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Characterization of Ice nucleation Bacteria and their Applications

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Abstract. Supercooled water can remain in liquid state at the temperatures below zero and needs catalysts for changing from liquid to solid state, and accumulation of water molecules causes nucleation and creates ice embryo. Then, other molecules are attached to this core and will make ice crystals "visible". These catalysts may be inorganic materials, bacteria and/or fungal spores. In this review article, bacteria such as P. Syringae have been studied in ice nucleation process and their industerial applications.

Keywords: Supercooled Water, Catalysts, Ice nucleation, Ice Crystals, P. Syringae

1. INTRODUCTION

Many plants, vertebrates and microbes are able to catalyze ice in supercooled water which are called heterogeneous ice nucleation. Supercooled water will not freeze homogeneously up to -38 °C without the presence of catalysts. [1]

These catalysts can be inorganic solids such as silver iodide, pollen, seeds of plant, dust and metal particles in the air or biological agents such as bacteria. [2] and provides creation of ice crystals, causes water molecules to bond together and forms hexagonal molecules. In the presence of these ice nucleations, freezing temperature is increased from -2°C to -10°C and number of necessary molecules is decreased with the reduced temperature. For example, for the creation of such ice at -5°C temperature, a number of 4500 water molecules are needed but in -40°C, number of water molecule is reduced to 70. [3]

After formation of this core, water molecules bind in the form of cascade and will form ice crystals. At least 10 species of bacteria have been identified as Ina^+ strains and involved genes in this phenotype is associated with "inak", "inav" and "inaz". [4]

These bacteria are aerobic gram-negative, and most abundant strain is plant pathogen epiphytic bacterium entitled "p. syringae" which are detached from lilac flower. [3]

Accordingly, other species were introduced in subsequent years, most of which had been isolated from filosphere. [5] and include pseudomonas species such as p. flurescens, p. viridiflava, p.antatrctica, pantoea agglomerans, pantoea ananas, Xanthomonas campestris. [1, 2 and 3]

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The main habitat of the bacteria is surface of leaf of plants and soil and can be identifiable from atmosphere, clouds and snowflakes as well.

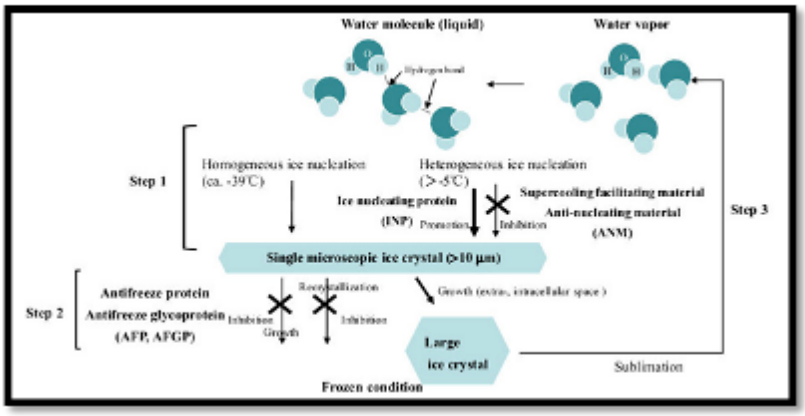

Figure 1. Cycle of Ice Crystals Formed by Water Molecules [6]ç

2. IDENTIFICATION OF SPECIFICATIONS OF ICE NUCLEATION PROTEINS (INP)

Ice Nucleation Proteins (INP) is a monomer composed of 1200 to 1500 amino acid, with molecular mass of 118 k/Dalton. [1, 4] and has three seprated domains.

1. The N-terminal end, which constitutes approx. 15 percent of total sequence, is hydrophobic part and is rich with asparagine, serine and threonine. [4] Which is capable of coupling with groups of mannan and phosphatidylinositol in the outer membrane through fittings of N glycan (Asp) or O glycan (Ser or Ter). [2]
2. The C- terminal end almost includes 4% of the sequences are hydrophilic.
3. The third part is Central Repeat Domain (CRD) which engulfs almost 81 percent of sequence and is the location of ice interactions. The third part is composed of 48 amino acids that can create three 16-amino acid parts and each of which is able to be divided and decomposed into two octameter peptides. The consequence of amino acid of this part is as follows:

Ala-Gln-Glu-Gly-Ser-Asn-Leu-Thr-Ala-Gly-Tyr-Gly-Ser-Thr-Gly-Thr-Ala-Gly-Ala-Asp-Ser-Ser-Leu-Ile-Ala-Gly-Tyr-Gly-Ser-Thr-Gln-Thr-Ser-Gly-Ser-Glu-Ser-Ser-Leu- Thr- Ala- Gly-Tyr- Gly- Ser- Thr- Gln- Thr

The generated octapeptides include Ala-Gly-Tyr-Gly-Ser-Thr-Leu-Thr [3, 4]. The central part can be attached by a glycosyl phosphatidylinositol (GPI) or phosphatidyl ethanol amine to the membrane.

The “N” and “C”-terminal INP part is free, exposed to the cellular surface, foreign and external proteins can be attached to these two areas and also can be placed on cell surface. INP also can remain productive of iced nucleation even when connected to external proteins.

Three different types of bacteria producing ice nucleation have been identified in various bacterial populations and are shown with the titles of I, II and III. These three groups are able to produce frozen embryo, the first type (I) at 2°C- to 5°C, the second type (II) at 5°C to 7°C and the third type (III) at the temperatures of 7°C to 10°C in order to catalyze the ice [3, 7]. The difference between these three locations of core is associated with glycolisation after the translation of N and C-terminal and multimerization of protein at the membrane.

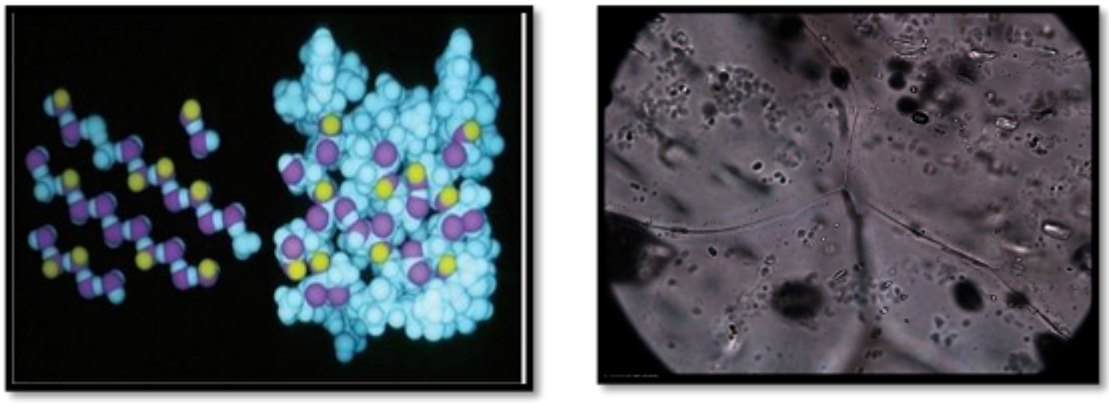

Figure 2. Left Side: INP at P. syringae, Right Side: P. syringae surrounded inside the network of ice crystals.

3. ICE NUCLEATION BACTERIA VERIFICATION METHODS

Various methods are applied to study ice nucleation activity, one of which is the Drop Freezing method in a way that some bacterial suspension is placed on floating surface covered with an aluminum foil and also a hydrophobic layer is placed with a cool surface in its below.

Given the observations, necessary studies are conducted. For example, through taking advantage of this technique, it became clear that almost all P. syringae bacteria cells are able to produce proteins type "I" and almost all cells are able to produce ice nucleation core at the temperatures of -1°C to -10 °C. [3]

Using wind tunnels and cloudy chambers are of the other methods that confirmed application of dried and powdered Ice + bacteria for climate change for the first time in 1980 [6, 7].

It was specified that destroyed bacteria are still able to produce ice nucleation, are used as a factor for seeding clouds and called as "Snowmax" [1-6].

Using "Free fall" droplet is another way [8] that these tests are done under certain conditions. In this technique, droplets is held as size as nanometer affected by vertical slope in a cylinder and freezing is identified by lighting drops on the verge of fall with a linear polarized laser and also by showing de-polymerizing the returned light.

"Drop freezing" is the most common and ordinary method for studying the bacteria and the results are usually studied using a thermo-analytical techniques or visual methods. [5.3]. Assuming time of nucleation is at the second rate, it can be grasped out that: cumulative spectrum kernel K (T) which represents as the number of active cores at volume unit per "T" temperature, in each volume of water, core cumulative spectrum is determined easily from particles of frozen drops (F_{ice}) at the given temperatures according to the following formula:

$$k(T) = \frac{-\ln(1-\text{fice(T)})}{V} \text{ [5]} \quad (1)$$

Wherein:

"V" represents volume of drop. If the number of cell in each volume of water (C_n) is specified, active site of nucleation in each particle (n_n) is as follows:

$$n_n(T) = \frac{-\ln(1-\text{Fice(T)})}{V\,Cn} \text{ [5]} \quad (2)$$

Based on CHESS Model, the following relationship holds that λ is the average number of active core in each drop and it can be calculated at the area that ice particles have been turned into the saturated form (f^*_{ice}).

$f_{ice}(T) = 1 - \exp(-\lambda(1 - \exp(-J_{het}(T)t)))$ [9] (3)

$\lambda = -\ln(1 - f^*_{ice})$ [9] (4)

The value of active heterogeneous nucleation in this condition (Jhet) is not dependent on the particles' size, and is calculable using another formula in a way that parameters of B= -2.032 $°C^{-1}$ and A= 1.55*10 8 s^{-1} is constant for active INA protein complexes.

$J_{het}(T) = A \cdot \exp(B.T)$[9] (5)

Given the aforementioned conditions, active ice nucleation bacteria and also "Snowmax", which was studied in 2013 by F. Stratmann using CHESS method, have maximum activity at the temperature of -1°C to -10°C.

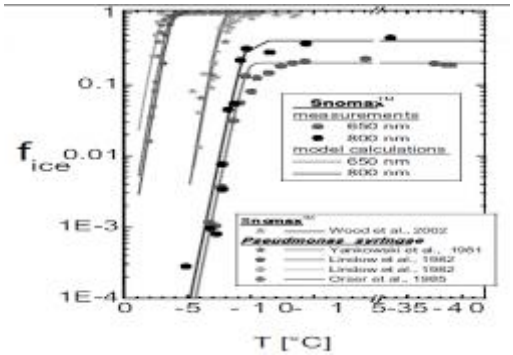

Figure 3. A. Formation of ice particles in various temperatures by Snowmax particles [9]

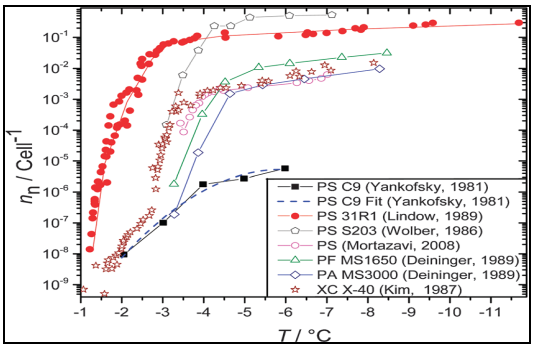

Figure 3. B. Formation of active nucleation site of cells of various bacteria with temperature changes [5]

4. EFFECT OF ENVIRONMENTAL FACTORS ON ICE NUCLEATION BACTERIA

Ice nucleation bacteria were first identified and recognized by meteorologists. These bacteria are isolated by wind and environmental factors from surface of leaves following bioprecipitation cycle and reach to clouds at atmosphere and cause creation of ice crystals and finally, generates rainfalls. These bacteria along with it will return to the surface of leaves at last. Consequently, this cycle of the group of bacteria is separable from atmosphere and are usually separable at rain and snow drops. [3]

When these bacteria are in atmosphere, they are affected by environmental factors such as UV- ray, NOX, PH acid, O3, etc., which may reduce the efficiency and activity of these bacteria and/or loss of efficiency of cells fully. Based on widespread research activities conducted in the field of study of the effects, the results obtained in this regard are as follows:

In 19th century and onset of industrialization period, increase of SO2, NOx and acidity PH at rain drops, which is a bed for P. Syringae, has increased. In other words, with increasing industrialization in the nineteenth century, SO2, NOX, PH acid rain as a bed for P.syringae has increased. Considering that these bacteria are able to survive, consistencies have created for them as a result of natural selection.

PH effect is evident at high temperatures and weaker effects have been reported on them at the temperatures of about -8°C to -10°C. The researchers observed that the number of INA bacteria reduces at the temperature of -4°C versus acidic PH greatly while it would be ineffective at the temperature of -9°C. PH acid operates with denaturation of the protein complex and binding rate and accumulation of ice nucleation specifies efficiency and performance of ice nucleation. Protein complexes are seen larger at warmer temperatures and smaller complexes are seen at colder temperatures as well and more effects are operated on larger complexes. Not only PH acid affects live cells but also it affects non-living bacteria such as bacteria which have turned into the form of dry ice and/or a part of membrane with IN properties. Then, effect of PH acid on combined living cells has direct effects on the structure of INP protein and also indirect impacts on metabolism of bacterium. [10].

In contrast, considerable effect has not been reported from NO2 or NO3 on strains of P. Syringae in the face of severe effects of acidity PH on INP and structure of INP will not change if exposed to these gases. Exposed to UV radiation is the other risky environmental condition for bacteria which have high impact on life of strains producing ice core especially P. Fluorescents. [11] Loss of viability is not due to the decrease in INA. Although wavelength of actual UV can cause destruction of INA, wavelength of UV-C is lethal in general and cause loss of viability and also severe increase of INA. It should be noted that this radiation is not observed at atmosphere. In general, P. Syringae bacteria are so strong in the face of environmental conditions and their vulnerability is less. [10,11].

5. PATHOGENICITY OF ICE NUCLEATION BACTERIA

It seems that INA bacteria are not able to cause disease in human but are able to cause cooling damage at plants. There are some routes at plants that bar creation of icy embryo and protect plant against cooling and frost injuries. In this respect, it can be referred to the mechanism of supercooled water at the temperatures below zero which prevent water from freezing. The supersaturated water which increases freezing temperature of water as a result of solution of sugar compounds, negative water suction at plant is created as a result of movement of water from root towards upper parts and/or adhesiveness of water molecules has been increased as a result of solution of compounds, based on which, creation of icy crystals is delayed [3].

But surface of leaves are considered as one of the most important ecosystems of ice nucleation bacteria in a way that leaves' freezing point depends on a number of bacteria existing on their surface. P. Syringae is able to produce phytotoxins which increase severity of plant diseases as a virulence factor of bacteria. The created disorders can be related to the necrosis of leaves and consequently, syringomycin and syringopetin phyto-toxins are created. Moreover, the disorders can be in the form of chlorosis which has been created by cronatine, fazeolotoxin and tabtoxin. As mentioned in above, these phytotoxins are not considered as main pathogenicity factor but they can affect severity of the injury to a great extent. [12]

6. APPLICATION OF P. SYRINGAE BACTERIA

P. Syringae is the factor of genesis of necrosis and chlorosis at leaves and are known as ice nucleation bacteria. P. Syringae has the capability of producing INP secreted protein as well as creating ice crystal at the temperatures between -2°C to -3°C. These INPs are at the focal attention due to several reasons. Among their applications, it can be referred to the production of highquality frozen products, production of snow, production of live vaccines, production of bio catalysts, etc [1, 7-9].

Since any adverse or pathogenic effects of INPs have not thus far been reported in human, it seems that these bacteria do not produce any toxicity for human. On the other hand, these INPs have been used in difficult tasks.

For example, INPs can be turned into lyophilized forms which do not perish during sterilization process and no change will be observed in number of cells as well.

Separation of external membrane of P. Syringae is the other method and/or ice nucleation proteins can be separated for being used as ice nucleation. The complete cell generates core at the temperature of -3°C to -4 °C and pierces of membrane carry out this operation at the temperature of -6°C to -8°C. [13]

Converting supercooled water to frozen water at food industries is one of the most important applications of bacteria P. Syringae and consequently, freezing quality of food products is increased using this method. [1]

The second application of bacteria P. Syringae is in the ice spray technology which was developed by Woerpel in 1980. In this study, he showed that P. Syringae is able to produce snow and in 1985, he showed that using inducers of Snowmax, which are powders of cells of bacteria P. Syringae, have been turned into dry-ice form and were used as the most efficient producer of ice core in supercooled clouds. [3, 13]

Among other applications of P. Syringae, they are used as copying report systems and Lindgren pointed to the said issue for the first time in 1989.

Copying and overwriting activity of a promoter is fused inside gene producing ice nucleation and can be studied by measurement of INPs. It should be noted that INP bacteria are applied in ammunition as well. Warren and Wolber in 1988 reported that ice nucleation genes are entered inside bacteriophages special of Salmonella and these bacteriophages with fluorescent green color are added to the food samples containing Salmonella. After incubation, Salmonella is observed, samples are frozen and are observed in non- fluorescent green color [13].

The last important application of INP (Ice Nucleation Protein) is in a surface display system. Explanation of external proteins on surface of cell is called “Surface display protein”[14].

Production of live compound vaccines, screening peptide library, production of antibody library, etc. are the other application of this method. [2, 9, 14 and 15] which is due to the attachment of P. Syringae protein to GPI surface protein.

7. DISCUSSION AND CONCLUSION

As bacteria catalyzing ice at the temperatures lower than that of temperature of supercooled water and usually at the temperature of -1°C to -3°C, ice nucleation bacteria are able to creation ice embryos which provide a pattern for the creation of ice crystals. To date, different strains have been identified that can carry out the said activity and are divided into three parts: I, II and III. It should be noted that these three groups of I, II and III carry out their catalyst operation at the temperatures of -3°C to -5°C, -5°C to -7°C and -7°C to -10°C respectively.

The genes are operating as codes for INA ice nucleation protein and phenotypes of this activity are called “Ice^{+}”. With producing plant pathogens and phytotoxins, these bacteria cause production of chlorosis and necrosis at plants but have not any toxicity effects in human. Nowadays, these bacteria have vast application in industrial fields such as in food industries, biotechnology and agriculture, etc.

Intervention of these bacteria in cycle of bio free falls is their most important application which is a significant factor for improving weather conditions and overcoming phenomenon of globalization warming. [16]

With producing products such as “Snomax”, these bacteria can be able to produce faked snow. It should be noted that “Snowmax” is the bacterial cell that has either been completed or destructed and have been turned into a dry ice form. It is worth mentioning that this product is usually used in ski resorts for reducing snow- related cost and energy.

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