et al. 2009). Thus, the inoculation of antagonist bacteria against INP-producing bacteria has also been proposed to diminish the frequency of biological ice nucleation damage on plants as shown by Lindow (1984, 1987) (Table 2). Moreover, as also shown in Table 2, active bacterial INPs have shown to be effective at pest control by reducing the capacity of potato beetles to resist freezing temperatures (Castrillo et al. 2001; Lee et al. 2001). In the environmental sciences, INP-producing bacteria have also been proposed as a cloud condensation inducer to produce artificial rainfall (Levin et al. 1986; Joly et al. 2013). Clearly, major efforts will be required to refine and implement these applications before it becomes effective and economically viable for use in the field.

Another mechanism that has been scarcely explored is the use of plant growth-promoting bacteria. The only example reported in the literature is the inoculation of P. putida GR12-2 isolated from the rhizosphere of Canadian High Arctic plants, which secretes an extracellular AFP (Sun et al. 1995). The inoculation of seeds of spring and winter canola with P. putida GR12-2 resulted in an increase in root elongation at 5°C. Bacteria, as epiphytic organisms living on the phylloplane of plants, have also been found to colonize intercellular and apoplastic spaces in leaves (Lindow and Brandl 2003), thus the inoculation of AFP-producing epiphytic bacteria could be applied to reduce frost damages in sensitive plants (Table 2). This possibility is supported by the finding that AFPs isolated from fish have been experimentally demonstrated to inhibit the ice-nucleation activity of an INP-producing E. herbicola, a common organism inhabiting the phylloplane of plants (Parody-Morreale and Murphy 1988). Similar results were found using type III AFP, which inhibit ice nucleation process by adsorbing onto both the surface of growing ice nuclei and dust particles (Du, Liu and Hew 2003). On the other hand, a recent article demonstrated that a plant AFP could also control ice growth, and act as a defensive strategy against bacterial ice nucleation (Tomalty and Walker 2014).

Recent advances in metagenome analyses have also suggested that the survival of Antarctic moss is achieved by the accumulation of bacterial AFPs activity (Raymond 2016). This occurs because homologs of the same domain (DUF3494) were identified in bacterial gene sequences discovered in metagenome analysis of the moss leaves (Davies 2016). The same domain (DUF3494) was reported to be transmitted by horizontal gene transfer between prokaryotic organisms such as Colwellia sp. SLW05 (ColAFP) and Flavobacteriaceae bacterium and eukaryotic microorganisms such as fungi and copepods (Hanada et al. 2014). The high expressions of AFP domains by eukaryotic microbial communities suggest an essential role for this protein in survival in Arctic and Antarctic sea ice (Uhlig et al. 2015).

In summary, in the context of significant economic losses produced by frost events in agriculture, the potential application of IBPs-producing bacteria or their IBPs to diminish damage by ice-crystal formation and promote growth at seasonal cold temperatures, presents an attractive strategy that should be explored in further research.

### IBPs IN PUBLIC DATABASES

Sequence databases are currently widely used in bioprospecting biotechnological and industrial attractive microorganisms (Lee and Lee 2013). The current UniProt database suggests that bacterial IBPs might be found in diverse bacterial groups (Table 3). In this context, database-based analyses can also be used to detect conserved regions in genes or proteins, which are very useful for the detection of unknown (or novel) microbial genes and proteins from environmental bacteria and predicting their function by molecular approaches. However, very limited information on IBPs is present in databases. Few phenotypically verified bacterial AFPs and INPs have been deposited, and most of IBP-like proteins are noted as ‘putative’, but their activities have not been confirmed. Figure 2 shows a phylogenetic analysis based on amino acid sequences from reported bacterial AFPs (phenotypically verified) and representative AFP-like proteins from plant- and soil-associated bacteria. The phylogenetic tree suggests clustering of three major branches. Branch 1 grouped the AFPs from M. primoryensis (MpAFP, A1Y1Y2) and P. putida GR12-2 (Q58VA9) with AFPS-like proteins from Gammaproteobacteria.

### Table 3. Summary of taxonomic affiliation of the phenotypically verified ice-binding proteins (IBPs) and putative IBPs present in the UniProt database.

<table>
<thead>
<tr>
<th>Class</th>
<th>Antifreeze proteins</th>
<th>Ice-nucleating proteins</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Branch 1</td>
<td>Branch 2</td>
</tr>
<tr>
<td>Alphaproteobacteria</td>
<td>Rhizobium Ensifer</td>
<td>Bradyrhizobium</td>
</tr>
<tr>
<td>Betaproteobacteria</td>
<td>Herbaspirillum</td>
<td></td>
</tr>
<tr>
<td>Gammaproteobacteria</td>
<td>Marinomonas*</td>
<td>Colwellia</td>
</tr>
<tr>
<td></td>
<td>*Pseudomonas</td>
<td>*Pseudomonas Serratia</td>
</tr>
<tr>
<td>Bacilli</td>
<td>Paenibacillus</td>
<td>Bacillus</td>
</tr>
<tr>
<td>Flavobacteria</td>
<td>*Flavobacterium</td>
<td></td>
</tr>
</tbody>
</table>

*Bold letters denote the presence of phenotypically verified strain within bacterial genus.*
Figure 2. Phylogenetic analysis of phenotypically verified AFPs (red) and putative AFPs (black). Amino acid sequences were taken from the UniProt database (http://www.uniprot.org) and aligned using Clustal Omega (http://www.clustal.org/omega/). In brackets, protein accession numbers were included. The neighbor-joining phylogenetic tree was built using the PHYLIP package (http://evolution.genetics.washington.edu/phylip.html), and interpreted using FigTree software (http://tree.bio.ed.ac.uk/software/figtree/). Marinomonas primoryensis (A1Y1Y2), Colwellia sp. SLW05 (A5XB26), Flavobacterium frigoris PS1 (H7FWB6), Flavobacteriaceae bacterium 3519-10 (B3GGB1), Pseudomonas putida GR12-2 (Q68VA9), P. syringae SM (S3MTC9), Pseudomonas sp. UW4 (KSNL46), Pseudomonas sp. Chol1 (KSVB0), Serratia fonticola AU-AFP2 (U2NA57), S. fonticola AU-P3 (U2LCA8), Xanthomonas campestris pv. campestris (A0A0C7DZ94), X. campestris pv. campestris ATCC33913 (Q8P6Q0), X. campestris pv. raphani 756C (G0CBK7), Rhizobium fredii HH103 (G9A7B7), Bradyrhizobium diazoefficiens SEMIA 5080 (A0A099IPF9), Ensifer adhaerens (Sinorhizobium adhaerens) OV14 (W8HVP5), Herbaspirillum frisingense GSF30 (R0G033), H. seropedicae SmR1 (D8IRJ0), Herbaspirillum sp. Gw103 (I3CTQ5), Bacillus sp. OxB-1 (A0A0A8JKC3), Paenibacillus alvei DSM29 (K4ZQG5), P. alvei TS-15 (S9SIL7), Paenibacillus sp. JDR-2 (C6D7M4), Paenibacillus sp. R7-277 (W4DV37).

(Xanthomonas and Pseudomonas) and Alphaproteobacteria (Ensifer and Rhizobium) classes. The second branch grouped members of the Bacilli class (Paenibacillus and Bacillus), but no reported AFP was present in this cluster. The third branch was subdivided into two minor branches. One of the branches grouped AFPs from F. frigoris PS1 (FJIBP; H7FWB6) and Colwellia SLW05 (ColAFP; A5XB26) with AFPs-like from Alphaproteobacteria (Bradyrhizobium) and Gammaproteobacteria (Pseudomonas), whereas the other minor branch grouped Flavobacteriaceae bacterium 3519-10 (B3GGB1) with AFPs-like proteins from Betaproteobacteria (Herbaspirillum) and Gammaproteobacteria (Serratia and Pseudomonas) classes.

Figure 3 shows the phylogenetic relation of reported bacterial INPs and representative INP-like proteins from plant- and soil-associated bacteria. The phylogenetic tree suggests clustering of three major branches. Branch 1 was formed by a reported INP from X. campestris (InaX; P18127) and INP-like proteins from Xanthomonas spp. The second branch was subdivided into two minor branches. One of the branches grouped INPs from P. ananas (InaA [P20469] and InaU [O30611], InaK [O30611], InaQ [B0FX3] and InaV [O33479]) with INP-like proteins from P. ananas and P. syringae. Notably, two INP-like proteins from P. savastanoi and P. syringae were not grouped with the other P. syringae sequences.

In addition, when MEME software (http://meme.nbcr.net/meme; Bailey et al. 2006) is used to discover novel motifs (recurring, fixed-length patterns) in amino acid sequences from reported bacterial INPs, the results suggest three motifs which were all based on the repetitive octapeptide region (Ala-Gly-Tyr-Gly-Ser-Thr-Leu-Thr) described previously by Wolber et al. (1986) (Fig. 4). The octapeptide region also contains a Thr-X-Thr motif (where ‘X’ is any amino acid), which has been found to be responsible for ice binding by AFPs from insects (Graether and Jia 2001). This finding has led to the suggestion that both AFPs and INPs can have similar structures yet different effects on ice (Graether and Jia 2001). It is clear that the first motif identified by the MEME tool is very similar to those previously described, while the other motifs were not particularly well conserved and have not been previously recognized in INPs. Therefore, these results must be taken with caution, because in fact represented
Figure 3. Phylogenetic analysis of phenotypically verified INPs (red) and putative INPs (black). Amino acid sequences were taken from the UniProt database (http://www.uniprot.org/) and aligned using Clustal Omega (http://www.clustal.org/omega/). In brackets, protein accession numbers were included. The neighbor-joining phylogenetic tree was built using the PHYLIP package (http://evolution.genetics.washington.edu/phylip.html) and interpreted using FigTree software (http://tree.bio.ed.ac.uk/software/figtree/).

- **Pseudomonas syringae** (K4WYP7),
- **P. savastanoi pv. savastanoi** (G7ZJR9),
- **P. syringae** SM (S3MD81),
- **P. syringae** (D2U789),
- **P. mandelli** JR-1 (A0A024E7B8),
- **Pseudomonas sp. GM30** (W6VKH0),
- **P. syringae pv. syringae** B728a (Q4ZW16),
- **Pantoaea ananatis** LMG 20103 (D4GJ13),
- **Xanthomonas gardneri** ATCC 19865 (F0C4Z6),
- **X. campestris pv. raphani** 756C (G0CIB0),
- **X. campestris pv. campestris** ATCC 33913 (Q8PD38),
- **Rhodobacter capsulatus** ATCC BAA-309 (D5AV30),
- **R. capsulatus** SB1003 (O68078),
- **Bacillus thuringiensis** MC28 (K0FS92),
- **P. ananas** InaA (P20469),
- **P. syringae** InaZ (P06620),
- **P. ananas** InaU (Q47879),
- **X. campestris** InaX (P18127),
- **P. fluorescens** InaW (P09815),
- **P. syringae** InaK (O30611),
- **P. syringae** InaQ (B0FX3),
- **P. agglomerans** iceE (P16239),
- **P. syringae** InaV (O33479).

motifs may simply be produced by tandemly-repeated amino acids.

Homology models can also be generated using protein database files (i.e. Swiss-Model and PDB) and AFPredictor (Doxey et al. 2006). This analysis can identify AFPs from a large set of structures with greater accuracy than sequence alone. However, when MEME and AFPredictor were used with reported bacterial AFPs, reliable results were not obtained (data not shown). This implies that current database is too small to provide adequate coverage of IBPs present in the bacterial world.

**CONCLUSIONS AND FUTURE DIRECTIONS**

The importance of IBPs as potential biotechnological tools has been recognized in various fields. However, only a limited amount of bacterial AFPs and INPs have been studied and characterized. Phenotypically verified IBPs, both AFPs and INPs,
Figure 4. Motifs in bacterial phenotypically verified INPs as represented by the motif discovery tool MEME (http://meme.nbcr.net/meme/). Black bars indicate the octapeptide repeat sequence. The UniProt accession numbers are given in parentheses.

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REFERENCES


Lee RE, Castrillo LA, Lee MR et al. Using ice nucleating bacteria to reduce winter survival of Colorado potato beetles, development of a novel strategy for biological control. In: Denlinger...
et al. Appl Environ Microb
et al. Pseudomonas syringae
The basis for hyperactivity
Molecular organisation of
J Mol biol
etal. Biophys J
sp. on red blood cells.
Bacterial com-
Methanogenium frigidum
et al. Identification of the
as an ice
Evol Bioinforma
1989; Extremophiles
Genome Res
Identification and pu-
Low temperature growth,
. New York: Elsevier Science B.V.,
Direct visualization of
Pseudomonas fluorescens
Process
Astrobiology
Possible application of bacte-
J Insect Physiol
etal. FEBS Lett
etal.
Raymond J. Dependence on epiphytic bacteria for freezing pro-
Pertaya N, Marshall C, Celik Y
Pearce R. Plant freezing and damage.
Pautler B, Simpson A, McNally D
Parody-Morreale A, Murphy K. Inhibition of bacterial ice nucle-
Middleton A, Marshall C, Faucher F
Michigami Y, Watabe S, Abe K
Margaritis A, Bassi A. Principles and biotechnological ap-
Lorv J, Rose D, Glick B. Bacterial ice crystal controlling proteins.
Scientifica (Cairo) 2014; 1–20.


