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Enhanced production of extracellular ice nucleators from *Erwinia herbicola*

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The effects of growth conditions and chemical or physical treatments on the production of extracellular ice nucleators (ECINs) by *Erwinia herbicola* cells were investigated. The spontaneous release of ECINs, active at temperatures higher than −4°C, into the environment depended on culture conditions, with optimal production when cells were grown in yeast extract to an early stationary phase at temperatures below 22°C. ECINs were vesicular, released from cell surfaces with sizes ranging from 0.1 to 0.3 μm as determined by ultrafiltration and transmission electron microscopy. Protein profiles of ECIN fractions during bacterial growth were examined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and Ina proteins were detected by Western blotting. ECIN production was enhanced 5-fold when cells were treated with EDTA and 20- to 30-fold when subjected to sonication. These conditions provide a means for large-scale preparations of ECINs by *E. herbicola.*

Key Words——*Erwinia herbicola*; extracellular ice nucleators; ice nucleation

Gram-negative bacteria such as *Pseudomonas syringae, Erwinia herbicola*, and *Xanthomonas campestris* are well known to have the ability to initiate ice nucleation at subzero temperatures as high as −1°C (Lindow, 1983; Margaritis and Bassi, 1991; Wolber, 1993). Heterogeneous ice nucleation caused by these bacteria is one of the most active and well-documented nucleation processes in nature (Upper and Vali, 1995). Most of these bacteria are plant pathogens, causing frost injury in the early spring or late fall that results in heavy economic losses (Lindow, 1983). The strong ice nucleation activity (INA) exhibited by bacteria, however, has important applications and potential benefits (Margaritis and Bassi, 1991). One successful application is in snowmaking with energy savings (Woerpel, 1980). The high sensitivity and low threshold for INA detection makes this nucleation property a valuable reporter gene in biotechnological studies (Wolber, 1993). This has served as a basis for the development of sensitive methods to detect foodborne bacteria such as *Salmonella.* In food systems, INA may be used to guarantee freezing at high subzero temperatures to save energy and modify food textures and to act as a processing aid (Arai and Watanabe, 1986; Li and Lee, 1995; Li et al., 1997; Watanabe and Arai, 1994).

Much work has been done on bacterial ice nucleation with the aim of understanding its biological mechanisms (Fall and Wolber, 1995; Gurian-Sherman and Lindow, 1993; Warren, 1995). It is generally believed that a single gene in each bacterium is responsible for ice nucleation, and at least six gene sequences have been published (Li and Lee, 1995; Wolber, 1993). Much effort has been devoted toward the identification and purification of ice nucleators. Thus far, however, a purified protein active above −3°C has not been isolated. Studies with whole cells revealed that bacterial ice nucleators are located at the outer membrane. They may consist of ice nucleation proteins at the core, which are associated with lipid and saccharides as lipoglycoprotein complexes (Govindarajan and Lindow, 1988a; Kozloff et al., 1984, 1991a, b; Lindow et al., 1989).

Phelps et al. (1986) investigated several INA bacteria and found that only *Erwinia herbicola* and other species in its genera could release cell-free ice nuclei into the environment. Since then, others have reported their release. These have been obtained from *Pseudomonas viridiflava*, *E. uredovora*, and *P. fluo-*

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rescens KUIN-1 (Kawahara et al., 1993; Obata et al., 1990, 1993). Research directed toward the production and chemical analyses of extracellular ice nucleators (ECINs) from *E. herbicola* is limited, and little information is available describing the effects of culture conditions or the way in which physical and chemical treatments of cells affect ECIN production.

The use of ECINs instead of large numbers of bacterial cells (10^7 cfu/ml) in food-freezing processes is preferred for sanitary purposes (Li and Lee, 1995). Therefore we investigated the production of ECINs by *E. herbicola.* Specifically, we evaluated different culture conditions and various types of treatments of the cells. We also tested ECINs for use in the freezing of selected foods. This paper reports the optimization of culture conditions for enhanced ECIN production. Furthermore, ECIN size and general morphology was examined by ultrafiltration and electron microscopy, and the effects of physical or chemical treatments on ECIN release are described.

Materials and Methods

Culture conditions. *Erwinia herbicola* subsp. *ananas* was obtained from the American Type Culture Collection (ATCC Cat. No. 11530; the new nomenclature under this catalog number after 1995 is *Pantoea ananas*). Bacteria were routinely grown in a yeast extract (YE) medium consisting of 20 g yeast extract, 10 g sucrose, 2 g L-serine, 2 g L-alanine, 8.6 g K_2SO_4, and 1.4 g $MgSO_4$, per liter of distilled water (Obata et al., 1990). Other media included nutrient broth, nutrient broth supplemented with 2.5% (v/v) glycerol, Koser citrate broth, *Pseudomonas* F medium, and Tryptic soy medium. Single colonies were used to inoculate 250 ml flasks containing 50 ml of any or all media. Cultures were incubated at 18°C with shaking at 200 rpm, and growth was monitored by measuring the OD of the culture broth at 660 nm. Bacteria were also grown in YE at 22°C and 25°C to study the effect of temperature on ECIN release. The activities of cell-free supernatants were also tested at various times of growth in YE at 18°C. Cell-free supernatants were obtained by centrifugation (10,000×*g*, 20 min, 4°C). Supernatants were filtered through 0.45 μm membranes (MSI, Westboro, MA) and evaluated for bacterial contamination by plating on nutrient agar.

INA assay. Cumulative INA, defined as the activity units at temperatures higher or equal to the bath temperature setting, was measured by the droplet-freezing method (Li et al., 1997; Vali, 1971). Samples were diluted so that the frozen fractions represented less than 40% of the total droplets. The activity units were calculated by the method of Vali (1971). Broth with cells or cell-free supernatants was serially diluted in sterile INA buffer (20 mM Tris, 10 mM $MgCl_2$, pH 7.6) and samples were seated on ice before droplet-freezing assays. Cell concentrations were routinely determined by absorbance measurement of OD at 660 nm. Approximately 1.2×10^9 cells/ml corresponds to 1 absorbance unit. INA was expressed as INA units per OD_{660} unit for broth or per ml for cell-free supernatants.

Ultrafiltration. Membranes of 100 and 30 kDa-MW cutoff were obtained from Sartorius (New York, NY) and used with a stirred cell (Model 8050, Amicon, Inc., Beverly, MA). The ultrafiltration unit was equilibrated with purified nitrogen gas at a pressure of 30 psi.

Physical and chemical treatments of cells. Cells of *E. herbicola* at log phase (from 20 ml of broth with an OD_{660} value of ~8) were resuspended in an equal volume of 20 mM Tris buffer and sonicated for 0, 30, and 90 s with a 10 s pause in every 10 s burst (Model XL-2020 Sonicator, Heat Systems, Inc., Farmingdale, NY). Cells from 20 ml of broth ($OD_{660}=10$) were suspended in an equal volume of 1 M NaCl, 0.5 M EDTA, or 2% CHAPS prepared in 20 mM Tris buffer (pH 7.6). After equilibration at room temperature for 2 h with occasional vortex mixing, cells were removed by centrifugation and supernatants were filtered through 0.45 μm membranes. ECINs were pelleted at 250,000×*g* for 1 h at 4°C and resuspended in an equivalent volume of Tris buffer. Protein and INA were measured.

Transmission electron microscopy. Cell suspensions or ECIN preparations of 10 μl were placed on carbon- and Formvar-coated nickel grids. These were stained with 2% aqueous phosphotungstic acid and examined with a JEOL 100CX II transmission electron microscope operating at 60 kV under standard conditions with a cold trap in place.

Protein assay and SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Protein concentrations of cell-free supernatants were determined by the Bradford method (Bradford, 1976) by using BSA as standard with a Pierce Coomassie Plus protein assay kit (Pierce, Rockford, IL). SDS-PAGE was performed with 8% linear slab gels and 3% stacking gels (Bollag and Edelstein, 1991).

Western blot analysis. After separation by SDS-PAGE, protein bands were transferred to 0.2 μm nitrocellulose filters (Bollag and Edelstein, 1991). Transfer electrophoresis was performed in 192 mM glycine, 20% v/v methanol, 25 mM Tris, and pH 8.3 (Bio-Rad Laboratories, Hercules, CA). The nitrocellulose filters were probed with an anti-Ina A polyclonal antibody (kindly provided by Prof. S. Arai, University of Tokyo, Japan). Immunochemical staining of the labeled band(s) was performed with an avidin-biotin alkaline phosphatase system designed for use with goat an-

Table 1. Effect of growth temperature on cell-free INA levels.[a]

Growth temp. (°C)	INA of cell-free supernatant (units/ml)[b]		
	−4°C[c]	−6°C	−8°C
18	1.3×10^5	1.0×10^6	3.3×10^7
22	30	7.5×10^2	6.7×10^6
26	ND[d]	25	8.3×10^3

[a] Cells ($\sim OD_{660}=10$) were grown in YE medium.
[b] Cells were removed by centrifugation (10,000×*g* for 25 min) and supernatants were filtered through 0.45 μm membranes.
[c] Indicates the temperature setting of the water bath.
[d] Not detectable.

tirabbit IgG. Labeled bands were visualized by colorimetric reaction with alkaline phosphatase by using 5-bromo-4-chloro-3-indolyl phosphate and the color development reagent nitroblue tetrazolium as substrate.

Results

Effect of growth temperature on ECIN release

The INA of ECINs at high subzero temperatures is strongly dependent on growth conditions. Cell-free supernatants were obtained from various culture broths ($OD_{660}=10$) grown in YE medium at 18, 22, and 26°C, respectively. INA at bath settings of −4, −6, and −8°C, corresponding to Types I, II, and III ice nuclei (Yankofsky et al., 1981), respectively, were measured (Table 1). Growth temperatures lower than 26°C induced higher levels of INA in cell-free supernatants. Activities measured at −4°C in the supernatant when cells were grown at 18°C were almost 4,000 times higher than for cells grown at 22°C. No INA at −4°C was detected in the cell-free supernatant when cells were grown at 26°C. When held at a bath temperature of −8°C, INA levels of cell-free supernatants when cell were grown at 18°C were only about 5-fold higher than 22°C, but they were 4,000-fold greater than that when cells were grown at 26°C. These results show that the INA of ECINs assayed at bath temperatures of −6 and −8°C are less dependent on growth temperatures than INA assayed at −4°C; in other words, INA at −4°C is strongly dependent on growth conditions.

Growth temperatures also affected threshold temperatures for the expression of INA by ECINs. For example, growth must occur at 22°C or lower for ECINs released into the supernatants to be active at −4°C. Similarly, growth at 26°C or lower is required for the expression of ECIN activities at −6°C. If we compare the INA at −4°C and −8°C in supernatants from cells grown at 18°C, the ratio of INA at −8°C to −4°C is 250. At 22°C, this ratio measures 2.2×10^5, a difference of almost 900-fold. These sharp increases in INA levels over the range of −4 to −8°C indicate that ECINs are much more effective ice nucleators if growth is at 18°C instead of at higher temperatures. In summary, the growth temperature of cells determines threshold temperatures and levels of ECINs in the supernatants.

Table 2. Effect of growth media composition on the INA of cells and supernatants.[a]

Medium	Growth (OD_{660})	INA ($\times10^{-4}$, −6°C)	
		Broth (units/OD_{660})	Supernatant (units/ml)
Nutrient broth	1.2	1.5	ND[b]
Nutrient broth with 2.5% glycerol	3.2	0.1	0.001
Koser citrate broth	0.1	7.7	ND
Pseudomonas F	4.1	49.0	0.1
Yeast extract with amino acids	10.4	2.2	2.3
Trypticase soy medium	6.8	6.9	0.07

[a] Cultures were grown at 18°C for 24 h with shaking.
[b] Not detectable.

Effect of growth media compositions on ECIN release

The effects of growth media composition on INA determined at −6°C of culture broth and cell-free supernatant fractions were investigated (Table 2). It is apparent that YE supplemented with L-Ser and L-Ala supported the most rapid growth of *E. herbicola* under these conditions. *Pseudomonas* F medium yielded the highest INA of cells, and Koser citrate medium was the least suitable growth medium. The least INA of cells was obtained with nutrient broth supplemented with 2.5% glycerol. It had been previously reported that growth in the presence of 2.5% glycerol significantly increased INA of *P. syringae* cells (Lindow, 1983). As shown here, although glycerol enhanced the growth of *E. herbicola*, it had no effect on INA levels of cells. The INA levels for cells grown in nutrient broth, Koser citrate broth, yeast extract, and Trypticase soy medium were all similar (Table 2). The INA values of the supernatant fractions from various culture media, however, significantly varied (Table 2). No INA was detected in cell-free supernatant fractions of cultures grown in nutrient broth or in Koser citrate broth, and very low activities were found in fractions from supernatants obtained from nutrient broth with 2.5% glycerol or Trypticase soy medium. The highest INA levels were obtained in supernatants from cells grown in YE. This experiment indicates that the *Pseudomonas* F medium provides abundant levels of INA in whole cells and in cell-free supernatants. The optimum bacterial growth medium for the production of highly active ECINs is therefore YE medium with L-Ser and L-Ala.

Effects of growth phase on ECIN release

The effects of growth duration on the release of ECINs into cell-free supernatant fractions was studied. Cells were grown in YE at 18°C for specified periods of time (Fig. 1). OD_{660} values were measured and the cells removed. The INA and protein levels of cell-free supernatants were tested. Cultures entered late log or early stationary phase (on the curve) after 24 h of growth. At a bath temperature setting of −5°C, INA was first detected in the supernatant at 16 h. From 16 to 20 h, a 380-fold increase of INA then occurred. From 20 to 24 h, INA increased 11-fold (Fig. 1). Maximum INA was achieved at 24 h, and after that, the activity declined rapidly. Protein levels of supernatants at various growth phases did not significantly change. It had previously been reported that cells of *P. syringae* S203 exhibited the highest INA levels during the stationary phase (Deininger et al., 1988). In contrast, *P. syringae* C-9 (Yankofsky et al., 1981) and 1105 (Pooley and Brown, 1991) had a relatively high number of ice nuclei in the middle to late phases of exponential growth.

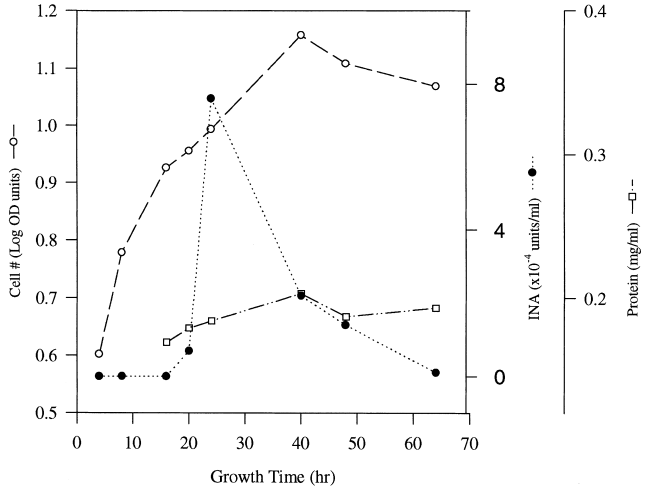

Fig. 1. Effect of growth time on INA levels of a cell-free supernatant of *E. herbicola.*

Bacteria were grown in yeast extract at 20°C. At each sampling point, the OD at 660 nm for 10 ml of broth was measured. Cell-free supernatant was prepared as described in MATERIALS AND METHODS. INA was measured at −5°C.

SDS-PAGE and Western blotting

Protein profiles in cell-free supernatant fractions as a function of time were examined by SDS-PAGE and Western blotting (Fig. 2, A and B). ECINs in the cell-free supernatant (~0.7 ml) at each sampling point were pelleted by ultracentrifugation, resuspended in Tris buffer (pH 7.6), and subjected to SDS-PAGE. As shown in Fig. 2A, the intensity of each protein band as stained with Coomassie blue increased with extended growth periods from 16, 20, and 24 to 40 h, corresponding to lane 1 to lane 5 in Fig. 2A. After 40 h (lane 5), the protein band with an apparent molecular weight of 112 kDa disappeared from supernatant fractions (from lane 5 to lane 6). The protein profiles at all stages of growth were generally similar, with intense bands of 112, 75, 66, 40, 34, and 29 kDa strongly stained in Fig. 2A.

Although Coomassie-stained gels revealed no proteins larger than 116 kDa, one with an apparent molecular weight of 185 kDa was clearly identified at each growth stage on the Western blot (Fig. 2B). The antibody also cross-reacted with many low-molecular-weight protein bands. Moreover, protein bands of 112, 75, 66, 40, and 29 kDa were strongly recognized by the antibody.

It is difficult to correlate the INA with protein band intensities either on SDS-PAGE or on Western blot. For example, INA was highest at 24 h of growth, but none of the prominent protein band could be identified

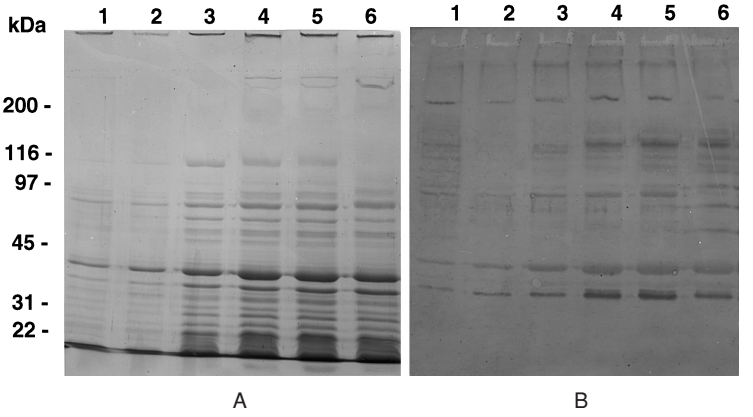

Fig. 2. SDS-PAGE (A) and Western blot (B) analysis of ECINs at various growth times.

Lanes 1–6 represent 16, 20, 24, 40, 48, and 60 h of cell growth, respectively. Each lane was loaded with pellet fractions obtained from 0.7 ml (~20 μg protein) of cell-free supernatant fractions. The antibody was anti-InaA protein with a dilution of 1 : 500 (see text for details).

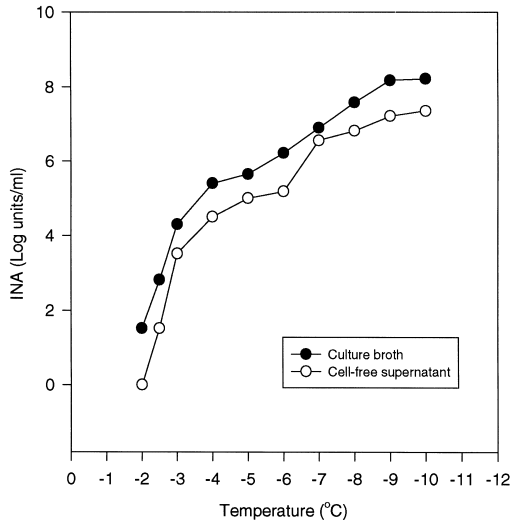

Fig. 3. Cumulative ice nucleation spectra of culture broth and cell-free supernatant.

Growth medium: yeast extract; $OD_{660}=14$; 18°C. Cell-free supernatant was prepared as described in MATERIALS AND METHODS.

on the corresponding lane 3 of both Fig. 2, A and B. It is interesting, however, to find that sizes of protein bands in these ECINs on both SDS-PAGE and Western blot showed a periodic pattern, meaning that every third band appeared denser than the adjacent two bands, which was reported with membrane preparations from strains of *P. syringae*, *P. fluorescens*, and *E. herbicola* by Deininger et al. (1988).

Association of INA with ECINs and whole cells

The ratio of INA between whole cells and ECIN fractions after 24 h of growth was examined (Fig. 3). INA was tested at supercooling temperatures ranging from −2°C to −10°C. No INA was observed in the medium at a bath temperature of −13°C or above. No viable cells were detected in culture supernatant filtrates; therefore INA was the result of ECINs. As the bath temperatures were decreased, INA for cells and cell-free supernatants increased dramatically. INA was found at −2°C with cells and cell-free supernatants. The ratio of INA between cells and supernatants increased as supercooling temperatures were raised from −3°C to −2°C (Fig. 3). Although shedding of ECINs was quite variable in quantity, usually 10 to 30% of the INA at −4°C was associated with ECINs when *E. herbicola* cells were grown on YE medium at 20°C (data not shown). Phelps et al. (1986) reported that an average of 10% of the total ice nuclei from *E. herbicola* M1 were cell-free and active at −3°C when grown on a minimal glycerol medium at 15°C.

Size determination

ECIN size distribution was determined by membrane filtration and transmission electron microscopy. The INA present in cell-free supernatants passing through 0.45 and 0.22 μm membranes was tested at bath temperature settings of −4, −6, and −8°C (Table 3a). By passage through 0.22 μm membranes, more than 80% of INA at −4 and −6°C of ECINs was removed. However, only 45% of INA at −8°C was removed. This means that the average particle size of ECINs active at temperatures of −6°C or higher is from 0.22 to 0.45 μm. Moreover, 55% of INA as measured at −8°C or lower was contributed by ECINs of 0.22 μm or less.

Table 3a. Effect of filtration on the INA of ECINs.[a]

Pass-through	INA ($\times10^{-4}$ units)		
	−4°C	−6°C	−8°C
0.45 μm filter	3.3	33.3	4.2×10^3
0.22 μm filter	0.6	3.5	2.3×10^3

[a] Cells were removed by centrifugation at 10,000×*g* for 30 min. Protein content was 0.17 mg/ml, and INA was expressed on a per ml basis.

Table 3b. Effect of ultrafiltration on the INA of ECINs.

Fraction	Protein recovered (%)	INA ($\times10^{-5}$ units)		
		−4°C	−6°C	−8°C
Supernatant (<0.45 μm)	100	9.9	99.1	1.3×10^4
Fraction I (>100 kDa)	39	3.5	25.2	1.4×10^5
Fraction II (30–100 kDa)	4	<0.01	0.02	3.3
Fraction III (<30 kDa)	57	ND[a]	<0.01	<0.01

[a] Not detectable.

After passage through a 0.45 μm membrane, supernatants (30 ml) were concentrated 10-fold by using a 100 kDa-cutoff membrane (Fraction I) (Table 3b). Filtrates were further concentrated 10-fold by using a 30 kDa-cutoff membrane (Fraction II). Total protein content and the INA of each fraction were measured. When measured at −4°C, a strong correlation was observed between total protein recovery and residual INA in Fraction I. Total INA level remaining in Fractions I, II, and III after ultrafiltration was only 35% of that in the supernatants. This indicates that 61% of the total protein that passes through the 100 kDa cutoff membranes does not significantly contribute to INA. Protein profiles of each fraction were analyzed by SDS-PAGE. Few differences in banding patterns between the ultrafiltration fractions were observed (data not shown). These results suggest that size differences between ECINs may be due to nonprotein additions such as lipid and carbohydrate.

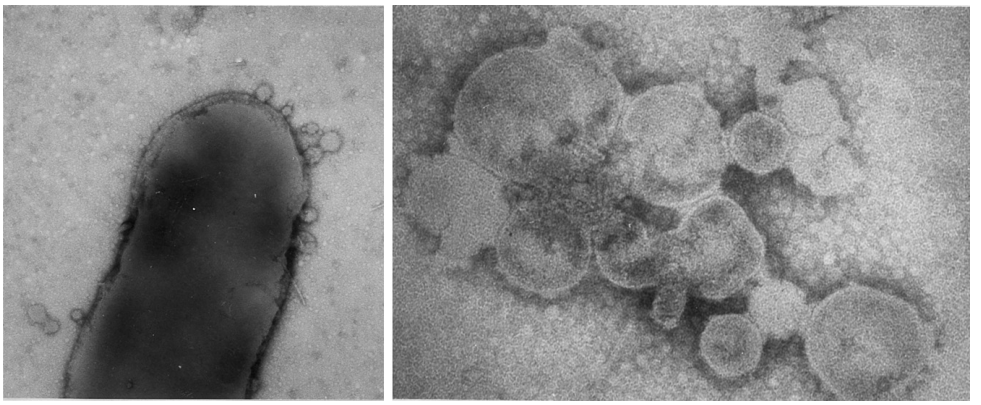

Fig. 4. Transmission electron micrographs of *E. herbicola* cell (A) and ECINs (B).
Samples were negatively stained as described in MATERIALS AND METHODS. Magnification=175,500×(A); 65,000×(B).

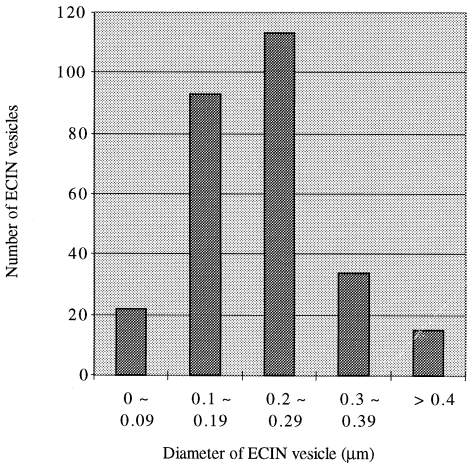

Fig. 5. Size distribution of ECIN vesicles as measured from micrographs of more than 300 vesicles.

Transmission electron microscopy

Figure 4 shows micrographs of *E. herbicola* cells and enriched ECINs. These establish that the ECINs are membrane vesicles shed from the outer membrane of cells. Some of vesicles were still attached to cells and some had already been released to the medium (Fig. 4A). A continuous membrane surrounds each vesicle. Vesicles and flagellar fragments are clearly visible (Fig. 4B). Most membrane vesicles were in the size range of 0.1 to 0.3 μm as measured from the TEM micrographs. Size distributions shown in Fig. 5 were obtained from the measurement of about 300 vesicles.

Release of ECINs by extraction with NaCl, EDTA, and CHAPS

The micrographs suggest that ECINs are chemically associated with cell surfaces (Fig. 4). Possible types of chemical forces include ionic and hydrophobic interactions and hydrogen bonding. To further probe the nature of these interactions, cell suspensions were washed and resuspended in buffer containing 1 M NaCl, 0.1 M EDTA, or 2% CHAPS. After centrifugation and washing, ECIN-enriched pellets were resuspended. Protein levels and INA at a bath temperature setting of −6°C were measured (Table 4). Cells treated with CHAPS released 10-fold more protein than buffer alone did. However, the cell-free supernatants exhibited very low INA. The addition of EDTA released around 5 times more protein than buffer alone did, and the INA was about 5-fold as great. Increasing ionic strength by the addition of NaCl induced a 2-fold increase in the amounts of protein and INA released from cell surfaces.

Table 4. Effect of NaCl, EDTA, and CHAPS on the release of ECINs.[a]

Chemical	Protein (μg/ml)	INA (−6°C) ($\times 10^{-3}$ units/ml)	Specific activity (units/mg protein$\times 10^{-5}$)
Tris buffer (pH 7.6)	2.1	1.1	5.1
+NaCl (1 M)	4.9	2.2	4.5
+EDTA (0.1 M)	9.7	5.4	5.5
+CHAPS (2%)	21.0	0.8	0.4

[a] Cells from midlog phase (OD_{660}=8.6) were thoroughly resuspended in 20 ml of each solution, incubated at room temperature for 2 h, then removed by centrifugation. ECINs in supernatants were pelleted by ultracentrifugation.

Sonication

The application of ultrasound dramatically increased the yield and specific activity of ECINs released from cells (Table 5). A 30 s burst resulted in a 20-fold increase in the total amount of INA, and a 90 s treatment led to a 33-fold enhancement. This activity increase of ECINs was accompanied by higher levels of extracellular protein. The specific activity for ECINs released at a 30 s burst is higher than the control. It de-

Table 5. Effect of sonication on the production of ECINs.[a]

Length of sonication (s)	Total protein (mg)	INA (−5°C) (units)	Specific INA (units/mg×10^{-2})
0	0.009	18	20.2
30	0.145	340	23.4
90	1.694	680	4.0

[a] After sonication as described in MATERIALS AND METHODS, ECINs were pelleted from 20 ml of cell-free supernatants by ultracentrifugation and resuspended in an equal volume of 20 mM Tris buffer (pH 7.6). Protein and activity units are expressed on a per ml basis.

clined to about 20% of the control for the ECINs with a 90 s treatment. This experiment provides us with a means for the enhanced production of ECINs from *E. herbicola.* In this way we obtained 840 mg of ECIN protein from cells of 1 L of culture broth, which exhibited an equivalent or higher specific activity. This is a yield 30-fold greater than the yield produced under direct isolation from cell-free supernatants.

Discussion

This work clearly demonstrates that growth temperatures strongly determine the natural release of ice nucleators into extracellular environment. Growing cells in YE medium at temperatures below 18°C to the early stationary phase is required for a maximum production of ECINs. These ECINs are membrane vesicles loosely attached to the cell surfaces that can be easily released by chemicals such as NaCl, EDTA, or CHAPS or by ultrasound treatments, which greatly enhanced their production 20- to 30-fold. ECIN sizes ranged from 0.1 to 0.3 μm, and their expression appears to be constitutive during bacterial growth.

The threshold growth temperature for the release of Type I ECINs by *E. herbicola* is approximately 22°C in YE medium. ECINs active below −4°C were also released under these conditions. Using a minimal growth medium, Phelps et al. (1986) found that cultures grown at room temperature do not release Type I nuclei. However, when cultured at 15°C, some strains of *Erwinia* spontaneously released cell-free ice nuclei into growth medium. Obata et al. (1990) also detected extracellular ice nuclei when *E. uredovora* was cultured at 18°C. ECINs exhibited strong INA in cell-free filtrates from cultures grown at 10°C, and no activity was recovered in filtrates from cells grown at 26°C (Michigami et al., 1995a).

The temperature-dependent release of ECINs may be similar to the phenomenon of low-temperature conditioning described by Rogers et al. (1987). That is, when bacteria grown at relatively high temperatures (e.g., 30°C) are shifted to lower temperatures (e.g., 5°C) for a brief period, new ice nuclei active at temperatures warmer than −5°C appear. Low-temperature conditioning did not occur if cells were removed from culture broths as found in this work (data not shown), confirming that conditioning requires the presence of whole cells. The appearance of ice nuclei may be the result of new protein synthesis (Rogers et al., 1987). Several Gram-negative strains spontaneously release membrane vesicles from their cell surfaces. *In vivo*, these vesicles may act as vectors for infection, provide bacteriocidal or proteolytic activities, or act as toxins (Fukuoka et al., 1992; Mayrand and Grenier, 1989). Thus it is possible that releasing ECINs into the environment is a means by which *E. herbicola* infects host plants. Apparently, a declining temperature is a key factor for inducing cells to release ECINs. These materials then promote ice nucleation and freezing of plant tissues, which in turn provide the bacterial cells with nutrients.

Growth medium composition significantly affected the expression of ice nucleation phenotype. As shown in this work, ECIN production required a nutrient-rich medium such as YE or *Pseudomonas* F media, suggesting that vigorous cell growth is needed. Obata et al. (1990) and Kawahara et al. (1993) obtained similar results with other strains of *Erwinia.* On the hand, Pooley and Brown (1991) found that large numbers of ice nuclei from *P. syringae* 1105 were obtained by using Koser citrate broth. Nemecek-Marshall et al. (1993) reported that nutrient limitation and low temperature induced high levels of ice nuclei by *P. syringae* T1. Phosphate starvation and low temperature also induced a high-level expression of ice nuclei in *E. herbicola* (Fall and Fall, 1998).

The effect of growth duration on ECIN release by *E. herbicola* had not been previously reported. This study demonstrates that maximal INA of cell-free filtrates was achieved at late log or early stationary phase. Comparable results were reported with *E. uredovora* (Michigami et al., 1995b). The timing of the expression of ice nucleation gene products and their correlation with the Ina^+ phenotype were examined in *P. syringae* S203 and *P. fluorescens* MS1650 (Deininger et al., 1988). The InaZ protein appeared in *P. syringae* S203 during a stationary phase, and its appearance was correlated with the Ina^+ phenotype. In contrast, both the InaW protein and INA occurred at relatively constant levels throughout the growth of *P. fluorescens* MS1650.

The immunoblot in Fig. 2 indicates that the 185-kDa protein is a minor component of the *E. herbicola* ECIN preparation and is maintained at a constant level throughout growth. Furthermore, many low-molecular-weight proteins were also recognized by the antibody throughout the growth period. Similar results were also reported by other investigators (Deininger et al.,

1988; Schmid et al., 1997; Southworth et al., 1988). These low-molecular-weight bands may represent degradation products of Ina proteins before and during preparation. No specific protein corresponded with the high levels of INA that appeared during an early stationary phase. This implies that proteins associated with INA in the *E. herbicola* are constitutively expressed and that INA may depend on nonproteinaceous materials associated with the ECINs, such as phospholipids or saccharides (Govindarajan and Lindow, 1988b; Kozloff et al., 1984, 1991a, b; Southworth et al., 1988).

Ice nucleation proteins from various bacterial species have related internally repetitive primary structures (Michigami et al., 1995a; Schmid et al., 1997; Warren and Wolber, 1991). Each protein has three domains: a central repeating domain of 80% total sequence with an 8-amino acid residue period of repetition, a unique N-terminal domain, and a unique C-terminal domain. Most investigators believe that Ina proteins form homoaggregate arrays that serve as templates for water binding and aligning into ice crystals (Fall and Wolber, 1995; Tsuda et al., 1997). The mechanisms of aggregation of proteins and their association with membranes and other nonproteinaceous materials, however, remain unclear. An additional discussion may be found in chapters 4–6 of the book "Biological Ice Nucleation and Its Applications" (R. E. Lee, Jr., G. J. Warren, and L. V. Gusta, ed. 1995).

Decreased INA yields in cell-free filtrates following passage through 0.45 and 0.22 μm membranes suggest that the ECINs released by *E. herbicola* are not uniformly sized. Their size distribution was further demonstrated in the range of 0.1 to 0.3 μm by ultrafiltration and TEM. Welch and Speidel (1989) estimated that the diameter of INA-containing particles ranges from 25 to 235 nm. The sizes of purified ECINs from *E. uredovora* KUNI-3 ranged from 200 to 400 nm (Turner et al., 1991). Govindarajan and Lindow (1988b) used radiation inactivation analysis and showed that the molecular mass for an ice nucleator ranged from approximately 150 kDa (active at −12°C to −13°C) to 8,700 kDa (−3°C). Filtrates from membranes of <30 kDa cutoff still retained INA-detectable at bath settings above −6°C (Table 3b). One possible reason is that Ina proteins still exhibit activity when degraded and in the presence of membrane structures (Wolber et al., 1986).

Direct negative staining of cells and ECINs (Fig. 4) showed that cells contained blebs of outer membrane protrusions, which are eventually shed as vesicles into the medium. Similar ECIN morphologies were also reported with thin sections (Phelps et al., 1986). A process such as minicell formation was suggested for the transport of the InaU protein from the inner membrane to the outer membrane, based on immunogold staining and electron microscopy (Michigami et al., 1995b). Vesicle formation may also result because the outer membrane grows more rapidly than the underlying peptidoglycan (Mayrand and Grenier, 1989).

Extracellular vesicles are derived from the outer membrane of the bacterial cells from which they are generated. Exactly how these membrane vesicles are associated with cell surfaces and under what conditions they are secreted to the environment is poorly understood. Salts, chelators, and detergents such as NaCl, EDTA, and CHAPS had not previously been used to release ECINs from *E. herbicola.* The high ECIN activities released by 1 M NaCl or 0.5 M EDTA, as found in this work, may suggest that ECINs are attached to the cell surface by a combination of noncovalent forces such as ionic interactions or hydrogen bondings. The low specific activity of ECINs following treatment with CHAPS may be due to a disruption of critical protein-phospholipid interactions within the membrane vesicles.

For application purposes, sonication provides a practical means to extract large amounts of ECINs. A light ultrasound treatment had previously been used to remove blebs attached to cell surfaces (Mayrand and Grenier, 1989). When this method was used to extract ECINs from *E. herbicola*, the efficiency was increased by a factor of 20- to 30-fold, yielding 800 mg/L of culture broth. This should provide significant advantages for a large-scale production of ECINs. A potential also exists that by using the cells grown in various media, especially *Pseudomonas* F, an even higher yield of ECINs may be obtained because higher INA was associated with these cells, as shown in this work (Table 2). Moreover, these results further confirm that ECINs are loosely attached to cell surfaces.

More work needs to be conducted regarding the isolation, characterization, and chemical analysis of ECINs. Their applications produced by the procedures described in this study in food-freezing experiments will be presented elsewhere.

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