Expression of a Bacterial Ice Nucleation Gene in a Yeast *Saccharomyces cerevisiae* and Its Possible Application in Food Freezing Processes

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A 3.6 kb ice nucleation gene (*ina*) isolated from *Erwinia herbicola* was placed under control of the galactose-inducible promoter (*GAL*1) and introduced into *Saccharomyces cerevisiae*. Yeast transformants showed increased ice nucleation activity over untransformed controls. The freezing temperature of a small volume of water droplets containing yeast cells was increased from approximately −13 °C in the untransformed controls to −6 °C in *ina*-expressing (Ina^+) transformants. Lower temperature growth of Ina^+ yeast at temperatures below 25 °C was required for the expression of ice nucleation activity. Shift of temperature to 5−20 °C could induce the ice nucleation activity of Ina^+ yeast when grown at 25 °C, and maximum ice nucleation activity was achieved after induction at 5 °C for ∼12 h. The effects of Ina^+ yeast on freezing and texturization of several food materials was also demonstrated.

Keywords: *Nucleation; activity; transformants; yeast; cells*

INTRODUCTION

Freezing is one of the best long-term preservation techniques used in the food industry. It is used for many agricultural commodities as well as processed foods (*1*). A typical freezing process consists of undercooling, ice nucleation, ice crystal propagation, and maturation, in which ice nucleation is the critical step preceding complete solidification and affecting the quality of frozen foods (*2*).

Bacterial ice nucleators, which primarily are present in certain Gram-negative bacterial strains of *Erwinia*, *Pseudomonas*, and *Xanthomonas*, have the ability to trigger ice formation in supercooled water at temperatures of −2 to −10 °C (*3*). Ice nucleation protein, a unique membrane-associated protein encoded by a single ice nucleation gene (*ina*) (*4*), has been demonstrated as the major component of bacterial ice nucleators. In general, it is believed that ice nucleation proteins promote ice nucleation by mimicking the structure of an ice crystal lattice and lowering the energy required for the initiation of ice formation (*5*).

Most of the known ice nucleation-active bacteria are either plant pathogens or plant epiphytes, and the ubiquitous presence of these bacteria on a plant surface makes them the principal cause of frost damage to plants (*6*). Because of their high ice nucleation activity (INA), ice nucleation-active bacteria, in particular *Pseudomonas syringae*, are currently used commercially for artificial snow-making (*7*), immunoassays (*8*), and reporter gene systems (*9*). They have also shown potential for applications in freezing concentration, freezing dry, and texturization of frozen foods (*10*−*13*). However, due to their plant pathogen nature, the application of bacterial ice nucleators in the food industry will face various safety, toxicity, and regulatory constraints. Expressing the *ina* gene in nonpathogenic and consumer-safe microbial hosts is one way to overcome these constraints, and *Saccharomyces cerevisiae*, a generally regarded as safe (GRAS) yeast, provides an ideal host for the production of food-grade ice nucleators.

In this paper we describe the expression of the *ina* gene from *Erwinia herbicola* in *S. cerevisiae* and the effect of the Ina^+ yeast on the freezing process and freezing texturization of some food materials.

MATERIALS AND METHODS

Strains and Culture Conditions. *Escherichia coli* M15 harboring pQTH21 was routinely grown in an LB broth (10 g of bacto-tryptone, 5 g of bacto-yeast extract, and 5 g of NaCl, per liter) containing 100 μg/mL ampicillin and 25 μg/mL kanamycin. *S. cerevisiae* Psyl was used in transformation experiments. Yeast cells carrying plasmid NT196 were grown at 25 °C in an Ura^- medium [0.72 g of dropout powder (without uracil), 6.7 g of yeast nitrogen base without amino acids (Difco), and 20 g of glucose, per liter].

Construction of Yeast Expression Plasmids and Yeast Transformation. The manipulations of DNA were carried out primarily as described by Sambrook et al. (*14*). A 5.1 kb DNA fragment containing *S. cerevisiae* galactose-inducible promoter (*GAL*1) and *URA*3 gene was released from plasmid YEp352 by digesting with restriction enzymes *Eco*RI and *Sma*I. Another 3.0 kb *Eco*RI/*Pvu*II-treated DNA fragment was obtained from plasmid pAC55 (*15*). These two DNA fragments were ligated together to yield plasmid NT195.

The DNA fragment containing the *ina* gene of *Er. herbicola* was released from pQTH21 (*16*) by digestion with restriction enzymes *Bam*HI and *Pst*I and then subcloned into plasmid

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NT195 previously digested with the same enzymes. The resulting plasmid was called NT196. Yeast transformation was carried out with alkaline cation treatment as described by Ito et al. (*17*).

Yeast Expression Analysis. Yeast cells containing plasmid NT196 were grown in Ura$^-$ medium with 2% glucose at 25 °C to a density of 5×10^7 cells/mL. Expression of *ina* was induced by the addition of 2% galactose to half of the culture broth; the other half of the culture was used as an uninduced control. The cells were allowed to grow for an additional 4 h, and then aliquots were collected for the INA assays.

INA Assays. INA of yeast cells was determined by droplet-freezing assays (*18*, *19*). All yeast cells were kept at 5 °C overnight before assay, to obtain the maximum INA. The INA of yeast cells at a given temperature was determined as follows: Whole cultures were serially (10-fold) diluted, and 10-μL droplets from each dilution were placed on the surface of an aluminum foil dish floating in an ethanol bath set at −8 °C (or as otherwise specified). INA was calculated by the equation $\text{INA} = -\ln(f)/v \times D$, where f is the fraction of unfrozen drops at a given temperature, v is the volume of each drop, and D is the dilution factor (*18*).

Food Materials. Foods including milk, juices, and soybean curd were obtained from local markets. Sucrose, egg white protein, and starch were purchased from Sigma Chemical Co. (St. Louis, MO).

Measurement of Freezing Curves. Water or food materials mixed with Ina$^+$ yeast culture (9:1 volume ratio) at room temperature were subjected to freezing in a custom-designed freezer (Scien Tem Co., Adrian, MI) at −6.5 °C. The changes of temperature in each sample were monitored with a Type T copper−constantan thermocouple (Omega Engineering Inc., Stamford, CT), which was placed at the geometric center of a 15-mL capacity test tube and connected to a WB-AAI data acquisition system (Omega Engineering Inc.). The data acquisition system was interfaced with an IBM computer through the QuickLog PC Data Acquisition and Control software program (Strawberry Tree, Sunnyvale, CA).

Microscopic Examination of Frozen Samples. Samples (100 μL) with or without Ina$^+$ yeast cells were placed on microscope slides, covered with a thin slide cover, and placed on a cold stage, which was connected to a cooling system (model TS-4 controller system, Sensortek, Inc., Clifton, NJ). The stage was observed under a dissection microscope (×10 magnification) and photographed with a Polaroid camera. Samples were cooled at a rate of ∼1 °C/min.

RESULTS

INA Ina$^+$ Yeast. Yeast transformants harboring plasmid NT196 (Ina$^+$ yeast) exhibited significantly higher INA than untransformed yeasts (Figure 1). The expression of the *ina* gene in yeast cells was also confirmed by Western blot analysis of Ina protein (data not shown). The threshold temperature of Ina$^+$ yeast is ∼ −6 °C, and no type I ice nuclei (active at temperature > −5 °C) were detected.

Effect of Growth Temperatures on the INA of Ina$^+$ Yeast. Table 1 shows the INA of Ina$^+$ yeast cells grown separately at 10, 15, 20, 25, and 30 °C. It is obvious that growth temperature significantly affects the expression of INA, which is inversely correlated with growth temperatures. Cells cultured at temperatures of ≥25 °C had a significantly lower INA compared to cells grown at temperatures <25 °C, and the lower the growth temperatures the higher INA in Ina$^+$ yeast cells. Differences in INA related to growth temperatures have been reported in ice nucleation active bacteria. Obata et al. (*20*) studied the effects of growth temperature on the INA of *Pseudomonas fluorescens*, and they found that cells grown at 25 °C had higher INA than cells cultured at 30 °C. Similarly, *Pseudomondas viridiflava* cells grown at 25 °C could nucleate ice at −4 °C, whereas cells grown at 30 °C could nucleate ice only below −4 °C (*21*). On the basis of our results and considering the low growth rate of yeast cells at temperatures <15 °C, a growth temperature between 15 and 20 °C is suitable for cultivation of Ina$^+$ yeast cells.

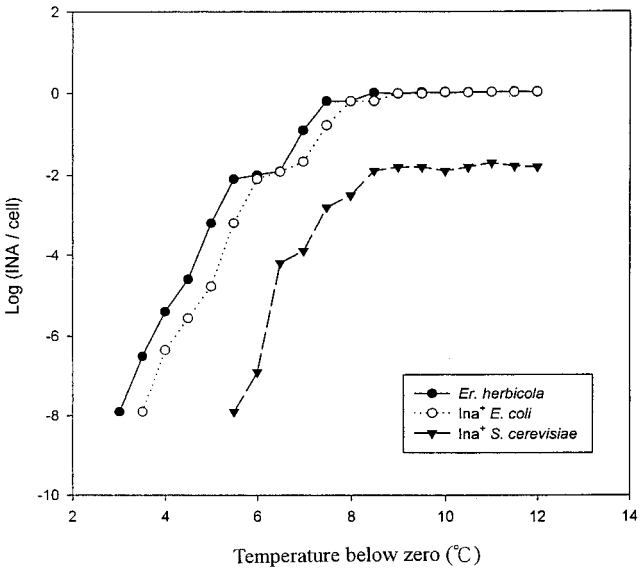

Figure 1. Cumulative ice nucleation spectra of cell suspensions of *Er. herbicola*, *E. coli* transformed with plasmid pQTH21, and *S. cerevisiae* transformed with plasmid NT196.

Table 1. Effect of Growth Temperatures on the Ice Nucleation Activity of Ina$^+$ *S. cerevisiae*[a]

	INA/cell	
growing temp (°C)	−4 °C	−8 °C
10	0	0.35
15	0	0.16
20	0	0.12
25	0	0.08
30	0	0.01

[a] Ina$^+$ *S. cerevisiae* was grown separately in Ura$^-$ medium containing 2% galactose at 10, 15, 20, 25, and 30 °C for 48 h. Aliquots of culture were taken and INA was measured at −4 and −8 °C, respectively.

Low-Temperature Induction of INA. When Ina$^+$ yeast cells were grown at 25 °C, there was a very low level of INA, as shown in Table 1. However, if these yeast cells were kept in a 5 °C refrigerator overnight, the INA significantly increased. To understand the kinetics of low-temperature induction, Ina$^+$ yeast cells grown at 25 °C were rapidly cooled to 5 °C, and the INA of aliquots of the culture broth were assayed at certain time periods. Figure 2 shows that the INA of Ina$^+$ yeast cells grown at 25 °C increases approximately linearly with time after this shift to 5 °C, and the maximum INA was achieved after induction at 5 °C for ∼12 h.

Effect of Ina$^+$ Yeast Cells on Freezing Processes. The effects of Ina$^+$ yeast cells on the freezing process of water and milk were studied (Figure 3). One milliliter of Ina$^+$ yeast culture was added to 9 mL of each solution to give a cell density of 10^7 cells/mL and mixed well on a mixer. The temperature of the freezer was set at −6.5 °C, and at this temperature distilled water did not freeze but remained supercooled through 260 min (Figure 3A). Addition of Ina$^+$ yeast cells initiated ice nucleation at ∼ −5 °C, and the whole freezing process (i.e., temperature of frozen samples reached the freezer temperature) was completed in ∼180 min. Similarly, freezing

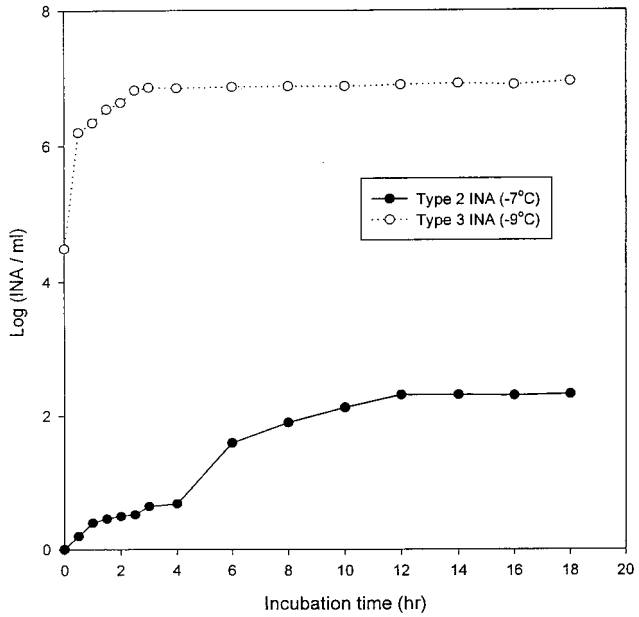

Figure 2. Induction of type 2 and type 3 INA in Ina$^+$ *S. cerevisiae* by low temperature. *S. cerevisiae* was grown in Ura$^-$ medium containing 2% galactose at 25 °C for 48 h. Cell culture was transferred to a 5 °C incubator. Aliquots of culture were taken at various times, and INA was measured at −7 and −9 °C, respectively.

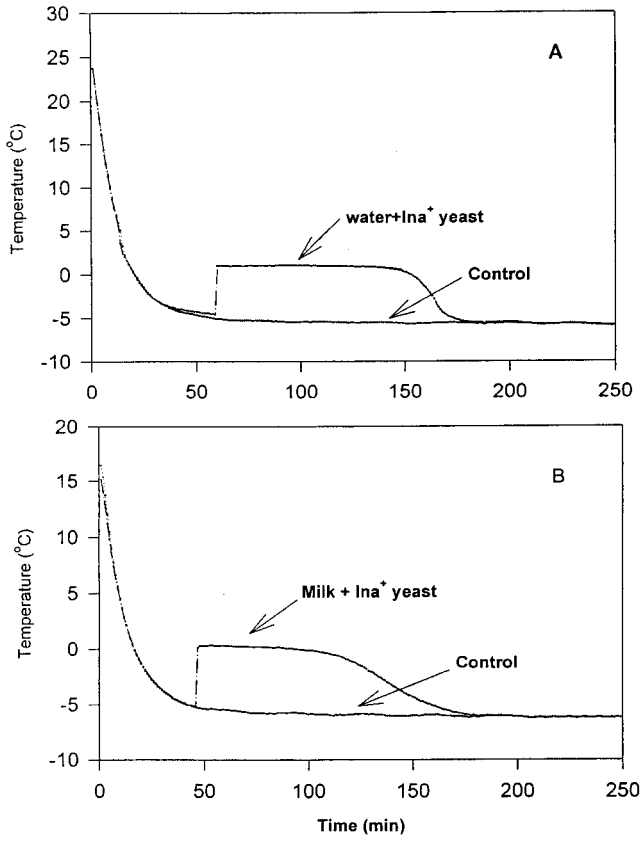

Figure 3. Freezing curves of water and milk in the absence or presence of Ina$^+$ *S. cerevisiae* cells. Sample size was10 mL, and freezer temperature was −6 °C.

of milk could be achieved at −6 °C with the addition of Ina$^+$ yeast cells, whereas controls remained unfrozen under the same conditions (Figure 3B).

Table 2 summarizes the effects of Ina$^+$ yeast cells on ice nucleation temperatures of various food materials.

Table 2. Effects of Ina$^+$ Yeast Cells on the Ice Nucleation Temperatures (Degrees Centigrade) of Various Food Materials[a]

sample	control	addition of Ina$^+$ yeast cells
water	DNF[b]	−4.7 ± 0. 2
sucrose (10%)	DNF	−4.9 ± 0.3
egg white (9%)	DNF	−5.3 ± 0.2
whole milk	DNF	−5.2 ± 0.3
2% milk	DNF	−4.7 ± 0.2
apple juice	DNF	−6.4 ± 0.8
grape juice	DNF	−6.5 ± 0.6
starch gel (5%)	DNF	−4.2 ± 0.3
starch gel (10%)	DNF	−5.6 ± 0.3
soybean curd	−3.8 ± 0.5	−3.5 ± 0.3

[a] Freezer temperature was set at −6.5 ± 0.5 °C. A sample of 10 mL or 10 mg was used. Results are an average of three separate tests. [b] DNF, did not freeze.

These results clearly indicate that the addition of Ina$^+$ yeast cells could effectively promote ice nucleation events in a −6.5 °C freezer. On the contrary, the ice nucleation events could not happen without ice nucleators under the same conditions.

Effect of Ina$^+$ Yeast Cells on Freeze Texturization. Freeze texturization is a process that utilizes freezing to produce a textured product from amorphous protein paste or a slurry. This process was developed in Japan to produce frozen soybean curd (kori-tofu) and has been used for several centuries (*1*). Kori-tofu, with a porous and texturized structure, has more strength and cohesiveness than the original tofu and can be dried for extended shelf stability at room temperature.

Ina$^+$ yeast cells in water solution not only elevate ice nucleation temperatures but also affect ice formation patterns and consequently produce a special freeze texture among food materials (Figure 4). Ice formation patterns without Ina$^+$ yeast cells show very fine particles with no directionality (Figure 4A). On the other hand, with the addition of Ina$^+$ yeast cells, an ordered, directional texture was formed in the food samples (Figure 4B).

DISCUSSION

Bacterial ice nucleation genes from *P. syringae*, *P. viridiflava*, *Er. herbicola*, and *Erwinia ananas* have been successfully cloned and expressed in *E. coli*, *Zymomonas utilis*, and plants (*22*−*25*).

The threshold temperatures of ice nucleation in recombinant hosts are normally lower than that in the wild-type Ina$^+$ bacteria (*26*). The threshold temperature of our Ina$^+$ yeast is ∼ −6 °C, which is ∼4 °C lower than that of *Er. herbicola*, the original source of the *ina* gene. The cause of the difference in threshold temperature is still not clear. One explanation is attributed to the composition of cell membranes (*26*). It is believed that ice nucleation proteins are located on the outer membrane of Ina$^+$ bacterial cells and the threshold temperature depends on the aggregation of ice nucleation proteins on the membrane surface. The difference in the fatty acid composition of the outer membranes of *E. coli* and Ina$^+$ bacteria confers the difference of threshold temperature. The reason for the much lower threshold temperature of Ina$^+$ yeast as compared with those of wild-type Ina$^+$ bacteria and Ina$^+$ *E. coli* is very likely due to yeast cells possessing a significantly different cell membrane structure compared to bacterial cells.

Low-temperature induction of INA in ice nucleation active bacteria was first reported by Rogers et al. (*27*).

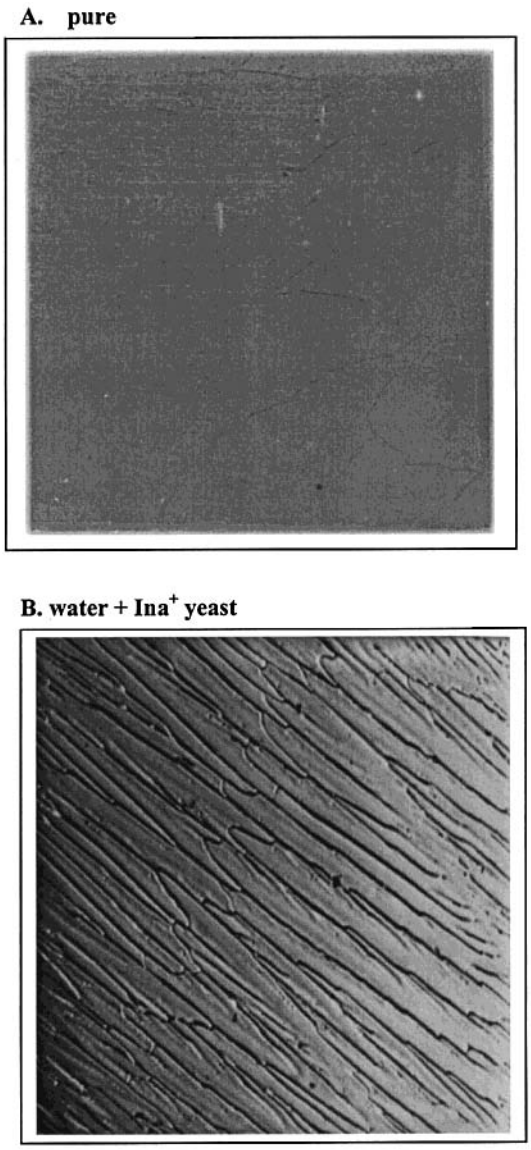

Figure 4. Ice formation patterns of water in the absence and presence of Ina^+ *S. cerevisiae* cells.

When Ina^+ bacteria grown at 30 °C are shifted to 5 °C, type I ice nuclei (active at temperatures >5 °C) start to appear. This phenomenon could be explained by the aggregation model of ice nucleation protein. According to this model, under lower temperatures, the ice nucleation proteins on the bacterial surface will aggregate into larger molecules that are more effective nucleation templates and are active at warmer temperatures (*28*).

Bacterial ice nucleators have the highest threshold temperature among the various inorganic and organic ice nucleators. Therefore, bacterial ice nucleators are effective triggers of ice nucleation events and have been successfully used as ice-nucleating agents for artificial snow-making and cloud seeding (*29*). Previous research work also demonstrated the potential applications of bacteria as nucleators in freezing concentration, freezing dry, and texturization of frozen foods (*12*, *13*). However, the utilization of bacterial ice nucleators in the food industry is restricted by their plant pathogen nature and safety concerns. Expression of the bacterial *ina* gene in nonpathogenic and GRAS host cells provides an alternative to resolve the restrictions.

Our studies demonstrate that the bacterial *ina* gene can also be expressed in yeast *S. cerevisiae* and the Ina^+ yeast cells can elevate ice nucleation temperatures and form a special freeze texture in several food materials.

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