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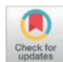

Review Article

# Bacterial Ice Nucleation Proteins: Features, Structure, and Applications

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**Abstract**
Some bacteria commonly found on plants can catalyze the freezing of water at a higher temperature than others, at or near 0 °C. The freezing point of pure water is about -40 °C and is initiated by creating ice nucleations. However, when ice nucleation proteins (INPs) are present, ice nucleations form at temperatures close to or above 0 °C. INPs are often attached to the outer membrane by a phosphatidylinositol anchor and are sometimes secreted extracellularly. The monomers of INPs in *Pseudomonas syringae* are 120 to 180 kDa. INP has three domains, and the central domain is highly repetitive. The central domain consists of the consensus sequence of eight amino acid repeats. Eight amino acid repeats create a 16-residue fragment, and three 16-residue fragments form the 48-residue fragment. Studies have shown that INPs may have a β-helical fold and interact with water through the repetitive motif. Most ice nucleation bacteria are gram-negative, including *P. syringae, Pseudomonas viridiflava, Pseudomonas fluorescens, Xanthomonas compestris, Erwinia ananas*, and *Erwinia herbicola.* For optimum protein activity, the presence of the complete bacterial cell is essential. INPs are influential in different aspects, including snowmaking, agriculture, freeze-concentration in the food industry, signal transduction, atmospheric applications as cloud condensation nuclei, and surface display (expression of a foreign protein on the cell surface for biotechnological purposes). This study provides a brief overview of ice nucleation proteins and their applications since ice nucleation is an important phenomenon that affects various aspects, from climate to biological systems.

**Keywords:** Ice-Nucleating Proteins, Supercooling, *Pseudomonas syringae,* Silver Iodide, Snow-Making

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## Introduction

Water is vital for all organisms, and it is essential for reactions in biological systems, transportation of substances, and other reciprocal activities. Various macromolecular structures are stabilized by water. Access to water is a crucial stage for most organisms. Consequently, the transformation of water from the aqueous phase to ice will be a threat to them. Pure water does not freeze spontaneously at 0 °C. Therefore, it remains liquid below 0 °C, and it is known as supercooled or undercooled.[1-3] Ice formation happens with the same water molecules that are arranged as crystals. Due to this process, the total energy increases, and in terms of energy, it is unfavorable. Therefore, these arrangements tend to break up. By further cooling, ice nuclei grow in size and number. These nuclei, otherwise known as ice embryos, gain the critical size/volume ratio. Further growth in size and number decreases the whole energy budget, which is a favorable process. The temperature that causes this phenomenon is called the supercooling threshold temperature, and it is about -40 °C. When an ice nucleus is formed by the simultaneous aggregation of water molecules, this nucleus is referred to as homogeneous nucleation. If the accumulation of water molecules is catalyzed by substances other than water molecules, their nuclei are considered heterogeneous nucleations.[2-5] These impurities are also called ice nucleation activators (INA). The ice formation is compatible with the dimension of the INA and provides templates for ice crystals. Therefore, heterogeneous ice nucleation happens at a greater temperature.[3] Various strategies are employed by microorganisms to survive in cold environments, including the Antarctic and the Arctic. To survive and reproduce in these conditions, bacteria accumulate some compounds, including trehalose, glucose, etc. Also, the production of several ice crystal-controlling substances such as ice-nucleating proteins (INPs) and antifreeze proteins (AFPs) with opposite functions are other methods that are applied by microorganisms. Therefore, they can make certain protein molecules that stop ice crystals from growing outside the cells.[2,3,6,7] At temperatures higher than -3 °C, ice-nucleating proteins can simplify the ice formation process. The ice-nucleating activity can be inhibited by anti-nucleating materials to facilitate the supercooling temperature for microorganisms. Antifreeze proteins suppress the growth of

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ice crystals by covering the surface of the ice crystals.[8-10] Another major issue related to the ice nucleation bacteria is the study of these biological agents as plant pathogens since they cause plant stress mechanisms. Some bacteria cause several disorders; such as frost injury at low temperatures. Due to ice formation, the disruption of cell membranes leads to the discoloration of the plants. Presumably, ice nucleation proteins can help the bacteria access nutrients released from the plant.[11-14] In addition, ice formation helps cold-tolerant insects survive in freezing temperatures by increasing the osmolality of their hemolymph.[15] Since ice nucleation is a significant phenomenon influencing several aspects, from climate to biological systems, this review gives a brief overview of ice nucleation protein and its applications.

## The Sources of Biological Ice Nucleators

Schnell, in 1970, found that the microbiological origin of decayed leaves had the potential for ice formation.[16] Proteinaceous ice nucleators in bacteria have become the focus of attention. Then several ice-nucleating bacteria were isolated. *P. syringae* was subdivided into a plethora of pathotypes or pathovars based on the primary plants to which they caused disease through extensive examinations of bacterial pathogens. For example, *P. syringae* pathovar *syringae* indicates strains isolated from lilac (a plant in the genus *Syringa*) and *P. syringae* pathovar *pisi* represents strains from pea (genus *Pisum*).[17] Several strains of *P. syringae*, as well as at least five additional bacteria, have since been identified as effective ice nucleators. Ice-nucleating activity has been found in *P. viridiflava*, *P. fluorescens*, *Xanthomonas compestris*, *Erwinia ananas*, and *E. herbicola* strains. The *ina* gene was then sequenced and cloned to identify ice nucleation protein genes. These genes were first identified and named in the following 5 species: the *ina*Z gene in *P. syringae*, the *ina*W gene in *P. fluorescens*, the *ina*X gene in *X. campestris*, the *ina*A gene in *E. ananas*, and the *ina*E gene in *E. herbicola*.[17-19] For the first time, Rostami et al. discovered two ice nucleation active bacteria, *Pseudomonas fragi* and *Pseudomonas moraviensis*, on pistachio trees isolated from distinct areas in Kerman Province, Iran.[20] *Lysinibacillus*, a gram-positive species not previously recognized as an Ina$^+$ bacterium, was found in two Ina$^+$ strains. *Lysinibacillus* strains' ice nucleation capability is owing to a nanometer-sized molecule that is heat, lysozyme, and proteinase resistant, as well as secretory.[21] Sadeghi et al. examine the properties of sugarcane endophytic and epiphytic bacteria, as well as their ice nucleation activity and function in frost damage. Only *P. fluorescens* strain Epi57-A4 was found to have an *ina* gene during amplification of the ice nucleation gene, showing that this gene has the ability to speed up frosting in sugarcane under cold conditions.[22]

Furthermore, ice nucleators in the hemolymph of the freeze-tolerant insect were regarded. Ice nucleators listed in Table 1 are induced at -2 °C to below -7 °C.

**Table1.** The Sources of Some Biological Ice Nucleators[15]

| Organisms | Protein molecular weight (kDa) | Lipids | Phospholipids | Carbohydrates |
|---|---|---|---|---|
| Bacteria | 120-180 | Yes | PI* and others | Yes |
| Insects | 74[1], 265[2] | Yes, No | PI | Yes |
| Molluscs | 16.6[3], 17.7[4] | No | No | No |
| Tardigrada | Native > 200[5] | | | |
| Vertebrates | Native > 300[6] | | | |
| Vascular plants | Native > 300[7] | Yes | Yes or other than PI | Yes |
| Lichen[8] | | No | No | No |
| Fungi (*Fusarium*)[9] | | No | No | No |

*Phosphatidylinositol; [1]*Vespula maculate*; [2]*Tipula trivittata*; [3]*Melampus bidentatus*; [4]*Melampus bidentatus*; [5]*Adorybiotus coronifer*; [6]*Rana sylvatica*; [7]*Prunus* spp.; [8]*Rhizoplaca chrysoleuca*; [9]*Fusarium acuminatum*

Phosphatidylinositol plays a major role in anchoring the protein into the cell membrane. Also, phosphatidylinositol is essential in the function of the ice nucleator in the insect, *Tipula trivittata*. The ice nucleation activities of the vascular plants are also related to phospholipids but not phosphatidylinositol. Non-proteinaceous ice nucleators such as silver iodide, and monolayers of aliphatic alcohols, and also calcium phosphate in the freeze-tolerant goldenrod gall fly, *Eurosta solidaginis*, were regarded.[15] The growth of snow molds was evaluated at temperatures from -5 to 30 °C, and then their ice nucleation and antifreeze activities were studied. *Coprinus psychromorbidus*, *Typhula phacorrhiza*, *T. incarnate*, *T. ishikariensis*, *T. canadensis*, and *Microdochium nivale* were isolated, and their growth temperature was at -5 °C, whereas *Sclerotinia homoeocarpa* and *Sclerotinia borealis* grew at temperatures above 4 °C. The ice nucleation activity's greatest temperature threshold was -7 °C. Snow molds cause plant damage at temperatures above -7 °C, implying that their harmful effects are unrelated to their ice nucleation activity. Most snow molds present antifreeze activities when they are growing at subzero temperatures. Therefore, they do not have a lot of ice-forming activity. As a result, snow molds can keep their environment from getting too cold, which helps them grow under snow at temperatures below zero.[23] Oktiningtiyas et al. studied ice nucleation active bacteria that cause frost injury on potato crops. The bacteria were identified based on the *16S rRNA* gene and the Ina$^+$ bacteria found in their study had

a neighbor-joining with $Ina^+$ bacteria discovered previously both in tropical and subtropical regions. Accordingly, 20 bacterial isolates indicated freezing activity. Each of the 5 isolates had similarities with *Pseudomonas jessenii* 96%, *Enterobacter ludwigii* 99%, *Stenotrophomonas maltophili* 94%, *Pseudomonas putida* 98%, and *Pseudomonas japonica* 99%.[24]

## Ice Nucleation Protein Features

The transition from liquid to solid occurs at supercooled temperatures, and the liquid will freeze at these temperatures. By adding a suitable catalyst known as ice nuclei (heterogeneous and homogeneous ice nuclei), this transition phase happens at temperatures only slightly below 0 °C.[13,25-27] Heterogeneous ice nuclei such as dust can promote the freezing temperature near -10 °C and warmer.[8,28] Threshold temperatures in some bacterial species that produce ice nucleation-active substances are -1.5 to -1.8 °C. Studies showed this feature is related to one of the proteinaceous cell membrane substances in *P. syringae*.[8,29] Furthermore, scientists could transform *Escherichia coli* by transferring the single small region of the chromosome from *P. syringae* to *E. coli*.[30,31] Ice nucleation activity in naive bacteria can be broken down by proteases and chemicals that change sulfhydryls. This suggests that ice nucleation activity is caused by a protein.[14,32,33] Phelps et al. evaluated the capability of *E. herbicola* to release ice nuclei into the growth medium. They showed that *E. herbicola* shed ice nuclei into the growth medium, being active at -2 to -10 °C. Also, they demonstrated that ice nuclei were associated with outer membrane vesicles released by bacterial cells.[34] *inaZ* is responsible for the ice nucleation feature in *P. syringae* S203. Its ice nucleation protein was assessed after overexpression in *E. coli*. After extracting the protein by gel filtration in a mixture of urea and the non-denaturing detergent octyl 1-D-thioglucopyranoside, it was found that the InaZ protein was responsible for the ice-nucleating property, and it was active even though there was no lipid.[31,35] To promote *ina* expression, *ina*Z was located downstream of the *tac* promoter. Consequently, it causes the accumulation of a large number of membrane-associated proteins. After translating the *ina*Z sequence to protein, it is demonstrated that there is a repetitive primary structure that might be a site for ice nucleation activity.[25] Ice nucleation protein is associated with the outer membrane in *P. syringae* and is considered to contain both protein and lipid components.[36] Some findings proved that the *inaZ* product is the phosphatidylinositol synthase, and the phosphatidylinositol is the water-binding site.[37] Generally, ice-nucleating agents are proteins or lipoproteins (such as insect hemolymph). The quality and quantity of the ice-nucleating agents and the osmolality of the fluid are the related factors that influence the nucleation temperature. One of the remarkable factors is the concentration of bacterial INAs in samples. Due to the increase in ice nucleation protein concentration, the chemical interaction between ice nucleation protein molecules would increase, thereby increasing ice nucleation protein aggregation and ice nucleation activity.[38,39]

## Silver Iodide and Other Heterogeneous Ice Nuclei

Silver iodide (AgI) is a well-known ice nucleator that is widely applied in cloud seeding. It was discovered in 1947. There is a similarity between β-AgI and ice (~3%) (Figure 1), and both rely on the hexagonal diamond lattice.[40] As ice nucleation, silver iodide was immobilized into the sodium alginate and calcium chloride and was applied to the freezing solution. Samples including AgI alginate-gel triggered latent heat at higher subzero temperatures than the control sample.[41,42] Another heterogeneous ice nucleation is caused by micrometersized $(NH_4)_2SO_4$ and maleic acid ($C_4H_4O_4$). Organic compounds (OC) such as pure dicarboxylic acid particles and organic aerosols (including bioaerosols) serve as cloud condensation nuclei (CCN). Some of these compounds have global effects (such as climate change)[43] and local impacts (such as toxicity and health hazards). The constituents of organic CCN, their chief sources, and their CCN features were reviewed by Sun et al. (2005). OCs (e.g., monocarboxylic acids, dicarboxylic acids, and humic-like substances) dominate in CCN. These molecules have many sources. Bioaerosols like bacteria, fungi, pollen, cellulose, and metabolites can also act as CCNs.[27]

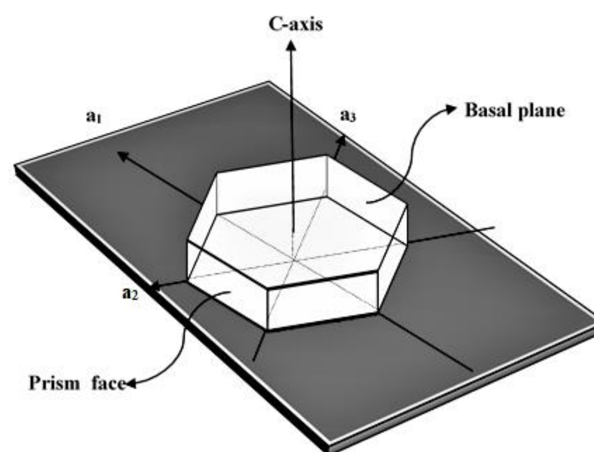


**Figure 1.** Schematic Illustration of a Hexagonal Ice Crystal. The basal plane, the c and three $a_1$, $a_2$ and $a_3$ axis and prism faces are displayed. Naturally, the growth of ice is formed on each prism face.[3]

Atmospheric ice particles (INPs) affect the global climate by changing cloud formation and precipitation efficiency. The function of secondary organic aerosol (SOA), which is a molecule produced via oxidation over several generations of a parent organic molecule, as a source of INPs in the atmosphere is not well defined. Wolf et al. concluded that

the ambient concentration of isoprene-derived SOA can compete with other INP sources. This suggests that isoprene and potentially other biogen-derived SOAs may affect the formation and properties of cirrus.[44] Data from the upper troposphere shows that particles with sulfate are often found in mixtures with organic compounds. Field measurements in both cities and remote areas show that there are large amounts of organic matter in the air, especially in the northern hemisphere, which is affected by human activity.[43,45] Knopf et al. showed that anthropogenic organic particles collected in Mexico City potentially provoke ice nucleation at laboratory conditions relevant to cirrus formation. The results show that organic particles could have an effect on how ice forms in clouds and how the climate changes.[43]

## Structure of Ice Nucleation Protein in Bacteria

The monomers of INA in *P. syringae* are 120 to 180 kDa.[46] DNA fragments from Ina$^+$ *E. herbicola* and *P. syringae* led to the recognition of 4.5 kbp and 5.7 kbp segments, respectively, responsible for INP production. The same 7.5 kbp DNA fragment was isolated from the Ina$^+$ strain of *P. fluorescens*.[47] These fragments transformed the Ina$^-$ *E. coli* into the Ina$^+$ *E. coli* with all the post-translational modifications that are required for active INA. The DNA sequences obtained from *ina* genes in the mentioned strains showed high similarity with a molecular mass of near 115 kDa and included a large number of consensus sequences of an eight-amino-acid repeat (Ala-Gly-Tyr-Gly-Ser-Thr-Leu-Thr). In addition, the 16-residue fragment is repetitive. Then a 16-residue fragment also becomes highly repetitive. Multiple replicates of 48-residue fragments (including three 16-residue fragments) (Figure 2) are also considered. The most constructional studies were conducted on bacterial INA by evaluating nucleation activities on Ina$^+$ cells and recombinant products isolated from transformed *E. coli*. Another method is mutational studies of any disruption in the 48-residue repeating fragment that influences nucleation activity by disrupting the periodicity of 48-residue repeats.[2,3,12,47,48] Activity at all temperatures is decreased by any deletions in the non-repeating domain of the N-terminal. Deletion in the non-repeating region at C-terminal ruins the activity very fast. Low activity can sometimes be attributed to poor INP translocation across the cell membrane. Therefore, it can be concluded that the N-terminal and C-terminal regions participate in the stabilization and assembly of the protein, and the repeating region is responsible for ice interaction. Recently, model structures have been developed. Warren et al. suggested two models of three-dimensional structures using the following clues: It is rational to suppose that the tertiary structure would be highly repetitive, and that the highly repetitive region contributes to ice interaction. Accordingly, the N-terminal and C-terminal sequences that are non-repeating regions do not get involved in the main

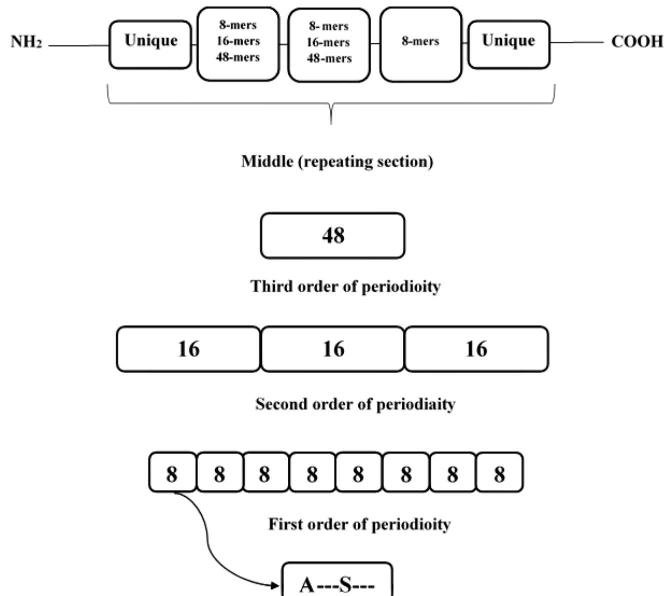


**Figure 2.** A Schematic Primary Structure of the Ice Nucleation Protein. The top shows that the protein consists of C-unique and N-unique terminals and a central repeating section. Consensus 8-, 16-, and 48-repeating amino acid residues are illustrated. A one-letter letter code is applied to amino acids; a dash demonstrates a variable amino acid.

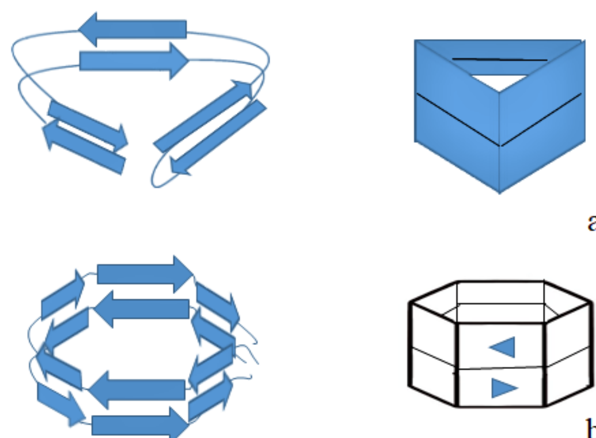


**Figure 3.** (a) In the trigonal model, a 48-residue repeating segment constructs three pairs of 8-sheets in an antiparallel model on each of three symmetric planes. (b) In the antiparallel double-helix model, a double helix is made from two antiparallel helices, one that goes clockwise and the other that goes counterclockwise.

conformation. There are some β-sheets with turns at Gly-Tyr-Gly in the 16-residue repeats, and thermodynamically, antiparallel β-sheets are stable. Other models have been suggested by using data. One of them recommended three pairs of β-sheets that are anti-parallel and have trigonal symmetry. Another model is hexagonal symmetry, which indicates it consists of two antiparallel helices to form a double helix formation (Figure 3). Other models based on minimum energy conformations are recommended, such as right-handed helical structures. Later ice-binding features were evaluated to find the best motifs, and the results showed

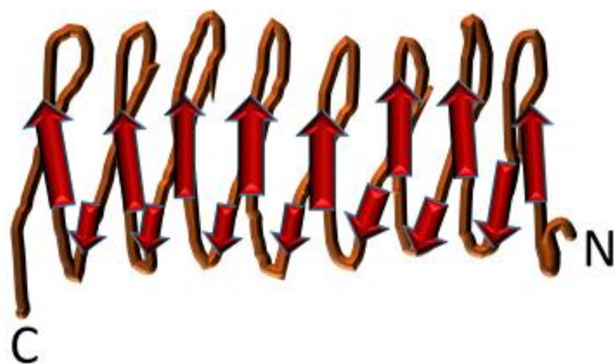


**Figure 4.** The Ribbon Model of a β-helical INP Displays the Structure of the 144-residue INP Model.

the stability of helical conformations for interaction with ice.[3,39,49,50] Further studies suggested that both this class of AFPs and INPs may include a similar β-helical fold (Figure 4) and that they could interact with water through the repetitive TXT motif (where X is any amino acid).[51]

## Ice Nucleation Activators Assembling

There is a particularly nonlinear correlation between the ice nucleation activity and the concentration of InaZ protein within the bacterial cell membrane. In terms of ice nucleation activity, as the InaZ protein concentration rises, the activity increases by 2-3 times. The interpretation is that ice nuclei are linked by InaZ protein aggregation and the power of ice nucleation activity depends on the concentration of InaZ protein. It seems unlikely that the opposite is true: that the number of proteins is controlled by ice nuclei. Also, the effective ice nucleus may be related to InaZ-containing particles. Results show that in *P. syringae,* the concentration -activity relationship may be similar to that of transformed *E. coli*. Due to the difference in the membranes (lipid compositions of the two species), the nucleation spectra of *P. syringae* start at moderately warmer temperatures than transformed *E. coli*.[3,52] The outer cell membranes are the locations of ice nuclei in some bacteria, such as *P. syringae*. Another site for locating the ice nuclei is microvesicles that are released from *E. herbicola*.[47] Studies show that high copies of INP are present in $Ina^+$ bacteria. INP residues aggregate to form INA (Figure 5). Further studies have demonstrated at least three copies of INP participate in ice formation at -8 °C.[3,47,53] The extent of aggregation is related to ice nucleation activity.[54] Filtration experiments demonstrate that cell-free ice-nucleating macromolecules from *Fusarium* are smaller than 100 kDa, and the aggregation of ice nuclei can be formed in solution.[55] The threshold temperature is associated with the number of copies. Accordingly, it has a variation from -13 °C as a monomer to -2 °C with the cooperation of more than 100 units. INP must be a cylindrical molecule with 48 residues along the cylindrical axis. This theory has been confirmed by substantial repeats, including 60-70 copies as helical conformations. The INP aggregation is likely to form a hexagonal impaction which is matchable with the ice lattice. INA stability will be raised by the anchoring of the INPs on the membrane because it reduces the entropy. Phosphatidylinositol is the main component that serves as a mediator for the anchoring of INA. Also, the N-terminal hydrophobic region would serve as an anchoring domain. In the population of the $Ina^+$ strains, the nucleation activities are not similar, and sometimes there is a small population that exhibits type A of ice nucleation, and the remaining cells show greater threshold temperatures (more than -13 °C). The reason for this is not obvious. One reason may be the various levels of expression of INP in cells. Another reason is the 'endergonic metabolism. Because of the variety of metabolic processes in every cell, INA can be varied. The interaction of each nucleus with impurities is another reason that causes varying threshold temperatures. Besides, the INA assembly needs a large number of INPs to produce effective ice nucleation templates that are not controllable among cells.[3,8,12,47] The measurement of ice fraction curves suggested that all particles do not possess ice nuclei, and the particles/droplets that include INP had functions, and the 100 nm particles showed a traceable ice nucleation ability. These findings suggest that an INP complex is associated with either intact cells or fragments of the outer membrane. It is thought that only half of the bacteria were able to nucleate ice. Articles show that the majority of *P. syringae* cells have either none, one, or just a few ice nucleators on their surfaces.[3,56]

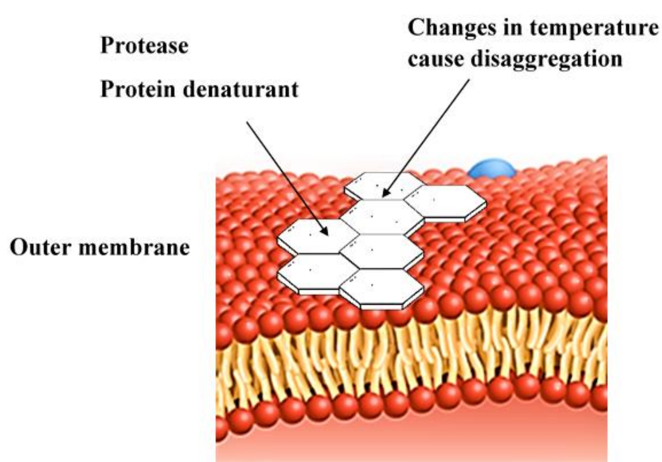


**Figure 5.** A Model of the Quaternary Structure of Bacterial Ice Nucleation Proteins. Copies of the ice nucleation protein are depicted as hexagonal prisms in order to highlight their function as an ice template and their tendency to aggregate into the outer membrane. The places where different treatments that break down bacterial ice nuclei are thought to work are also shown.

## Cloning and Mutation

Arai et al., (1989) showed the capability of ice nucleation-activity of *E. coli* MM294 after cloning an approximately 7 kbp

genomic DNA fragment of *E. ananas*. The truncated 5'-end that was 0.7 kbp and 1.7 kbp truncation from the 3'-end was expressed effectually in *E. coli*. Thus, an approximately 5 kbp fragment was an *ina* gene, and it was named *ina*A.[57] One of the most effective approaches for measuring gene expression is using ice nucleation as a reporter system. In comparison to β-galactosidase activity, which is expressed by *lacZ*, the ice nucleation activity was at least five times greater in sensitivity. As a result, fewer InaZ protein molecules are required for the production of measurable signals, and its sensitivity is at least 105-fold greater than β-galactosidase. Therefore, it is comparable with bacterial luciferase.[58] In a different study, the quantification of ice nucleation activity and the production of *ice*C genes were measured in the fractions of *P. syringae* and transformed *E. coli*. Ice nuclei were almost preserved during the separation of cell envelopes. Also, ice nucleation activity was observed when the membrane fragment was insoluble in Triton X-100. Almost all ice nucleation activity was connected with the outer membrane because the partitioning of 3-ketodeoxyoctonate (a lipopolysaccharide ingredient) and ice nuclei in cell portions was alike and opposite that of NADH oxidase (a cytoplasmic membrane component).[59]

*Nicotiana tabacum,* which is susceptible to freezing, and the freezing-tolerant *Solanum commersonii* were transformed by the ice nucleation gene (*inaZ*) from *P. syringae* pv. *syringae*. Results demonstrated that these transformed species could express ice nucleation activity. The results also showed that the ice nuclei activity could remain in transformed plants. The freezing temperature in untransformed controls increased from nearly -12 °C to -4 °C in the transformed samples.[53] Watabe et al. over-expressed 15.3 mg of ice nucleation-active protein of *E. ananas* IN-10 (InaA) as inclusion bodies in 60 mg of *E. coli* dried cells. Protein purification was carried out using solubilization with surfactants, thereby becoming free from sugar and lipid. With this preparation, InaA could act as a nucleus to start the formation of ice.[60]

Hwang et al., assessed the ice nucleation activity in transformed *Saccharomyces cerevisiae*. An ice nucleation gene (*ina*) isolated from *E. herbicola* was located near the galactose-inducible promoter (*GAL1*). Then it was inserted into *S. cerevisiae*. The transformed yeast showed an increase in ice nucleation activity compared to untransformed controls. The results showed an increase of approximately -13 °C in the untransformed yeast to -6 °C in the transformants. For the expression of ice nucleation activity, lower temperatures than 25 °C were needed. The induction of ice nucleation activity was observed by shifting the temperature to 5-20 °C, and the maximum activity was shown at 5 °C for ~12 h. $Ina^+$ yeast can be used for freezing and texturing in some food products.[61]

Wu et al. examined summer collected soils for their ice-affinity selection of bacteria. Then *P. borealis* was recovered and characterized. Ice nucleation activity was shown in this isolate. Psychrophilic growth was needed for ice nucleation activity. *E. coli* was used to create the *inaPb* recombinants. *E. coli* showed ice nucleation activity above -5 °C, and its ice nucleation activity was classified as a high-activity nucleator. This strain showed no significant identity to other sequences, and the C-terminal was divergent but in some aspects of residues had similarity to the *P. fluorescens* protein. Phylogenetic trees displayed more divergence from the plant epiphytes and were related more closely to *P. fluorescens* as a soil microbe. There is no evidence that it is the result of horizontal gene transfer or that the differences in sequence are an adaptation to a soil habitat.[62]

Research was conducted by Yu et al. (2013), and recombinant *E. coli* expressed ice nucleation protein from *Pantoea ananatis*. Ice nucleation protein expression caused an increase in the freezing point by a few degrees. Deletion in 3-ketoacyl-ACP synthase III (FabH), which is responsible for the start of fatty acid elongation and is a major enzyme in fatty acid biosynthesis, prevented the ice-nucleation function. When saturated fatty acids were added, the ice-nucleation activity increased. In contrast, adding unsaturated fatty acids showed the reverse effect. As a result, membrane fatty acids play a significant role in INP function in *E. coli*. Consequently, *E. coli* with a lot of saturated fatty acids was much better at starting ice crystals.[63]

Lagzian et al. transformed the *ina* gene from *Fusarium acuminatum* into *E. coli* and subsequently purified and characterized it. The optimum pH was 5.5 and was stable between pH 5 and 9.5 and up to 45 °C. The protein's 3D structure model consisted of three distinct domains, as seen in other ice nucleation proteins.[64]

## Molecular Mechanism of Water Binding to Ice Nucleation Proteins

In nature, a hexagonal ice crystal ($I_h$) is created in the supercooled water and causes crystal expansion by adsorption of water molecules. The repetitive peptide of INP is capable of shaping an ice crystal. The data suggest that the repetitive domain of INP supplies a structural surface that can interact complementarily with the hexagonal ice crystal surfaces.[65] The findings of Graether *et al*. indicated that both classes of AFPs and INPs may contain a similar β-helical fold and they could interact with water via the repetitive TXT motif (where X is any amino acid). The defining characteristic between AFPs and INPs is the size of the ice-interacting surface. For INPs, the more considerable surface area serves as a template and is larger than the critical ice embryo needed for ice growth. In contrast, AFPs are small enough to bind to ice and inhibit additional growth without acting as nucleators.[51] As mentioned, to provide the ordered water molecules into the crystal of ice, the repetitive region of the

protein is needed as a template for ice crystal formation. The mutations disrupting the periodicity (dysperiodic mutations) reduce the quality of ice nuclei due to degradation of the regularity in templates rather than reducing their quantity. The non-disruptive removal of several repeats had few deleterious effects. The results showed the number of repeats in some protein genes is related to their optimum activity. The functions of ice nucleation proteins include: firstly, providing the binding sites of water molecules; secondly, self-assembling for conformational water binding sites; and thirdly, the aggregation of the monomers with other membrane ice nucleation proteins as their neighbors. This co-operation is essential for ice nucleation activity at warmer temperatures. Any N-terminal mutants cause the lack of this cooperativity function. The non-repetitive N-terminal acts as the aggregation domain. At all temperatures, every mutation in the C-terminal reduces or eliminates activity. The non-repetitive C-terminal plays as self-assembly. The C-terminus may play a role in protein localization to the appropriate part of the cell membrane.[3,66]

### Measuring Ice Nucleation Activity

The experiments for measuring freezing drops were used for evaluating ice nucleation in water and other substances, and the classification of ice nuclei was shown by freezing drops (Figure 6 and 7). There are two different nucleus spectrums: differential and cumulative nucleus spectrums. The differential nucleus spectrum exhibits the concentration of nuclei having activity at a certain temperature. The cumulative nucleus spectrum represents the activeness of nuclei at all temperatures, which are warmer than the specific one.[67] The antibodies were produced against a synthetic peptide according to the consensus repeats of the *ina*Z and *ina*W genes. The antibody technique shows that the INPs are in clusters in bacterial cells. The sizes of the clusters vary with the growth temperature and the type of nuclei. Usually, the high threshold temperature is related to larger ice nuclei. The concentration of INP in the cell membrane (the intensities of bands on Western blots) causes the differences in the protein activity at the temperatures of 15 to 37 °C. Consequently, the decrease in protein concentration near 10,000-fold occurs by growth at 37 °C. INPs also form intracellular inclusion bodies and some particles. Despite other inclusion bodies, the growth at 15 °C shows the interaction of the INPs with the cell wall.[68]

The bacterial ice nucleation activity is evaluated in the cell.[32] 10-1000 µl of a known number of bacterial cells (Z) were cooled to a specific temperature to determine the presence of ice nuclei (N) at that threshold temperature (t). Cumulative ice nucleation frequency (f) is the number of ice nuclei/cell in the given temperature (equation 1).

$f = N/Z$  Eq (1).

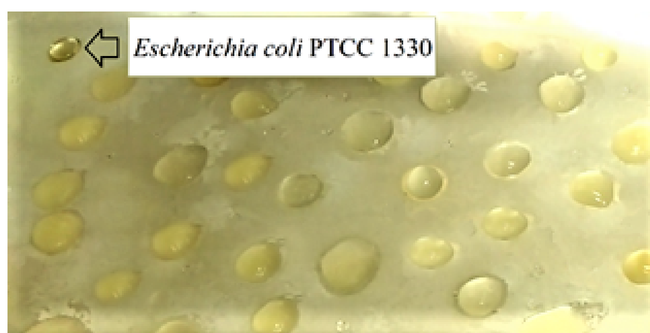


**Figure 6.** Ice Nucleation Assay by the Frozen-Droplet Technique. The LAUDA® Alpha RA-8 Refrigerating Circulator is used for ice nucleation activity evaluation. All *Pseudomonas syringae* droplets froze at 5 °C. The negative control (*Escherichia coli* PTCC 1330) has not frozen at the given temperature.

**Figure 7.** Vials containing fragments of outer membrane proteins were placed on an aluminum plate, and ice nucleation was evaluated using the frozen droplet technique. The LAUDA® Alpha RA 8 Refrigerating Circulator was employed to evaluate ice nucleation activity. At -5 °C, all droplets containing the outer membrane of the *Pseudomonas syringe* were frozen. Distilled water was used as a negative control, shown in the red circle.

A cumulative ice nucleation spectrum is gained when log f against t is plotted.[3,69] Cumulative spectra show that bacterial nuclei can be classified into three types: types I, III, and III at temperatures of -5 °C or higher, -5 °C to -8 °C, and -10 °C or lower, respectively.[70] After general revision of these types, it turned to types A, B, and C with ranges of -4.4 °C or greater, -4.8 °C to -5.7 °C, and -7.6 °C or lower, respectively.[71]

### Optimization of Medium Compositions

INA is produced at the end of the log phase and the beginning of the stationary phase. The conditions for optimizing the type I of INA bacteria have been evaluated. *Pseudomonads* can use many substrates such as carbohydrates, hydrocarbons, lipids, and alcohol for growth and production of different bacterial byproducts. The INA enhancement was gained by using glycerol and sorbitol as the carbon sources.[72-75] Sugar beet molasses was used by Afendra et al. to make xanthan gum and 108 ice nuclei/ml by *Xanthomonas campestris* pv. *Campestris*.[76] The starvation of some mineral

components, including phosphorus, sulfur, nitrogen, and iron, led to the high production of INA. To simulate the condition of the leaves, which are poor in nutrients, programmed fermentation was carried out. Some experiments were conducted to evaluate the components that are required in the assembly phase of ice-nucleating, including mannose, glucosamine, inositol, and $Mn^{2+}$. INA production was increased by preculture in phosphate-starved medium and then culturing in wheat bran, phytate, and phytic acid, which are the precursors for Myo-inositol.[72-75] Nemecek et al. looked into how starvation affects the process of making ice nuclei.[77] Iron, sulfur, phosphorous, or nitrogen starvation, combined with a temperature of 14-18 °C, induced type I ice nuclei that were active at temperatures warmer than -5 °C. The induction was promoted in the stationary phase when sorbitol was utilized as the carbon source in the cool phase. [35S]-Methionine demonstrated only shifting the temperature to low temperature-induced protein production. The result showed that ice nucleation protein was stable at 40 °C after 8 days. However, when the temperature was raised again, the ice nuclei vanished. The results proved that ice nucleation properties may be regulated by nutrient starvation and low temperatures.[75,77]

## Optimization of Environmental Agents

pH has a significant impact on bacterial growth. Class A ice nucleation proteins are highly sensitive to low pH (pH 5.5 or lower) and cannot be restored by increasing pH. The configurations of classes B and C are more resistant to pH changes. Class A and B are highly resistant to the higher pH, and class C is approximately stable between pH 3.5 and 9.5.[71] The *in vitro* culture conditions affect INA. $Ina^+$ bacteria inhabit plants. Therefore, it is correct to assume that INA environmental situations such as hygrometry affect them. Studies showed high humidity was needed for the growth of *P. syringae* on plants. But for in-vitro growth, a low level of hygrometry was required.[78] In addition, the relationship between INA, water activity, and the hydrophobic medium has been proven. Vegetable oil that has low fluidity, like olive oil, causes the high production of INA. Due to the numerous substances in olive oil, it is a suitable option for many biotechnological purposes. The basis of the use of olive oil is that INA depends on an outer membrane protein, which is bonded to the membrane by a glycosylphosphatidylinositol (GPI) anchor. Olive oil can be hydrolyzed to produce fatty acids as the precursors for bacterial ice nucleation protein anchors. Vegetable oils are composed of different proportions of unsaturated and saturated fatty acids. These fatty acids are probably metabolized and integrated into the bacterial membrane and lead to the ice nucleation protein anchor.[75,79,80] Also, the fluidity of mono-unsaturated and saturated fatty acids is low. Since olive oil contains a high amount of mono-unsaturated fatty acids, it leads to the ice nucleation sites' assembly. Temperature is another important factor in enhancing INA induction. Ice nucleation proteins, particularly type I, are profoundly affected by temperature. The fast shift from 32 °C to almost 16 °C in the stationary phase led to the high expression of type I INA.[32,72,73,75,79,80] The modification of the temperature from 30 °C to 18 °C during the cultivation increased the biomass and the INA of *Xanthomonas ampelina* TS206.[81] The characterization of airborne ice-nucleation-active bacteria was evaluated by Santl et al. INA induction was seen in the late exponential growth phase and when the temperature was set to 4 °C and then 15 °C overnight, one after the other.

## The Treatments of Ice Nucleation

Heat treatment was evaluated for suppressing the ice nucleation activities in bacteria and degradation of the cell.[82] The result showed that the ice nucleation activity remained after heating in the microwave for 3 min. Another method is irradiation to achieve a sterile or decreased viability, but it does not cause the loss of ice nucleation activity. In addition, lyophilization is a beneficial method for the storage of these bacteria with no change in ice nucleation activity.[83] Heat treatments at 40 to 98 °C strongly decreased the observed ice nuclei concentrations in *Fusarium*, assuring earlier hypotheses that the ice-nucleating macromolecules in *Fusarium* mainly consist of a proteinaceous compound. The freeze-thaw cycle and long-term storage tests indicated that the fungal ice nuclei in an aqueous solution remain active over several months. Also, exposure to nitrogen dioxide and ozone at levels that are normal for the atmosphere did not change the activity of ice nucleation.[55]

Ice-nucleating protein has been studied by protein extraction. The research showed the outer membrane contains ice-nucleating protein and the whole cell has more activity than isolated protein.[75,84] As mentioned, some bacteria are able to release ice nucleators into their medium as vesicle forms. If *P. syringae* could release extracellular ice nucleators, it would be more efficient than the bacteria that do not release them. Furthermore, the issue of using living ice nucleation bacteria would be solved. However, there is no evidence that *P. syringae* is toxic.[2,8]

## Applications

Ice nucleation proteins can be applied in various fields such as snowmaking, agriculture, food processing, signal transduction, atmospheric applications, and surface display (Figure 8).

## Snow-Making Technology

Snow-making is useful for recreational ski resorts and oil exploration in the Arctic. Using bacteria as a snow inducer was suggested by Woerpel (1980). Later, the first commercial product of *P. syringae*, which was named Snowmax® in the

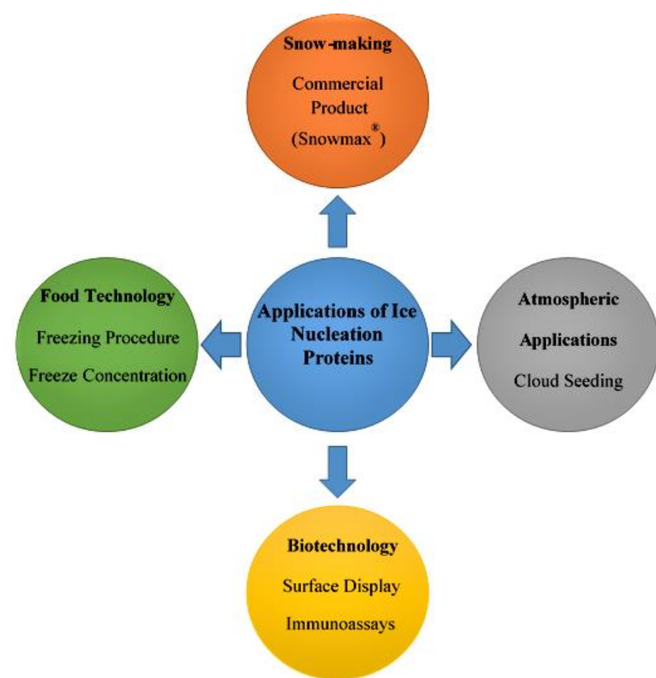


**Figure 8.** Applications of Ice Nucleation Proteins in Various Industries.

USA, was developed. This product contains freeze-dried powder of *P. syringae* that makes snow production much easier (as high as 1.1 °C). Despite the inadequate activity in most ice nuclei at the lower temperatures, the Ina$^+$ *P. syringae* is active under various environmental conditions.[85] The inoculation of *P. syringae* for inducing precipitation in supercooled clouds can initiate ice nucleation in clouds. Research has shown Snowmax® can be used as an inducer for this purpose.[86] In addition, it is more active than silver iodide aerosols. The usage of a *P. syringae* nucleating agent decreases energy demand dramatically. In Arctic regions, paths and aircraft runways are fabricated from seawater at low temperatures. The formation of ice in the Arctic regions can be initiated without ice nucleation. But *P. syringae* is applied to decrease supercooling temperatures efficiently in seawater streams. Accordingly, a large volume of water will be frozen. Therefore, ice construction can occur in higher temperatures.[2, 87-89]

## Freezing Procedure

The quality of frozen food may be promoted by using *P. syringae*. In the freezing procedure, the temperature applied for freezing is lower than the temperature that is theoretically required for freezing. The moisture in food causes a decrease in the textural properties of frozen food. For this reason, a temperature as low as -40 °C is given, which is not cost-effective. Using bacteria such as *P. syringae,* the crystallization temperature (Tc) was higher than the control sample. Similar results were gained for distilled water, fish fillets, and meat steaks containing membrane fractions from *P. syringae*. Also, the stability of frozen food could be promoted throughout cold storage. Moreover, degradation of the texture did not occur. In conclusion, several main effects are created by using *P. syringae*: crystallization in subzero temperatures; increasing freezing temperatures; stability enhancement; reducing freezing time, and being cost-effective.[90,91]

## Freeze-Concentration

Freeze-concentration is a method for removing excess water from some liquid-based products. In this process, the water turns into ice and is then followed by the elimination of ice from the liquid. Compared with evaporation and reverse osmosis, this method leads to minimal loss of aromas and volatile flavors. Therefore, this approach is used for juices, wine, coffee, and vinegar. In the freeze-concentration method, symmetrical and large crystals are essential parameters for enhancing the efficiency of the process. High supercooling is needed to achieve this purpose. In contrast, lower rates of supercooling are suitable to suppress excessive nucleation. By adding *P. syringae* to the liquid-based products, both advantages are obtained: The remarkable reduction in supercooling and large ice nucleators. Therefore, this approach leads to reducing the time and cost of freezing and also efficient productivity.[2,92,93] Due to the stability of bacterial ice-nucleation activity below 25 °C, it is possible to seed at room temperature. This technique is used for raw egg white, fresh milk, and lemon juice to obtain a concentrated product with their original flavor. In other research, a non-heated strawberry jam was obtained, which was comparable with conventional jam in terms of texture, flavor, and color.[18,94] In another study, the effectiveness of ice nucleation temperature on the primary drying rate during lyophilization was implemented. Overall, the ice nucleation temperature is the main factor in the primary drying rate. But important factors like the amount of particles and the condition of the vial must be controlled so that they don't change by accident during scale-up and process development.[95]

## Signal Transduction

A promising application of ice nuclei is their property of signal transduction (the ability to convert one type of signal into a visible one, for example, in immune assays).[32,75,80] In this case, the presence of a submicroscopic body is transduced to a macroscopic physical change during a second. For this approach, the extraction of protein from bacteria would be necessary.

## Immunoassays

In immunoassays, the ice-nucleating gene is inserted into a *Salmonella*-specific bacteriophage (containing a green fluorescent dye). This manipulated bacteriophage is added to evaluate food contaminated with *Salmonella.* By incubation at -5 °C, ice-nucleating proteins are allowed to form. If *Salmonella* is

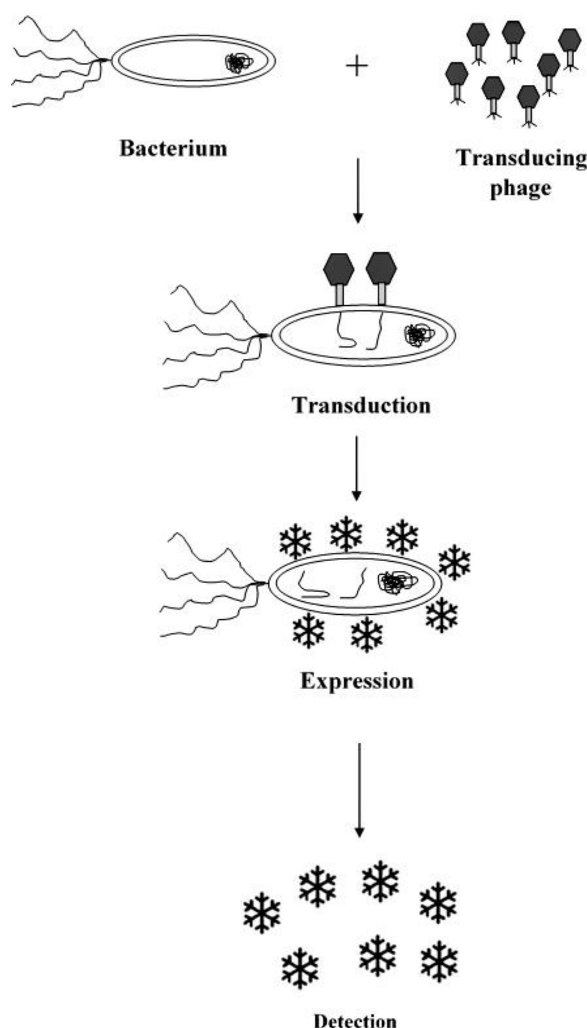


**Figure 9.** The Bacterial Ice Nucleation Diagnostic (BIND) Assay System. The figure depicts a schematic diagram of the assay protocol. The bacteriophage, which can make ice nucleation proteins and is specific to a single species, can change a bacterium that can't make ice nucleation proteins into one that can.

present in the sample, the green dye will become dark (Figure 9) without fluorescent.[75,96]

## Atmospheric Applications

Currently, ice-nucleating (IN) bacteria are being employed as a replacement for silver iodide in cloud seeding.[97] About 20% of the particles in the middle and upper parts of the troposphere are living bacterial cells. Bacteria are one of the most important particles in the atmosphere. Bacteria, especially those with ice nuclei, are dispersed in the air due to natural factors such as wind and human activities such as harvesting. In the upper atmosphere of agricultural lands, the concentration of bacteria is even higher. Among them, *Pseudomonas* is the predominant bacterium in fog and clouds. About 4% of bacteria in snowfall have ice nucleation activity at -4 to -7 °C. *P. syringae* is uniquely present in rainfall and freshwater and is part of the water cycle. The highest frequency of bacterial ice nuclei is related to class III ice nuclei, and one in every 300 cells is in this class, but the frequency of class I and II is one in a thousand to one million.[56] Atmospheric evaluations demonstrate that biological particles have the potential for ice nucleating activity. 50% of the fungal spores in the atmosphere are from *Cladosporium.* Bacteria, pollen, and fungal spores are the most biologically diverse particles that can serve as ice nuclei. Water droplets containing fungal spores such as *Cladosporium* are one of these biological particles. There is a direct association between freezing temperature and the number of spores of *Cladosporium* entrapped within a droplet. Droplets that contain 1-5 spores show mean freezing temperatures of approximately -35.1 ± 2.3 °C. By comparison with the surface of *P. syringae*, the weakness of the ice nucleation ability of *Cladosporium* sp. could be explained by its surface, which is coated with a class of hydrophobic proteins in filamentous fungi (hydrophobins).[98]

## Surface Display

A surface display is an expression for displaying a foreign protein on the cell surface due to its biotechnological potential, such as enzyme-coated microbes or antibody libraries. Ice-nucleating protein is almost a novel approach that is used as the anchor for these biotechnological methods.[75] Kwak et al. (1999) designed a new system for the surface display of eukaryotic viral proteins by recombinant proteins on *E. coli*. The fusion of gp120 of HIV-1 to the C-terminal of the ice nucleation protein could be expressed on the membrane of *E. coli*. As a result, the expression of eukaryotic viral proteins can be performed by the INP system. This approach can be used in AIDS diagnostics, expression of weight proteins, and oral vaccination.[99] Bae et al. (2001) investigated synthetic phytochelatins (ECs) as metal-binding proteins. Using the Lpp-OmpA anchor, EC20 was expressed on the surface of *E. coli*. Therefore, the bioaccumulation of cadmium and mercury, which are heavy metal contaminants, was improved by this novel technique. EC20 was also expressed on the membrane of *Moraxella* sp. by applying the truncated ice nucleation protein as an anchor, and mercury-binding capacity was improved 10-fold more than *E. coli*.[100] The ability of the N-terminal domain of InaK from *P. syringae* KCTC1832 to act as an anchor was studied by Li et al. (2003). In the comparison between the expression level of green fluorescent protein (GFP) that was fused to the N-terminal domain of InaK (the InaK-N/GFP) and truncated InaK containing both N-terminal and C-terminal regions (InaK-NC/GFP), the InaK-N/GFP fusion protein demonstrated more yield than InaK-NC/GFP. Therefore, the N-terminal region is the only part that can be responsible for the translocation of proteins to the cell surface.[101] Another investigation into surface display was pursued by Li Wu et al. (2005). By the means of the N-terminal region of the ice nucleation proteins (INPN),

chitinase 92 (Chi92) from *Aeromonas hydrophila* JP10 was exposed on the cell surface of *E. coli,* and the catalytic activity of Chi92 to chitin was enhanced. Accordingly, chi92-displayed cells (as biocontrol agents) can influence phytopathogenic fungi such as *Fusarium decemcellulare*, *F. oxysporum* sp. *melonis*, and *Rhizoctonia solani Kuhn*[102] To overcome the diffusion of organophosphorus pesticides, Latifi et al. (2012) used a new anchor system derived from the N-terminal domain of ice-nucleation protein from *P. syringae* InaV (InaV-N) to display organophosphorus hydrolase (OPH) on *E. coli*.[103] In the other study, they compared the ability of an engineered *E. coli* for chlorpyrifos (Cp) degradation using an organophosphorus hydrolase enzyme encoded in both *Pseudomonas diminuta* or *Flavobacterium* sp. ATCC 27551 using the N-terminal domain of the ice nucleation protein and Lpp-OmpA chimera as anchoring motifs. They concluded that Lpp-OmpA displayed more functional OPH protein but less protein.[104]

In another study, xylose dehydrogenase (XDH) was anchored by an ice nucleation protein from *P. borealis* DL7 on the surface of *E. coli*. The catalysis of the oxidization of xylose was facilitated by the XDH-displayed bacteria (Figure 10). The growth of the recombinant *E. coli* was not banned by the XDH surface displaying system. The optimization for temperature and pH was performed, and the results showed that 30 °C and pH 8.0 were the optimum temperature and pH, respectively. Enzyme extraction and purification decrease the stability of the enzyme. In addition, detection of D-xylose in food and degradation products of lignocellulose can be done by the recombinant cells rapidly and cost-effectively. Besides, genetically engineered cells can be a promising approach for biosensors and biocatalysts.[105]

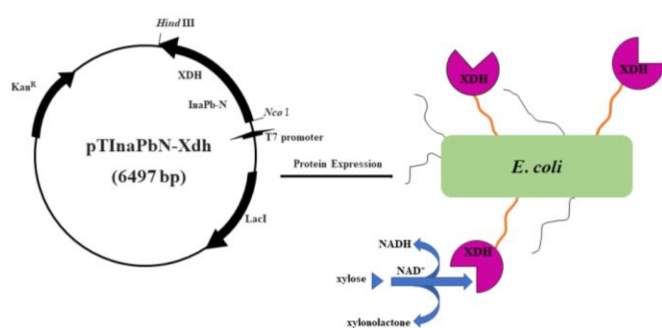


**Figure 10.** Xylose Dehydrogenase (XDH) Was Anchored by an Ice Nucleation Protein from *Pseudomonas borealis* DL7 on the Surface of *Escherichia coli.*

## Conclusion

Ice nucleation bacteria are the catalyst for ice. We described how they initiate the freezing of supercooled water. Also, the environmental and industrial implications and the structure of ice nucleation protein were explained. INP is able to control water molecules and causes a phase change. Thus, it is a powerful tool for INA bacteria to form ice nuclei rapidly among plant tissues. The role of INA bacteria in frost damage to crops has been recognized. In other words, ice is formed in the plant tissue, leading to cell death, and bacteria utilize the substances released from the plant. For optimal protein activity, a complete and healthy bacterial cell is essential. The research in bacterial ice nucleation has focused on various approaches: (1) the production of effective ice-nucleating bacteria for different applications, many of which have not yet been discovered; (2) the research on the structure, composition, and location of the ice nucleation site; and (3) the construction of effectual frost controllers. Meteorologists, physicists, and biochemical engineers are interested in the first topic. Biochemists and genetic engineers are looking into the second issue, and scientists in the field of agriculture are interested in the third approach.

## Authors' Contributions

Study concept and design: NS and AM.L. Drafting of the manuscript and critical revision of the manuscript for important intellectual content: NS, AM.L, SM, and A.AS.

## Conflict of Interest Disclosures

The authors declare that they have no conflicts of interest.


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