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MINI-REVIEW

N. Cochet · P. Widehem

Ice crystallization by *Pseudomonas syringae*

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Abstract Several bacterial species can serve as biological ice nuclei. The best characterized of these is *Pseudomonas syringae*, a widely distributed bacterial epiphyte of plants. These biological ice nuclei find various applications in different fields, but an optimized production method was required in order to obtain the highly active cells which may be exploited as ice nucleators. The results presented here show that *P. syringae* cells reduce supercooling of liquid or solid media and enhance ice crystal formation at sub-zero temperatures, thus leading to a remarkable control of the crystallization phenomenon and a potential for energy savings. Our discussion focuses on recent and future applications of these ice nucleators in freezing operations, spray-ice technology and biotechnological processes.

Introduction

At least ten species of bacteria are known to include ice nucleation-active strains (Ina^+). Hence, not all strains within a species are active as ice nuclei. Among all these aerobic, Gram-negative bacteria, the most abundant and widely distributed Ina^+ bacteria appear to be strains of the epiphytic plant pathogen *Pseudomonas syringae*, which is one of the most active Ina^+ strains (Hirano et al. 1978, 1985; Paulin and Luisetti 1978; Li et al. 1997). For these reasons, *P. syringae* was chosen as a model for biological ice-nucleating agents. In this review, its role in ice crystallization and its expected applications are discussed.

P. syringae is able to synthesize a membrane protein that confers on cells the ability to catalyze ice formation at warm, sub-freezing temperatures (−2 °C to −4 °C; Maki et al. 1974). The structure of the protein involved has been elucidated (Turner et al. 1991). The ice-nucleating activity (INA) depends on an outer membrane lipoglycoprotein, INaZ, which could be linked to the membrane by a glycosylphosphatidylinositol (GPI) anchor (Govindarajan and Lindow 1988a; Kozloff et al. 1991a, b) and is able to mimic the structure of an ice nucleus, acting as a template for ice formation. Nucleation threshold temperature and nucleation frequency of Ina^+ bacteria depend upon the dynamic assembly of multiple, membrane-bound aggregates of ice-nucleating proteins. In a recent study, Palaiomylitou et al. (1998) assumed the involvement of phosphatidylethanolamine rather than phosphatidylinositol (PI).

As bacterial ice nuclei are active at widely varying frequencies and temperatures, this variation has been used for defining three types of bacteria in the same population a function of their INA (Yankofsky et al. 1981). These sub-populations of nuclei have been termed types I, II and III, with threshold ice-nucleating temperatures in the ranges −2 °C to −5 °C, −5 °C to −7 °C and −7 °C to −10 °C respectively. The most active ice-nucleating structures possess the INaZ protein linked to mannose, probably as a mannan, glucosamine or PI complex. It seems that only 10^{-6} bacteria of a population possess the entire and most active ice-nucleating structure (type I). The other bacteria have the INaZ protein, but not as the GPI complex. More recently, Turner et al. (1990) distinguished three classes by freezing-difference spectra in D_2O versus H_2O: class A is active above −4.8 °C, class B between −4.8 °C and −5.7 °C, and class C is active at −7.6 °C or colder.

Numerous studies have confirmed the ice-nucleating site to be a flat disk, 300 Å in diameter, containing 53 INaZ proteins (Green and Warren 1985; Warren et al. 1986; Govindarajan and Lindow 1988b). Kozloff et al. (1984, 1991b) proposed that there is a molecular maturation by a stage of glycosylation and anchorage of INaZ proteins to the outer membrane. Thus, type III ice nuclei play a precursor role for the formation of type II

N. Cochet (✉) · P. Widehem
Université de Technologie de Compiègne,
Département Génie Biologique, BP 20,
529-60205 Compiègne, France
e-mail: nelly.cochet@utc.fr
Tel.: +33-344-234454
Fax: +33-344-235276

and type I ice nuclei; and it seems therefore that this maturation may be inducible by culture conditions (Rogers et al. 1987; Ruggles et al. 1991).

Three attempts have been made to predict the spatial structure of the site. Warren et al. (1986) proposed a model describing the secondary structure of the non-repetitive N- and C-terminal domains as α-helices and β-sheets, whereas the repeating domains were predicted as alternating β-sheets and random-coil regions. To predict the 3-D structure, they proposed two models, having triangular and hexagonal shapes. Mizuno (1989) neglected the two higher orders of periodicity found in the ice-nucleating protein and assumed a helicoidal structure. Nevertheless, a more likely 3-D structure was proposed by Kajava and Lindow (1993) as an arrangement of stacked β-hairpins.

As these microorganisms are already used in snow-making (Woerpel 1980) and currently have potential applications in the production of frozen foods (Watanabe and Arai 1987; Watanabe et al. 1989) and in freezing processes like freeze-drying, cryopreservation and freeze-concentration, the production of these biological nucleating agents must be optimized at low cost. These applications need a strategy for optimizing the ice-nucleating activity and for developing a large biomass production process (Lawless and LaDuca 1992).

Ice-nucleating activity

Ice crystallization occurs after breakdown of supercooling. For pure water, the degree of supercooling may be around −40 °C. Crystallization starts either when suitable conditions are reached for the aggregation of a group of water molecules into particles of a certain order, called ice nuclei of crystallization (homogeneous crystallization), or when solid particles serve as sites for crystal formation (heterogeneous crystallization). Many organic and inorganic substances are well-known ice nucleators: silver iodide (Vonnegut 1947), kaolinite, cholesterol, amino acids. Biological ice nucleators are also well established; and among these, Ina^+ bacteria are the most active (Vali 1995).

Different methods may be used to study the INA of *P. syringae* but the most generally used is the drop-freezing assay described by Vali (1971).

Bacterial cells were suspended in distilled water or in phosphate buffer and were serially diluted. Forty droplets (10 µl each) of these dilutions were placed individually on a paraffin-coated aluminium foil sheet which was then floated on the surface of a bath being progressively cooled (from 0 °C to −10 °C). The number of drops that froze within 5 min for a given temperature was recorded. The proportion of drops frozen was evaluated at each temperature step and the qualitative aspect of INA (type I, II or III) was expressed as the temperature at which 90% of the drops were frozen (T_{90}). The numbers of ice nuclei per milliliter [$n(T)$] were calculated by the equation of Vali (1971) and normalized for the number of cells present in each suspension, in order to estimate nucleation frequency (FN) in logarithm units of ice nuclei/cells): $FN = n(T) \times 10^D \times C^{-1}$. The cumulative number of ice nuclei per unit volume is: $n(T) = -\ln(f) \times V^{-1}$, where f is the fraction of droplets unfrozen at temperature T, and V is the volume of each droplet used (0.01 ml).

$n(T)$ in each dilution was normalized to the concentration in the original suspension (10^D). FN is the fraction of cells active at a given temperature and is determined as the number of ice nuclei/ml [$n(T)$] divided by the cell density (C) in cells/ml.

Heterogeneous and homogeneous crystallization have also been studied by calorimetry. Differential scanning calorimetry (DSC) is a well-known method for determining crystallization temperature at the onset of the freezing peak, using a DSC thermogram. The ice-nucleating activity of *P. syringae* in microsize droplets dispersed within emulsions has already been studied by DSC (Franks et al. 1983; Charoenrein and Reid 1989; Clausse et al. 1991; Ozilgen and Reid 1993). This is an easy and rapid method for determining the freezing and melting points of microsized liquid samples. The thermodynamic and kinetic aspects of ice nucleation by bacteria have to be well known in order to determine the temperature at which the freezing will surely occur.

Optimization of INA

The nucleation efficiency of *P. syringae* depends on the cell concentration. If the cell concentration is high, the temperature required for nucleation will also be high. However, at very high cell concentrations ($>10^6$ cells/ml), nucleating activity becomes independent of the cell concentration (Maki et al. 1974).

Moreover, economically viable applications of Ina^+ bacteria require that the ice-nucleating frequency of type I nuclei be as high as possible. Consequently, the composition of the medium and the environmental parameters of the culture must be studied.

Composition of the culture medium

The most effective INA is expressed in the late log-phase and at the beginning of the stationary phase (Lindow et al. 1982; Hirano et al. 1985; Deininger et al. 1988; Luquet et al. 1991; Pooley and Brown 1991). The aim of several works was to adapt the composition of the growth medium in order to obtain a high biomass production in the late growth phase, characterized by a higher proportion of type I bacteria in the population (Hendricks et al. 1987; Lynn and Noto 1987; Lawless and Laduca 1992; Nemecek-Marshall et al. 1993).

Enhanced INA was obtained with either glycerol (Lindow et al. 1982) or sorbitol (Nemecek-Marshall et al.

1993) as the carbon source. Nutritional starvation for nitrogen, phosphorus, sulfur or iron was showed to lead to highly active nuclei (Nemecek-Marshall et al. 1993).

Batch cultivation of *P. syringae* has shown that increasing growth rates leads to decreased INA (Blondeaux and Cochet 1994b).

Programmed fermentation strategies were also designed with the idea that the Ina^+ phenotype might be related to nutrient-limited conditions existing on leaves (Lawless and LaDuca 1992). Nemecek-Marshall et al. (1993) noted high INA in cells grown with both low nitrogen and low phosphorus levels.

Components involved in the stepwise assembly of the ice-nucleating site (inositol, mannose, glucosamine and Mn^{2+} ions) might also be added to the culture medium, as they maximize the INA (Turner et al. 1990; Kozloff et al. 1991a, b). In this respect, we looked for a natural substrate, wheat bran, containing a PI precursor as an essential component of the complex (Blondeaux and Cochet 1994a).

In all of the experiments we performed with inorganic phosphate-starved preculture and culture media containing a precursor of myo-inositol (phytic acid, phytate and wheat bran), we obtained increased INA.

Moreover, the influence of wheat bran, a cheap agro-food byproduct, seems to be related to the number of type I structures in the population, leading to 1000-fold more of this type of cell.

Environmental factors

It was observed that both the frequency of ice nucleation by the cells and their ice nucleation temperatures were profoundly affected by the in vitro culture conditions (Lindow et al. 1982; Lindow 1983). As related by O'Brien and Lindow (1988), the INA is generally higher when *P. syringae* cells were grown on plants rather than when they were grown in vitro.

In this context, since *P. syringae* is a natural inhabitant of plants, it is reasonable to assume that INA is influenced by environmental factors such as atmospheric hygrometry. Dickinson (1986) showed the importance of a high relative humidity for the growth of *P. syringae in planta*, while Hirano and Upper (1989) found a higher growth and INA when the atmospheric hygrometry was low.

In a recent study, we focused on the relationships between hydrophobic environment, water activity of the culture medium and INA (Blondeaux et al. 1999). It was interesting to notice that addition of a vegetable oil characterized by its high content in low fluidity fatty acids (olive oil) led to the highest INA. Govindarajan and Lindow (1988a) and Lindow (1995) have already pointed out that highly fluid lipids were less effective in reconstituting INA in delipidated bacterial membranes than lipids having a lower fluidity. In our study, silicone oil was also shown to act both as an a_w-depressor and as an INA-improving agent.

From a general point of view, we observed the enhancement of INA under water-stress culture conditions.

Temperature was also shown to have a profound impact on the induction of type I ice nuclei (Gurian-Sherman and Lindow 1995). A rapid shift of stationary-phase *P. syringae* from 32 °C to 14–18 °C was found to lead to an increased expression of type I ice nuclei (Lawless and LaDuca 1992; Nemecek-Marshall et al. 1993). A recent patent (Sung et al. 1994) selected a deregulated mutant able to grow and to synthetize active ice nuclei at 30 °C.

Kozloff et al. (1983) established the pH range for optimal INA expression (pH = 5.0–9.2); and Turner et al. (1990) proved that the optimal activity for the three population classes A, B and C is obtained at pH = 6.5.

Application of *P. syringae* as an ice nucleator

Treatments of *P. syringae* for further applications

For any food application using bacteria, containment or sterilization of the active cells is required. With this aim in view, various physical treatments were tested on different biological ice nuclei (*Xanthomonas campestris*, *Erwinia ananas*, *E. herbicola*, *P. fluorescens* and *P. syringae*; Table 1).

Concerning *P. syringae*, Zamecnick et al. (1991) prepared entrapped cells in alginate beads for use as ice nucleators in the cryopreservation of plant material.

As heat treatment resulted in the degradation of the cellular membrane and a suppression of activity, different alternative sterilization techniques were examined. Surprisingly, Lee (1990) obtained unchanged activity when cells of *P. syringae* were subjected to microwave heating for 3 min.

Low-dose beta irradiation of freeze-dried cells is routinely performed either to reduce viability or to obtain a sterile product, with minimal loss of activity (LaDuca et al. 1995).

Lyophilization processes provide a good storage method for biological ice-nucleating agents. No change in cumulative active ice nucleus concentration at any given temperature was observed during the lyophilization of *P. syringae* (Govindarajan and Lindow 1988b). Cells can therefore be stored for several months in a dried state and then used as a source of ice nuclei (LaDuca et al. 1995).

Isolation of ice-nucleating protein has also been considered, but ice-nucleating protein has less activity than the whole cell or outer membrane of *P. syringae* (Lindow 1982). For that purpose, membrane fractions were prepared (Sprang and Lindow 1981) and tested (Kim et al. 1989; Luquet et al. 1991). Whole cells were active at −3 °C to −4 °C and the membrane fractions were active at −6 °C to −7 °C.

Pantoea ananas is an Ina^+ bacteria well known for its ability to release membrane vesicles into the culture

Table 1 Physical treatments applied to different ice nuclei species leading to the preservation of ice-nucleating activity. *E*, *Erwinia*; *P*, *Pseudomonas*; *X*, *Xanthomonas*; *ND* data not determined or not shown, – viability reduced but data not shown

Treatments	Species	Viability	References
High pressure	*X. campestris*	0%	Honma et al. (1993)
	E. ananas	0%	Watanabe et al. (1991)
β-Irradiation	*P. syringae*	–	LaDuca et al. (1995)
Microwave	*P. syringae*	–	Lee (1990)
UV radiation	*P. syringae*	0%	Turner et al. (1991)
Lyophilization	*P. syringae*	ND	Govindarajan and Lindow (1988b); LaDuca et al. (1995)
Entrapment	*X. campestris*	ND	Watanabe et al. (1996)
	E. ananas	ND	Watanabe et al. (1989)
	P. syringae	ND	Zamecnick et al. (1991)
Vesicles	*E. herbicola*	ND	Phelps et al. (1986)
(ECIN)	*E. herbicola*	ND	Li and Lee (1998)
	Pantotea ananas	ND	Zasypkin and Lee (1999)
	P. fluorescens	ND	Obata et al. (1993)
Active membrane fractions	*P. syringae*	ND	Sprang and Lindow (1981); Kim et al. (1989)

medium (Phelps et al. 1986). Addition of these extracellular ice nucleators to liquids elevated the ice nucleation temperature and promoted freezing (Li and Lee 1998; Zasypkin and Lee 1999). If extracellular ice nucleators could be prepared from *Pseudomonas syringae* culture, it would be more active than those coming from *Pantoea ananas* and would solve the problem of adding whole living bacteria to food products.

However, no adverse effect on humans is currently known for bacteria ingested in the amounts likely to be present in such foods; and Lee (1990) proposed that *Pseudomonas syringae* may be considered a non-toxic microorganism.

Application to freezing operations

Application to frozen food

Freezing operations have to be carried out at temperatures well below those at which food products would theoretically be expected to freeze, in order to avoid problems of supercooling. When supercooling occurs, the moisture present in solid foodstuffs and biological products leads to a failure of the process, diminishing the organoleptic and textural properties of the frozen food. It has led food freezing practitioners to operate plants at temperatures as low as −40 °C, which results in very high energy costs.

For improving the quality of frozen foodstuffs, the freezing may be facilitated by applying *P. syringae* as an ice-nucleating agent. This approach was tested by Ryder (1987, cited by Li and Lee 1995), who showed the impact of decreasing the degree of supercooling on the quality of frozen salmon muscle.

We studied the impact of adding *P. syringae* cells to liquid broth; and the results obtained after the addition of *P. syringae* cells were compared with two references: sterile culinary broth and culinary broth with *P. putida* as an inactive bacterium. DSC analysis was used as the first step in determining the thermodynamic parameters for a culinary broth chosen as a real food system (Widehem et al. 1998).

Addition of *P. syringae* in broth has a significant effect on the crystallization phenomenon (Table 2). The melting point (T_m) of culinary broth is around −0.6 °C for the three conditions tested, but the crystallization temperature (T_c) is markedly higher in the presence of *P. syringae* ($T_c = -3.1$ °C, while $T_c = -17.5$ °C and −16.2 °C for the references).

From these results, *P. syringae* may lead to three main impacts on the crystallization of broth: (1) initiating the crystallization phenomenon at sub-zero temperatures, (2) lowering the degree of supercooling and (3) decreasing the dispersion of values.

The standard errors obtained by DSC analysis were ±4.1 °C and ±4.5 °C with sterile broth and *P. putida* respectively, but were only ±0.5 °C with *P. syringae*. The ice crystallization event is a well-known uncertain phenomenon when it is carried out without nucleating agents. When added to aqueous solutions, cells of *P. syringae* are able to exert accurate control of the nucleation, which is the key step of the crystallization phenomenon ($Tc = -3.1 \pm 0.5$ °C).

Carrying out time–temperature profiles is the second part of the study (Widehem et al. 1999a). The freezing

Table 2 Freezing (T_c) and melting (T_m) temperatures and degree of supercooling (ΔT) for culinary broth, determined by calorimetric analysis. All values are given in °C (with SE results from an average of 20 samples for each condition)

		Sterile broth	Broth with *P. putida*	Broth with *P. syringae*
T_c	T_{min}	−7.0	−7.9	−2.2
	T_{max}	−22.1	−22.1	−3.4
	$T_{mean} \pm$ SE	−16.2 (±4.1)	−17.5 (±4.5)	−3.1 (±0.5)
T_m	T_{min}	0.0	0.0	0.0
	T_{max}	−1.3	−1.7	−1.5
	$T_{mean} \pm$ SE	−0.6 (±0.4)	−0.8 (±0.4)	−0.6 (±0.4)
$\Delta T \pm$ SE		15.6 (±4.2)	16.8 (±4.3)	2.3 (±0.6)

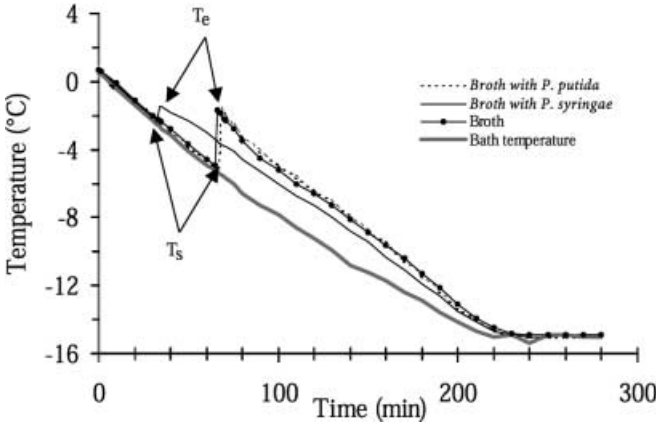

Fig. 1 Time–temperature profiles during freezing for three samples of culinary broth. *P*, *Pseudomonas*

point (T_e) for the three samples of culinary broth is around −2 °C, but the supercooling break (T_s) is different for treated and control samples: −2.5 °C and −5 °C respectively (Fig. 1). This means that the degree of supercooling was reduced by 2.5 °C in the case of *P. syringae* addition (Table 3).

With salmon muscle, Ryder (1987, cited by Li and Lee 1995) also showed a decrease in total freezing time. At a temperature of −5 °C, freezing time was reduced 33% by adding Ina^+ cells.

The total freezing time of distilled water containing crude membrane fractions from *P. syringae* was reduced by 57%, as compared with untreated distilled water (research cited by Li and Lee 1995). Similar results were obtained by Lee (1990) on fish fillets and meat steaks.

Moreover, *P. syringae* may improve the stability of the frozen product during cold storage, by enhancing the formation of small crystals. These small crystals do not cause membrane deterioration or degradation of the product texture.

Therefore, *P. syringae* may be successfully applied to freezing processes with: (1) supercooling reduction, (2) high freezing temperature, (3) crystallization control, (4) freezing time reduction, (5) improved stability and consequently (6) decreasing the energy costs.

Application to the freeze-concentration process

The freeze-concentration process is based on the partial solidification of water into ice within a fluid product, followed by the removal of the solid ice phase from the concentrated liquid phase (Thijssen 1970). This process is characterized by minimal loss of volatile flavors and aromas, resulting in a high quality product. Already used in the coffee, vinegar, juices and wine industries, further advances in the technology are required to reduce operating costs and to make the process more competitive with evaporation and reverse osmosis.

In the freeze-concentration process, crystal size appears to be an essential parameter for ensuring process efficiency. Thus, large and symmetrical crystals are easier to separate by mechanical means (Karel 1975). Very high supercooling can create a large number of smaller new crystals, whereas lower rates of supercooling are desirable in order to prevent excessive nucleation (Deshpande et al. 1984). Several authors have shown that supercooling was limited when *P. syringae* was added to the solution, but there are few data on its application to freeze-concentration processes (Table 4). Lee and Song (1995) have carried out the concentration of soy protein isolate and apple juice with the addition of a *P. syringae* cell suspension. In a recent study, we pointed out the limitation of supercooling in a culinary broth and sucrose solutions when *P. syringae* cells were added (Widehem et al. 1999a, b).

However, at a temperature of −6 °C, we noted that 100% of the sucrose solution samples froze after the addition of *P. syringae* cells, compared with only 50% of the reference solutions. These results confirm those published by Lee and Song (1995), who noticed that apple juice samples did not freeze at −5 °C, while they were entirely frozen within 10 s after the supercooling was broken by *P. syringae* cells. As the addition of

Table 3 Supercooling (T_s), equilibrium ice/liquid water temperature (T_e) and degree of supercooling ($\Delta T = T_s - T_e$) for culinary broth determined from freezing curves. All values are given in °C (with SE results from an average of ten samples for each condition)

		Sterile broth	Broth with *P. putida*	Broth with *P. syringae*
T_s	T_{min}	−4.9	−4.0	−2.1
	T_{max}	−5.8	−5.5	−2.8
	$T_{mean} \pm$ SE	−5.1 (±0.3)	−4.9 (±0.5)	−2.4 (±0.2)
T_e	T_{min}	−1.5	−1.4	−1.3
	T_{max}	−1.9	−2.9	−2.1
	$T_{mean} \pm$ SE	−1.7 (±0.2)	−2.0 (±0.5)	−1.6 (±0.3)
$\Delta T \pm$ SE		3.2 (±0.5)	2.9 (±0.6)	0.8 (±0.2)

Table 4 Freeze-concentration parameters obtained with and without *P. syringae* as ice nucleators

Material	Solids content (%)	Freezing temperature (°C)	Concentrate content (%)	Ice nucleators	References
Apple juice	10.0	−4.0	30.0	Small ice crystals	Thijssen (1970)
	11.5	−2.2	15.2	*P. syringae*	Lee and Song (1995)
Sucrose solution	10.0	−6.0	21.0[a] Not frozen[a]	None	Widehem et al. (1999b)
			21.0	*P. syringae*	

[a] 50% of the samples

P. syringae leads to a remarkable decrease in supercooling, the crystals formed in the liquid food may be larger and may be used as ice nucleators to replace the seeds used in conventional processes during heterogeneous nucleation.

The reduction of the degree of supercooling by Ina^+ bacterial cells and/or their products implies that there is great potential for shortening freezing times, reducing refrigeration costs and making production more efficient (Li and Lee 1995).

Application to spray-ice technology

Woerpel (1980) was the first to suggest the application of *P. syringae* in snow-making. Then, in order to satisfy the growing demand, the commercialization of *P. syringae* started in 1985 with the development of a snow inducer (Snowmax; Genencor, Rochester, USA). This product is a freeze-dried powder containing *P. syringae* cells and facilitates the efficient production of snow at temperatures as high as 1.1 °C.

Artificial ice nuclei, such as silver iodide, are currently introduced through conventional weather modification techniques in order to enhance the precipitation process.

Since most naturally occurring ice nuclei are not active at sub-zero temperatures, there is interest in searching for ice nuclei active at the lowest degree of supercooling. Interestingly, Ina^+ *P. syringae* appears to be an aerosol-stable microorganism under a range of environmental conditions (Constantinidou et al. 1990).

For this reason, *P. syringae* inoculation was introduced to induce precipitation from supercooled clouds, and was shown to be the most efficient ice-nucleating agent. Ward and Demott (1989) have shown that the Snowmax snow inducer can be easily dispersed to initiate nucleation in naturally supercooled clouds. The activity of Snowmax with little supercooling is greater than the activity of silver iodide aerosols.

Man-made ice ponds equipped with pumping systems and a heat exchanger are used as natural thermal storage systems, replacing the conventional chlorinated fluorocarbon chiller and associated cooling tower system. The application of a *P. syringae* nucleating agent dramatically reduces both power and energy requirements, thus improving the economic feasibility of such plants (LaDuca et al. 1995).

Since many inorganic substances are preferentially excluded from ice crystals during the freezing process, natural thermal storage may also be used for water purification. The addition of *P. syringae* in these ponds should enhance the efficiency of the system.

In arctic regions, fabricating ice stuctures such as aircraft runways or roads from seawater can be initiated without ice nucleants, but only when temperatures are sufficiently low to cause rapid freezing of the seawater. Owen et al. (1987) have shown that *P. syringae* can be successfully applied to effectively reduce supercooling in sprayed seawater streams. Rapid freezing of large amounts of seawater have been obtained, resulting in enhanced efficiency of ice formation and extended temperature ranges for ice construction applications.

Application to biotechnological processes

The use of an ice-nucleating gene as a transcriptional reporter was first explored by Lindgren et al. (1989). INA reporters differ from conventional reporters in the detected signal, which is not due to enzymatic catalysis but to a physical phenomenon. The transcriptional activity of a promoter fused to a promoterless ice-nucleatign gene can be assessed by measuring INA. Compared to conventional reporters, the speed and simplicity of INA assay and its high sensitivity make the use of INA reporters attractive. This system provides a useful molecular tool for promoter activity and gene expression studies (Arvanitis et al. 1995; Drainas et al. 1995; Panopoulos 1995); and it becomes extremely useful in studies evaluating in situ gene expression by bacteria inhabiting natural environments (Loper and Lindow 1997).

Bacterial ice-nucleating assay

Bacterial INA has been applied in immunoassays (Warren and Wolber 1988), and in the bacterial ice-nucleating assay for *Salmonella* developed by Wolber and Green (1990a, b) and Gutterson et al. (1993). The ice-nucleating gene is incorporated in a *Salmonella*-specific bacteriophage. The bacteriophage (together with a green fluorescent dye) is then added to test samples of food that may contain *Salmonella*. After incubation to allow the formation of the ice-nucleating proteins, the test samples are chilled to −5 °C. When *Salmonella* is present, the sample freezes and the green dye becomes dark and non-fluorescent.

The total time for the test does not exceed 4 h, compared with 2–5 days required by the currently used method.

Surface display system

The expression of foreign proteins on the cell surface, called the surface display system, has recently gained much attention because of its biotechnological potential. It can be used in producing recombinant live vaccines, screening peptide libraries, constructing antibody libraries, bioconverting whole cells with enzyme-coated microbes and producing whole cell adsorbents (Georgiou et al. 1997). A novel surface display system was recently proposed using the ice-nucleating protein of *P. syringae*, which is known to be a GPI-anchored surface protein (Kozloff et al. 1991a, b; Turner et al. 1991; Wolber 1993).

It has also been demonstrated that a single DNA fragment from *P. syringae* confers Ina^+ phenotype (INP) to *Escherichia coli* (Wolber et al. 1986). These results indicate that INP itself has the necessary secretion and anchoring signals.

In a recent study, Jung et al. (1998) reported the cloning of the *inaK* gene and its expression in *E. coli*, using carboxymethylcellulase (CMCase) as a reporter enzyme fused to the C-terminal of INP. CMCase activity was mainly detected on the cell surface, whereas no enzyme activity was detected in the culture supernatant. As the INA was maintained, it was also proved that the fusion protein is functionally expressed. It was proved that the INP could be secreted and targeted to the outer membrane of *E. coli*.

That new anchoring system may be very useful for the design of a novel bioconversion system with more stable cell surface-anchored enzymes, thereby introducing a novel in vivo immobilization technique.

Conclusion

Nutritional and environmental signals seem to be the key physiological effectors required for optimal expression of INA in the bacterium *P. syringae*.

The major interest in *P. syringae* as an ice nucleator is its ability to minimize supercooling of aqueous solutions and facilitate ice crystal formation at high sub-zero temperatures, thereby elevating nucleation temperatures and reducing the amount of energy required to complete the freezing process.

Related to the enhancement of the formation of small crystals, an improvement in the quality of the final product was also indicated, both in snow-making applications and in solid food-freezing processes.

Concerning food applications, food-grade ice nucleators have to be prepared. With the advancements of recombinant DNA technology, the ice-nucleating gene could be cloned in edible food-grade microorganisms like *Lactobacillus* or yeasts (Hwang et al. 1998). However, studies are still needed for the preparation of extracellular ice nucleators from *P. syringae* culture, similar to those already made from *Pantoea ananas* cells.

Concerning liquid foods, it was shown that *Pseudomonas syringae* can provide interesting advantages for the freeze-concentration process. Not only applied just to food products, this process is also currently under development for the recovery of hazardous traces during wastewater treatment (Ruemekorf 1994). Since the supply of drinking water appears to be a major concern all over the world, the development of new purification strategies is needed. For that purpose, seawater treatment and the separation of hazardous traces would be studied as priority subjects. The discussion presented here indicates that *P. syringae* could be applied to freeze-concentration wastewater treatment and could play a significant role in enhancing the treatment yield.

Whatever the application (freezing operations, freeze-concentration processes, spray-ice technology), cells of *P. syringae* appear to be highly active ice nucleators. Since the crystallization of ice is well known to be an uncertain event, it is shown that the initiation of the crystallization of liquid water into solid ice happens at a reproducible temperature after the addition of *P. syringae*.

Moreover, ice-nucleating genes from *P. syringae* have been cloned in *E. coli* and other hosts. These genes appear to be new tools for molecular biology studies, especially as reporter genes. They also allow the development of a new surface display system, providing great scope for exploring their biotechnological applications.

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