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Release of Cell-Free Ice Nuclei by *Erwinia herbicola*

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Several ice-nucleating bacterial strains, including *Erwinia herbicola*, *Pseudomonas fluorescens*, and *Pseudomonas syringae* isolates, were examined for their ability to shed ice nuclei into the growth medium. Only *E. herbicola* isolates shed cell-free ice nuclei active at −2 to −10°C. These cell-free nuclei exhibited a freezing spectrum similar to that of ice nuclei found on whole cells, both above and below −5°C. Partially purified cell-free nuclei were examined by density gradient centrifugation, chemical and enzymatic probes, and electron microscopy. Ice-nucleating activity in these cell-free preparations was associated with outer membrane vesicles shed by cells and was sensitive to protein-modifying reagents.

The unique ability of certain bacteria to catalyze ice formation in supercooled water at relatively warm temperatures has been a fairly recent observation (16, 28, 31; L. R. Maki and D. M. Garvey, EOS Am. Geophys. Union Trans. **56**:994, 1975). While small volumes of water free of heterogeneous ice nuclei generally will not freeze until temperatures approach −40°C (1, 26), the presence of dust particles can raise the freezing temperature to −10°C, and mineral particles such as silver iodide can cause ice nucleation at −8°C (18, 34). Bacterial isolates, on the other hand, have been found to cause freezing at temperatures as warm as −1°C (11).

There are at least three species of ice nucleation-active (INA) bacteria: *Pseudomonas syringae*, *Pseudomonas fluorescens*, and *Erwinia herbicola*. They have a worldwide distribution and are found in soils, on plant leaf surfaces, and in leaf mulch (11). Plant pathologists have found that these bacteria play an important role in initiating frost injury to plants at temperatures above −5°C (12, 14).

The practical implications for being able to prevent frost damage in agriculture have stimulated interest in ice-nucleating bacteria. Little is known, however, about the nature of the ice-nucleating site itself. Research has been hampered by a low frequency of expression of ice nuclei in normal bacterial cultures (12, 16, 32) and by failure to isolate highly active ice nuclei from cells (12, 17, 29, 32, 33).

Work with intact bacteria has shown ice-nucleating activity to be susceptible to proteases and sulfhydryl-modifying chemicals, suggesting that a protein is responsible for the activity (10, 12). More recently it has been established that cloned ice nucleation genes from *P. fluorescens* and *P. syringae* encode a 180-kilodalton (kDa) protein (2, 6). This protein contains a highly repetitive amino acid sequence that probably provides the necessary water-binding array characteristic of ice nuclei (6). Other evidence has been presented that ice nuclei are located in the outer membrane of these gram-negative bacteria (11) and that the phospholipid phosphatidylinositol is involved in the ice nucleation site (9). By inactivation of cell-associated ice nuclei with gamma radiation, their size has been estimated to vary from 620,000 Da for −9°C activity to 19,000,000 Da for activity at −2°C (A. G. Govindarajan and S. E. Lindow, Plant Physiol. **75**:(Suppl.)94, 1984). These results are consistent with the view that the ice nucleus contains an aggregating ice nucleation protein located in the cell membrane.

We have been working to isolate bacterial membranes with functional ice nuclei. In the course of this work we have found that the majority of INA *E. herbicola* isolates will shed ice nuclei into the growth medium when grown at 15°C. These nuclei, when released from the cells, are termed cell-free ice nuclei. This communication describes some of the properties of these cell-free ice nuclei and suggests that these structures are useful for the further biochemical characterization of bacterial ice nuclei.

MATERIALS AND METHODS

Chemicals. Elastase (porcine pancreas type I), mitomycin C, Percoll, pronase (type XIV), and thermolysin (type X) were purchased from Sigma Chemical Co., St. Louis, Mo. All other chemicals were reagent grade.

INA bacteria. Bacteria were obtained from the sources listed in Table 1 and were grown either in TYG (5 g of tryptone [Difco Laboratories], 2 g of yeast extract, 25 g of glycerol per liter) or in a minimal medium (3) with either 0.1 M glycerol or 0.1 M lactate as the carbon source. Frozen stocks were prepared by diluting log-phase TYG cultures 1:1 with sterile 14% dimethyl sulfoxide, followed by rapid freezing to −70°C. Bacteria were classified as described in detail elsewhere (4).

Ice nucleation activity assay. Ice nucleation activity was assayed by a drop-freezing assay described previously (10, 30), with a thermoelectric cooling plate. Type I, II, and III ice nuclei are defined as the population of ice nuclei that catalyze the freezing of drops above −5°C, between −5 and −7°C, and between −7 and −10°C, respectively (32). The concentration of ice nuclei was determined by serial dilution into buffers containing 10 mM $MgCl_2$ and recording the freezing temperature of 10 to 100 10-μl drops of each dilution. The concentration of ice nuclei in each dilution was calculated as described in detail elsewhere (14, 30).

Induction and isolation of cell-free nuclei. Cultures were routinely grown to log phase in minimal medium with shaking at 15°C. Cells were pelleted by centrifugation at 4°C, and the supernatant was filtered (0.22-μm-pore-size filter, Millex-GV; Millipore Corp., Bedford, Mass.). The filtrate was checked for contaminating bacteria by plating on TYG agar.

Mitomycin C was used to induce a higher expression of ice nuclei in *E. herbicola* M1, with a procedure described

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previously (33). Cells were grown in minimal glycerol medium at room temperature with shaking at 150 rpm. Mitomycin C was added to a log-phase culture to a final concentration of 1 μg/ml. The culture was returned to the room-temperature shaker for 4 h and then transferred to a 4°C chamber overnight. This procedure was found to increase type I ice nuclei equally in both the total culture and cell-free fraction.

Density gradient centrifugation. Cell-free ice nuclei were obtained from strain M1 following induction with mitomycin C in minimal glycerol medium as described above. Following filtration, ice nuclei were concentrated by centrifugation at 200,000 × *g* for 2 h at 1°C. The supernatant was discarded, and a sable hair paint brush moistened with minimal medium was used to loosen the pellet. The cell-free ice nuclei could then be suspended evenly, free of clumps, without measurable loss of activity.

This preparation was mixed with Percoll to give a final density of 1.049 g/ml and volume of 40 ml. A control was prepared from Percoll mixed with 10 mM $MgCl_2$ and density marker beads (Pharmacia, Piscataway, N.J.) to the same density. Centrifugation at 18,500 × *g* for 100 min in a Sorvall SS-34 rotor generated a gradient. Density across the tube was measured by the distance of the density beads from the meniscus. One-milliliter fractions were collected after puncturing the bottom of the tube.

Electron microscopy. (i) Whole cells. Strain M1 cultures (50 ml) were grown in mimimal glycerol medium and induced by mitomycin C as described above. Cells were centrifuged at 10,000 × *g* for 15 min at 4°C, suspended and incubated for 1 h at 4°C in 3% glutaraldehyde (EM grade; Polysciences, Warrington, Pa.) in 0.1 M sodium cacodylate buffer (pH 7.2), rinsed in 0.1 M sodium phosphate (pH 7.2), and postfixed with 1% OsO_4 in 0.1 M sodium phosphate for 2 h. During the second hour, the samples were warmed to room temperature. Samples were then dehydrated in a graded ethanol series, followed by two changes of propylene oxide and embedding in Epon-Araldite resin (20). Thin sections were stained with 2% aqueous uranyl acetate and lead citrate.

(ii) Cell-free preparations. Samples (30 to 50 ml) of unfiltered and filtered (0.22-μm pore size) culture supernatants were fixed by adding 70% glutaraldehyde to give a final concentration of 3%. After 30 min at 4°C, samples were centrifuged at 27,000 × *g* at 4°C for 40 min. Postfixation of the pellet in OsO_4 and subsequent processing were the same as for whole cells.

(iii) Percoll gradient fractions. Fractions were pooled by sets of three, diluted threefold with 10 mM $MgCl_2$, and centrifuged at 200,000 × *g* at 2°C for 2 h. Flocculent material just above the Percoll pellet was withdrawn (0.5 ml) and suspended in 3 ml of 2% glutaraldehyde in 10 mM $MgCl_2$–20 mM sodium phosphate (pH 7.2). Fixation took place during centrifugation at 200,000 × *g* at 2°C for 30 min. Samples were rinsed in buffer and postfixed in 1% OsO_4 for 2 h. After a final centrifugation, very small pellets remained intact for dehydration and embedding (as above).

Chemical and enzymatic modification. All incubations were done on ice and in the presence of 10 mM $MgCl_2$. All serial dilutions were done in cold 10 mM $MgCl_2$. Cell-free ice nuclei were isolated from strain M1 log-phase cultures grown in minimal lactate medium at 15°C, as described above. Identical treatments were carried out on log-phase M1 cultures grown at 22°C in minimal lactate medium. The loss in ice nucleation activity was determined by comparison with controls incubated under conditions identical in every respect, but without added reagent or enzyme. In the case of enzymatic digestions, boiled enzyme was used as the control. Loss in viability was determined by plating each treated sample and its control on TYG agar at the end of the incubation period. The chemical modification data represent the average of at least two trials.

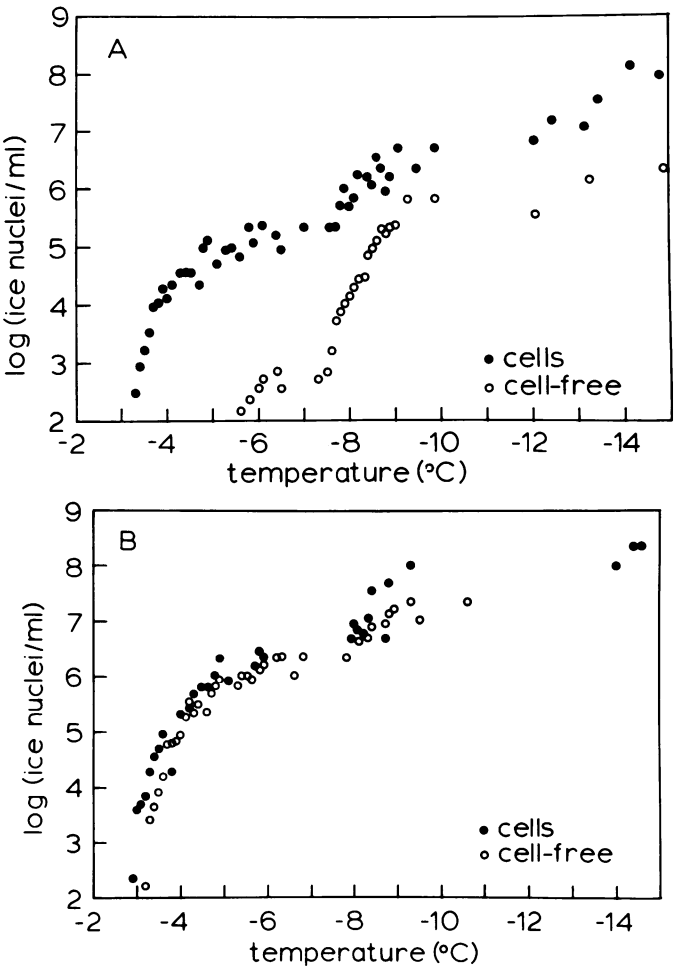

FIG. 1. Ice nucleation spectra of *E. herbicola* M1 grown at (A) 22°C and (B) 15°C to log phase in mimimal lactate medium. Symbols: ●, activity found by serial dilution of the entire culture in 10 mM $MgCl_2$; ○, activity found following removal of cells by centrifugation and filtration, as described in the text.

RESULTS

Detection of cell-free ice nuclei. Several INA bacterial isolates were found to produce cell-free ice nuclei when grown at or below 15°C, in either rich or mimimal medium, and with a variety of carbon sources. The criteria for cell-free ice nuclei were: (i) the activity passed through a 0.22-μm filter and (ii) the filtrate contained no viable bacteria. Although cultures grown at 22°C produced cells capable of nucleating ice formation at temperatures above −5°C, such ice nuclei were not found in cell-free preparations (Fig. 1A). Cell-free nuclei from cells grown at 15°C, however, exhibited populations of nuclei that froze both above −5°C (type I) and below −5°C (type II and III), similar to nuclei found associated with cells (Fig. 1B). In the experiment shown (Fig. 1B), a large percentage of the total nuclei were released from the cells. More typically, when grown at 15°C

TABLE 1. Characteristics of ice-nucleating strains studied[a]

Strain	Source or reference	Release of cell-free nuclei	
		Type I	Type II + III
E. herbicola			
GrA	Grape (Calif.)	−	−
GrB	Grape (Calif.)	++	++
GrC	Grape (Calif.)	+	++
GrE	Grape (Calif.)	+	++
GrF	Grape (Calif.)	++	++
Ex5-A	Grape (Calif.)	+	++
Fl1-B	Grape (Calif.)	+	+
P136C	Rye (Ga.)	+	++
P141C	Clover (Ga.)	+	+
M1[b]	Citrus (Israel); S. Yankofsky (33)	++	++
Eh26	Corn (Wis.) (13)	+	++
EK1	Aspen; L. Kozloff (10)	++	++
P. syringae			
PS10	Tomato (Ga.)	−	−
PS16	Tomato (Ga.)	−	−
5F	Tomato (Ohio)	−	−
1M	Tomato (Ohio)	−	−
LJ12	Natal plum (Calif.)	−	−
R9U	Radish (Ohio)	−	−
B3628	Snap beans (Wis.); S. Hirano	−	−
T2304	Oats (Wis.); S. Hirano	−	−
F33	Peach (Fla.); R. Stall	−	−
P. fluorescens			
F/CN	Marine isolate (4)	−	+
F-12	Fresh water (17)	−	−

[a] All cultures were grown at 15°C in minimal glycerol medium. Cell-free nuclei in the culture fluid were detected as described in the text and scored as follows: ++, $>10^5$/ml; +, 10^2 to 10^5/ml; −, not detectable. Type I and type II and III nuclei are defined as ice nuclei active at −2 to −5°C and −5 to −10°C, respectively.

[b] Bacterium M1, isolated from citrus leaves by Yankofsky et al. (33), is listed here as *E. herbicola* since it is virtually identical with strain GrB by a variety of biochemical and phage-typing tests (C. Kack, L. Fall, M. Prochoda, and R. Fall, unpublished observations).

strain M1 released an average of 10% (12 experiments) of the total nuclei as cell-free nuclei.

Although none of the INA pseudomonad isolates from a variety of sources and locations produced cell-free type I nuclei, most of the *E. herbicola* INA strains did so when grown at 15°C (Table 1). Although shedding of cell-free ice nuclei was quite variable, 10% of the total activity of *E. herbicola* cultures grown on minimal glycerol medium at 15°C was often found in the cell-free fraction.

Induction and isolation of strain M1 cell-free ice nuclei. Mitomycin C has been found to induce expression of ice-nucleating activity of *E. herbicola* M1 cells (33). We confirmed these results and observed a parallel increase in the production of cell-free nuclei by strain M1. Typically, we observed a 200-fold increase in type I nuclei in both the total culture and cell-free fractions.

Mitomycin C appeared to increase the expression of ice nuclei without affecting the degree of shedding of ice nuclei into the growth medium. The following experiments illustrate this point. Cultures of M1 were grown to log phase at room temperature, incubated with and without mitomycin C (1 μg/ml) for 4 h, and transferred to a 4°C incubator overnight. Mitomycin C-treated cells released 19% (average of six experiments) of type I nuclei into the cell-free fraction, compared with control cells that released 24% (average of four experiments) of type I nuclei. Although the degree of shedding of ice nuclei was variable, in all cases it was clear that temperature is an important factor since cultures grown at 22°C never shed type I nuclei.

Cell-free type I ice nuclei were routinely isolated from strain M1 as described in Materials and Methods. The nuclei were pelleted from the culture filtrate by centrifuging at 200,000 × *g* for 2 h. Following suspension and centrifugation in a Percoll self-generating density gradient, type I ice nuclei banded at a density of 1.03 to 1.05 g/ml (Fig. 2). This suggests that cell-free nuclei are associated with large particles of discrete composition. It is noteworthy that cell-free ice nuclei obtained from induced and noninduced cultures banded at the same density on Percoll gradients.

Electron microscopy of vesicles and whole cells. Electron micrographs suggested that cell-free nuclei are borne on outer membrane vesicles shed into the growth medium. Small vesicles, 50 to 200 nm in diameter, were abundant in cell-free (filtered) supernatants of induced strain M1 cultures (Fig. 3a). The trilaminar structure of the limiting membrane of the vesicles was characteristic of osmium-fixed bilayer membranes (35). Residual cells and large cell envelope fragments present in the crude supernatants (data not shown) were completely removed by the filtration step without any significant loss of ice nucleation activity. In addition to vesicles, cell-free preparations contained fibrous material, presumably fragments of the flagella (Fig. 3a). Electron microscopy of the Percoll gradient fractions showed that the most active fractions consisted mainly of membranous vesicles with slight contamination by flagellar fragments (Fig. 3b). Higher-density fractions with less than 1% the amount of activity (Fig. 2) were enriched in flagellar fragments and depleted of vesicles (Fig. 3c).

Micrographs of whole cells in actively shedding cultures revealed frequent outfoldings or blebs in the outer membrane as well as small vesicles still attached to the cells (Fig. 3d and e). Induced (Fig. 3e) and uninduced (Fig. 3d) cultures exhibited vesicle formation at a roughly equivalent fre-

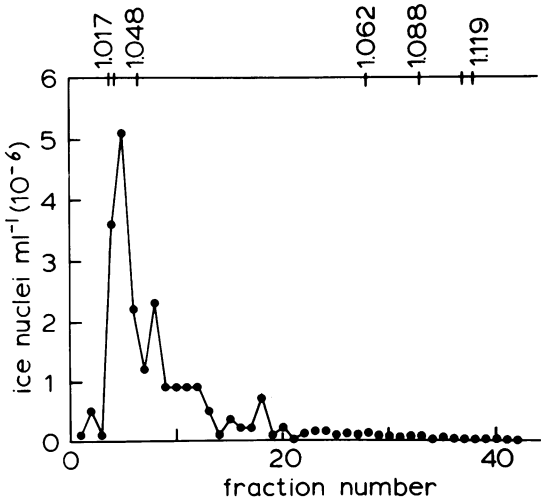

FIG. 2. Percoll gradient of cell-free ice nuclei from *E. herbicola* M1. Cell-free nuclei were obtained from mitomycin C-induced cells and concentrated by ultracentrifugation as described in the text. Percoll was added to give an initial density of 1.049 g/ml, and the gradient was generated by centrifugation for 100 min at 18,500 × *g*. Fractions (1 ml) were collected, and the density was measured by the distance of density marker beads from the meniscus.

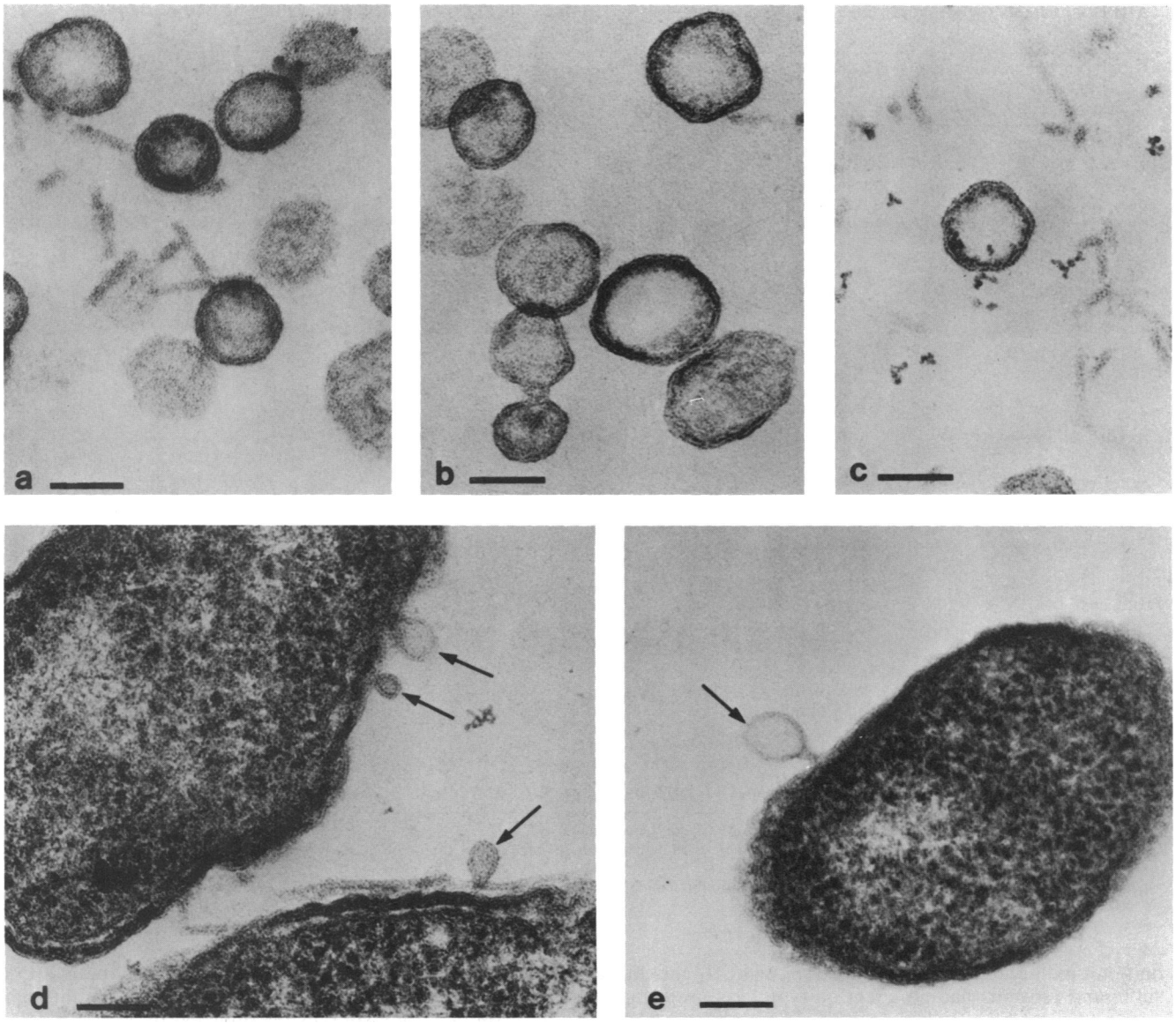

FIG. 3. (a) Preparation containing cell-free ice nucleation activity, obtained by passing a culture supernatant through a 0.22-μm filter. Membrane-limited vesicles and flagellar fragments are the two major visible constituents. (b) Combined fractions from Percoll gradient (see Fig. 2) containing the peak ice nucleation activity (ρ = 1.03 to 1.05 g/ml) are enriched in vesicles and depleted of flagella. (c) Percoll fractions collected at higher densities ($\rho \cong 1.055$ g/ml) exhibit little ice-nucleating activity (see Fig. 2), are enriched in flagellar fragments, and contain very few vesicles. (d and e) Thin-section electron micrographs of *E. herbicola* M1, showing outer membrane blebs and vesicles (arrows). (d) Uninduced culture. (e) Culture induced by addition of mitomycin C (1 μg/ml). Bars, 0.1 μm.

quency (morphometric analyses were not done). No evidence of outer-membrane blebbing was found in an INA strain of *P. syringae* which did not shed ice nuclei.

Some characteristics of *E. herbicola* M1 cell-free ice nuclei. While nuclei shed into growth media could be stored for weeks at 0 to 4°C with no loss in activity, they were rapidly and irreversibly inactivated by heating at temperatures over 30°C. Incubation at 35°C for 5 min destroyed 90% of type I nuclei and 60% of type II nuclei, while type III nuclei were virtually unaffected. Type I cell-free nuclei were found to be stabilized by divalent cations such as Ca^{2+}, Mg^{2+}, and Mn^{2+}. By serially diluting nuclei into various concentrations of $MgCl_2$, 10 mM was found to maximize the concentration of type I nuclei. Nuclei diluted in 0.1 mM $MgCl_2$ had only 26% of the level of type I nuclei found with dilution in 10 mM $MgCl_2$. This effect was reversible in that addition of either $MgCl_2$ or $CaCl_2$ to 10 mM resulted in 100% recovery of type I nuclei.

Previous work by Kozloff et al. (10) showed that cell-associated ice nuclei in an *E. herbicola* and in *P. syringae* strains were susceptible to a variety of sulfhydryl-modifying reagents. In agreement with that work, cell-associated type I nuclei in *E. herbicola* M1 were found to be susceptible to low levels of *N*-ethyl maleimide and *p*-hydroxymercuribenzoate (Table 2). Hydrogen peroxide and 5,5′-dithio(2-nitrobenzoate) also had a small effect on cell-associated nuclei, and iodine was very effective in inactivating type I nuclei. In contrast to these results, cell-free nuclei were susceptible

TABLE 2. Effects of sulfhydryl modification and proteolysis on cell-associated and cell-free ice nucleation activity (type I) from *E. herbicola* M1

Reagent	Concn	Incubation conditions[a]	% Loss of type I nuclei		% Loss of viability (complete[c])
			Cell-free[b]	Complete[c]	
Sulfhydryl reagents					
Iodine	0.001 mM	0.024 M KI, 0.05 M borate, pH 8.0	20	27	60
	0.005 mM		30	95	100
	0.010 mM		44	97	100
	0.020 mM		65	100	100
N-Ethyl maleimide	0.05 mM	0.04 M Tricine, pH 7.5	0	50	70
	0.50 mM		0	71	95
	5.00 mM		0	93	100
p-Hydroxymercuribenzoate	0.05 mM	0.04 M Tricine, pH 7.5	0	0	0
	0.50 mM		0	64	50
	2.50 mM		0	94	72
Hydrogen peroxide	1.0 mM	0.04 M lutidine, pH 8.4	0	0	51
	4.0 mM		0	21	91
	10.0 mM		0	36	100
5,5'-Dithio (2-nitrobenzoate)	0.5 mM	0.04 M lutidine, pH 8.4	0	0	29
	2.0 mM		0	18	37
	5.0 mM		0	47	52
Proteases					
Elastase	42 U/ml	0.05 M Tris, pH 8.8	62	ND[d]	ND
	83 U/ml		85	0	0
Pronase	0.22 U/ml	0.05 M Tris, pH 7.5	40	ND	ND
	0.56 U/ml		80	ND	ND
	1.12 U/ml		96	0	0
	11.2 U/ml		100	12	0
Thermolysin	90 U/ml	0.05 M Tris, pH 7.5	79	0	0

[a] All incubations were done in the presence of 10 mM $MgCl_2$ for 1.5 h on ice.
[b] Cell-free ice nuclei were obtained by growth of strain M1 in minimal lactate medium at 15°C to log phase, followed by filtration of cells, as described in the text.
[c] Cell-associated nuclei were prepared by growth of M1 in minimal lactate medium at 22°C to log phase; the complete culture was used.
[d] ND, Not determined.

only to iodine treatment. Incubation with all the other sulfhydryl reagents had no effect on type I cell-free nuclei, even under conditions which inactivated greater than 90% of the cell-associated nuclei (Table 2).

For each sulfhydryl reagent, the viability of cells following treatment was assessed by plating on TYG agar. In every case, cell viability decreased with increasing concentration of reagent (Table 2).

Proteases have been reported to inactivate bacterial ice nuclei (12). Cell-free nuclei and parental *E. herbicola* M1 cells were tested for sensitivity to some proteases. Although cell-free type I nuclei were susceptible to all the proteases tested, only pronase had even a small effect on the cell-associated nuclei. In addition, the proteases were found to have no effect on cell viability (Table 2).

DISCUSSION

Earlier workers have failed to separate ice nuclei active at temperatures warmer than −5°C from INA bacterial cells (12, 17, 29, 32, 33), leading to speculation that a physically intact or physiologically normal cell is required for expression of type I nuclei. In screening a large number of *Pseudomonas* and *Erwinia* INA strains, we found that although room-temperature cultures do not release type I nuclei, 11 of 12 *Erwinia* isolates do so when cultured at 15°C. In each case, these cell-free type I nuclei passed through a 0.22-μm filter and were uncontaminated by viable cells.

Cell-free nuclei were isolated from *E. herbicola* M1 for further studies. Following filtration, the nuclei were pelleted by ultracentrifugation and banded by isopycnic centrifugation on a Percoll density gradient. Following removal of the Percoll, electron microscopy showed that ice nuclei copurified with membrane vesicles and separated from flagellar components. Since type I nuclei both sedimented with vesicles under high centrifugal force and banded with vesicles on a density gradient, it is likely that they were associated with the vesicles.

Shedding of outer membrane particles has been reported in normally growing cultures of a variety of gram-negative bacteria, including *Escherichia coli*, *Salmonella typhimurium*, *Aeromonas* sp., *Bacteroides succinogenes*, *Vibrio cholerae*, *Neisseria* sp., and *Actinobacillus actinomycetemcomitans* (5, 7, 15, 21, 27). Electron microscopy of *E. herbicola* M1 showed that the cells were blebbing outer membrane protrusions and shedding vesicles into the medium.

Unfortunately, sucrose at the concentrations used in density gradients was found to inactivate type I cell-free nuclei, and too little material was obtained from analytical-scale

Percoll gradients for definitive detection of outer membrane markers. Large-scale isolation and characterization of these nuclei will be reported elsewhere. The ice nucleation vesicles banded at 1.03 to 1.05 g/ml on a Percoll gradient, a density considerably lower than that expected for outer membranes from most gram-negative strains isolated on sucrose gradients (23). Percoll gradients, however, have consistently been shown to give buoyant densities considerably lower than those on sucrose gradients (24). Cell walls from a number of gram-positive bacteria were found to band in a number of density gradient media between 1.25 and 1.50 g/ml, but banded on Percoll between 1.03 and 1.055 g/ml (8). Percoll gradients have not been routinely used to isolate membranes from gram-negative bacteria (M. J. Osborn, personal communication). We have isolated *E. herbicola* M1 membranes by spheroplasting, lysis, and differential centrifugation (23), but were unable to separate cytoplasmic from outer membrane fractions on Percoll, as determined by an assay of keto-deoxyoctanoate and NADH oxidase, outer and cytoplasmic membrane markers, respectively (22). The total membranes from *E. herbicola* M1 banded at 1.02 g/ml on Percoll, a value lower than that of the cell-free ice nuclei. Since cytoplasmic membranes of gram-negative bacteria usually have a lower buoyant density than outer membranes (23), this observation indirectly supports the idea that cell-free ice nuclei are associated with outer membrane vesicles. The direct measurement of membrane markers will ultimately prove whether these ice nucleation vesicles are derived from cytoplasmic or outer membranes.

The level of outer membrane shedding detected by electron microscopy did not appear to be related to the ice nucleation frequency of the culture. Cultures induced only by growth at low temperature (about 1 ice nucleus per 1,000 cells) and cultures induced by addition of mitomycin C (about 1 ice nucleus per cell) both exhibited about the same frequency of blebbing. In each case, 10 to 20% of the total ice nuclei in the culture were recovered in the cell-free fraction. We suggest that only a fraction of the resulting vesicles contain an ice nucleus, since the probability that a vesicle will "pick up" an ice nucleus may depend on the level of expression of ice nuclei in the outer membrane.

Isolation of cell-free nuclei should facilitate biochemical characterization of these structures and also provide a system that uncouples physiological effects on a whole cell from direct effects on the nuclei. For example, Kozloff and co-workers (10) have shown that ice nucleation activity in intact *P. syringae* and *E. herbicola* strains is sensitive to sulfhydryl reagents such as *N*-ethyl maleimide, *p*-hydroxymercuribenzoate, and iodoacetamide, suggestive of a role for an essential protein sulfhydryl group in the ice nucleation site. Here we have shown (Table 2) that although the ice nucleation activity of *E. herbicola* M1 whole cells was extremely sensitive to many cysteine-modifying agents, the cell-free activity was inert to all but iodine. Iodine is a less specific sulfhydryl modification reagent than the other reagents tested; tyrosine and histidine are as susceptible to iodination as is cysteine (19). Since inactivation of cell-associated ice nuclei was better correlated with loss of cell viability than with loss of ice nuclei in cell-free preparations (Table 2), it is likely that the sulfhydryl reagents exert their effect on cell-associated ice nuclei indirectly by their toxicity to the cells.

The susceptibility of cell-free ice nuclei to proteases and amino acid modification reagents (Table 2) indicates that proteins participate in the ice nucleation event. This is consistent with recent reports that cloned ice nucleation genes from *Pseudomonas* strains encode a 180-kDa protein that presumably is integral to the ice nucleation site (2, 6). We are working to characterize the molecular components of bacterial ice nuclei, including the 180-kDa protein and other membrane components, and to investigate how they order surface water molecules and trigger ice formation. The ability to isolate cell-free ice nuclei as stable outer membrane vesicles is a key step towards this goal.

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