Localization of Ice Nucleation Activity and the *iceC* Gene Product in *Pseudomonas syringae* and *Escherichia coli*

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Ice nucleation activity and the *iceC* gene product were quantified in different subcellular fractions of the *Pseudomonas syringae* source strain and in *Escherichia coli* containing the cloned *iceC* gene to determine the activity of this protein in different subcellular locations. Ice nuclei were nearly completely retained during isolation of cell envelopes but exhibited a decrease in the temperature at which they were expressed. Ice nucleation activity was found in Triton X-100 insoluble membrane fragments as well as in slowly sedimenting and high-density membrane fragments. Nearly all ice nucleation activity was associated with the outer membrane because the partitioning of 3-ketodeoxyoctonate (a lipopolysaccharide component) and ice nuclei in cell fractions were similar to and opposite that of NADH oxidase (a cytoplasmic membrane component). The *iceC* gene product had an apparent mass of 150,000 Da based on migration in SDS-polyacrylamide gels. This protein was not found in soluble cell components. Nearly all of the *iceC* gene product, which occurred in low abundance, was associated with the outer membrane of both *P. syringae* and *E. coli*. Therefore, the *iceC* gene product is located at and is maximally active in or on the outer membrane of cells of the source strain and heterologous strains.

Heterogeneous ice nucleation, the catalysis of ice formation in supercooled water, is exhibited by some strains of *Pseudomonas syringae* van Hall, *P. viridiflava* (Burkholder) Dowson, *Erwinia herbicola* (Lohnis) Dye, *Xanthomonas campestris* pv. *translucens* (Jones *et al.*) Dye, and *P. fluorescens* Migula (Hirano *et al.* 1978; Kim *et al.* 1987; Lindow *et al.* 1978b; Maki *et al.* 1974; Maki and Willoughby 1978; Paulin and Luisetti 1978; Yankofsky *et al.* 1981a). These bacteria are among the most efficient biological ice nucleating agents in nature, catalyzing ice formation at temperatures as warm as −1 to −2° C (Kim *et al.* 1987; Lindow *et al.* 1978b; Lindow *et al.* 1978c).

Many ice nucleation active (INA) pathovars of *P. syringae* (Dye *et al.* 1980; Hirano *et al.* 1978; Paulin and Luisetti 1978) and strains of *E. herbicola* occur in large numbers as epiphytes on healthy plants in nature (Lindow *et al.* 1978a; Lindow *et al.* 1978c; Yankofsky *et al.* 1981a). These epiphytic bacteria can injure the frost-sensitive plants on which they reside by initiating ice formation (Arny *et al.* 1976), which causes frost damage (Burke *et al.* 1976; Levitt 1972). Ice formation may facilitate the invasion of plants by *P. syringae* (Süle and Seemüller 1987) or permit the colonization of damaged plant tissues (Panagopoulos and Crosse 1964). Although some woody plants may contain nonbacterial sources of ice nuclei (Ashworth *et al.* 1985), most frost-sensitive plants are devoid of nonbacterial ice nuclei active at temperatures above −5° C and INA bacteria are thus a major limiting factor in the survival of these plants at temperatures above −5° C. These INA bacteria may also have significance in global climatology by contributing atmospheric freezing nuclei that are important in precipitation processes (Lindemann *et al.* 1982; Vali *et al.* 1976).

Some characteristics of bacterial ice nucleation sites have been reported. Ice nucleation activity of some *P. syringae* strains is associated only with intact cells and is not found in culture filtrates (Maki *et al.* 1974). The ice nucleation activity of *E. herbicola* occurs in membranous vesicles shed by some strains (Phelps *et al.* 1986). When *P. syringae* cells are disrupted, qualitative tests showed that ice nucleation activity was associated with the cell envelope and not with soluble cell contents (Maki and Willoughby 1978). The ice nucleation site of intact *P. syringae* cells is sensitive to lipases and phospholipases, 2-mercaptoethanol, sodium dodecyl sulfate (SDS), quaternary ammonium compounds, borate compounds, and lectins (Kozloff *et al.* 1983; Maki *et al.* 1974; Sprang and Lindow 1981; Wolber *et al.* 1986) indicating that although the site is at least partially proteinaceous, lipids and other membrane components may be a part of, or act to stabilize, the active catalytic site.

Genes determining the ice nucleation (Ice) phenotype have been cloned from two different *P. syringae* strains (*inaZ* and *iceC*) (Green and Warren 1985; Orser *et al.* 1985), *P. fluorescens* (*inaW*) (Warren *et al.* 1986), and *E. herbicola* (Orser *et al.* 1985). The *P. syringae* ice nucleation genes share considerable sequence homology (S. E. Lindow, unpublished). The translation products predicted from the nucleotide sequences of two ice nucleation genes, *inaW* and *inaZ*, are proteins with a repetitive primary structure and sizes of 120,000 and 119,000 Da, respectively; their apparent sizes as estimated by SDS-PAGE, however, are 180,000 and 153,000 Da, respectively (Green and Warren 1985; Warren *et al.* 1986; Wolber *et al.* 1986).

Knowledge of the location of the ice nucleation activity in the bacterial cell will be of critical importance in understanding mechanisms of ice nucleation by these bacteria. The catalysis of ice formation in different locations within or on bacterial cells would involve different mechanisms

of ice propagation to extracellular sites, and may also require different self-defensive mechanisms against freezing. Knowledge of the subcellular location of ice nucleation would also help to determine the regulation of ice nucleus formation and function and to identify other cellular components that influence ice nucleation activity in these bacteria. The cell envelope of gram-negative bacteria is composed of a cytoplasmic membrane and an outer membrane that are separated by a thin peptidoglycan layer (Costerton et al. 1974). Both membranes contain protein and phospholipids, with the outer membrane also containing lipopolysaccharide (LPS). The inaZ gene product and ice nucleation activity were detected in both membranes when overexpressed in Escherichia coli (Migula) Castellani and Chalmers (Wolber et al. 1986). In wild-type P. syringae strains, the localization of the ice nucleation protein is unknown, and the efficiency of active ice nucleus formation and expression may differ greatly.

We report here the isolation and characterization of the cytoplasmic and outer membranes of P. syringae and E. coli containing the iceC gene by three different procedures, as well as the quantity of the iceC gene product and ice nuclei in different cellular locations in these cells, in order to ascertain the efficiency of expression of this phenotype in different sites in native cells.

MATERIALS AND METHODS

Organisms and culture conditions. The characteristics of P. syringae strains 31R1 and Cit7, and the cloning of the iceC gene from strain Cit7 and its expression in E. coli HB101 have been previously reported (Arny et al. 1976; Orser et al. 1985). In all studies, Ice$^+$ E. coli HB101 (pICE1.2) was used (Orser et al. 1985). Cells were grown with vigorous agitation on a reciprocating shaker at 21° C to an absorbance of 1.8 at 600 nm in glycerol mineral broth (GMB) containing 0.5% glycerol, 0.1% (NH$_4$)$_2$SO$_4$, 0.01% MgSO$_4$, 0.1% KH$_2$PO$_4$, 0.35% KH$_2$PO$_4$, pH 7.0. Cells were also grown for 3 days at 21° C on plates of mannitol-aspartic acid agar (MAA) containing 1% mannitol, 0.5% L-aspartic acid, 0.17% Na(NH$_4$)HPO$_4$·4 H$_2$O, 0.01% MgSO$_4$, 0.75% NaH$_2$PO$_4$, 0.075% KCl, and 1.5% Bacto agar, pH 6.8. Cells were harvested from GMB by centrifugation and from MAA by scraping the plates, and they were washed once in 0.05 M Tris-HCl buffer (pH 7.5). Cells grown in GMB were used fresh for isopycnic sucrose density gradient centrifugation. Cells harvested from MAA and GMB were frozen at −20° C until used in differential sedimentation separations and Triton X-100 extractions, respectively.

Cell disruption and membrane isolation. Cells harvested from MAA were suspended at a concentration of 50 mg of cells per milliliter of 0.01 M Tris-HCl, 0.01 M MgCl$_2$ (pH 8.0), and 20% sucrose. After the addition of 3.0 mg of lysozyme (30 μg/ml), the cell suspension was gently stirred for 1 hr at 20° C. Spheroplasts were harvested by centrifugation at 13,000 × g for 10 min and resuspended in 20 ml of 0.01 M potassium phosphate and 0.01 M MgCl$_2$ (pH 6.2). Resuspended spheroplasts were disrupted by sonication for 2 min at 0° C (Heat Systems Ultrasonics, Farmingdale, NY, Model 350). Intact cells and spheroplasts were removed by centrifugation at 1,800 × g for 10 min. Crude membranes were collected by centrifugation of the

supernatant at 68,000 × g for 90 min and stored at −20° C.

Cells harvested from GMB were suspended in 50 mM Tris, 0.5 M sucrose, 0.2 mM dithiothreitol (pH 7.5). Ribonuclease and deoxyribonuclease (each at 33 μg/ml) were added, and cells were disrupted by passage through a French pressure cell at 18,000 pounds per square inch. Intact cells were removed by centrifugation at 2,500 × g for 5 min. Crude membranes were collected by centrifugation of the supernatant at 125,000 × g for 1.5 hr.

Separation of inner and outer membranes. Three different protocols were used to separate the cytoplasmic and outer membranes of INA bacteria. Cytoplasmic and outer membranes were separated by differential sedimentation velocity, based on a procedure of Matsushita et al. (1978). Cytoplasmic and outer membranes were separated due to differential Triton X-100 solubility, similar to the method of Schnaitman (1971). Sucrose density gradient centrifugation was performed by a method similar to that of Osborn et al. (1972). Crude membranes were resuspended in 0.05 M Tris-HCl buffer (pH 7.5), containing 25% sucrose, to a concentration of approximately 10 mg of protein per milliliter. Approximately 1 ml of this solution was layered on a sucrose gradient and centrifuged in a SW41 rotor at 37,000 rpm for 18 hr. Sucrose gradients were prepared by layering 1.8 ml of sucrose solutions of 55, 50, 45, 40, 35, and 30%, respectively, on top of 0.5 ml of 60% sucrose. All sucrose solutions were in 0.05 M Tris, pH 7.5. Gradient tubes were stored at 2° C for 8 hr before use. Gradients were fractionated by collecting 0.6-ml samples from the top of the gradient by displacement using an ISCO fractionator.

Chemical analysis. Protein content was determined using the Bio-Rad (Richmond, CA) protein dye binding assay (Bradford 1976). 2-keto-3-deoxyoctonate (KDO) was determined by the thiobarbituric acid assay of Osborn (1963). NADH oxidase activity was measured as the rate of decrease in absorbance at 340 nm at 20° C after the addition of NADH (Osborn et al. 1972).

Polyacrylamide gel electrophoresis. Protein composition was analyzed by SDS-PAGE using the method of Laemmli (1970). Samples were heated at 100° C for 2 min in a sample buffer containing SDS and β-mercaptoethanol before loading. The gels were stained with Coomassie Brilliant Blue R-250.

Measurement of ice nucleation activity. Ice nuclei active at −9° C were quantified by droplet freezing assays similar to that described previously (Lindow et al. 1978b; Vali 1971). Measurements of ice nucleation activity of membrane fractions at different temperatures were made by a procedure similar to that reported previously (Lindow et al. 1978b).

Protein identification in E. coli minicells. Plasmids pBR322, pICE1.2 (pBR322 containing the iceC gene BglII-EcoRI fragment), and pICE1.2d (pICE1.2 containing a 1.5-kilobase (kb) internal deletion to iceC) (Orser et al. 1985) were transformed into E. coli minicell-producing strain P678-54 (Meagher et al. 1977). Minicells were harvested, purified on sucrose gradients, and analyzed essentially as previously described (Roozen et al. 1971; Meagher et al. 1977).

Construction of pTB5. The P*tac* vector used, pTB5, was derived from plasmid pKK223-3 (Pharmacia 27-4935-01) by the following steps: the 1.1-kb HindIII fragment

encoding the *lacI*^{Q} repressor was excised from plasmid pMMB22 (Bagdasarian *et al.* 1983) and inserted in the *Hin*dIII site of pKK223-2 to produce plasmid pTACIQ. This vector ensured the production of high amounts of *lac* repressor and, therefore, more efficient repression of potentially deleterious proteins in hosts not containing a *lacI*^{Q} allele. The plasmid was further modified by replacing the pUC8 multilinker with a portion of the multilinker from plasmid πVX (Maniatis *et al.* 1982). To accomplish this, the pTACIQ plasmid was first linearized with *Eco*RI and then partially digested with *Pst*I (a second *Pst*I site is present in the pBR322-derived ampicillin-resistant gene of pKK223-3 and pTACIQ). The πVX multilinker was derived from plasmid pWB5a (Ronson *et al.* 1987) after digestion with *Eco*RI plus *Pst*I and extraction of the 44-bp multilinker fragment from an acrylamide gel. The molecules mentioned above were ligated and transformed into HB101. Plasmids from ampicillin-resistant transformants were tested for multilinker substitution by digestion with *Bgl*II (present in the πVX multilinker portion, but absent from pTACIQ) and *Sal*I (only one site expected in the final construct). One plasmid, pTB5 (Fig. 1), met the criteria described above, and its structure was further confirmed by digestion with other appropriate enzymes.

tac **promoter-*iceC* fusion.** A clone containing the 4.8-kb *iceC Bgl*II-*Eco*RI fragment inserted between the *Bam*HI and *Eco*RI sites of the pUC8-derived multilinker in pLAFR3 (Staskawicz *et al.* 1987) was initially mutagenized

by insertion of Tn*3*HoHo1 to produce *iceC::lacZYA* fusions by the procedure of Stachel *et al.* (1985). An in-frame fusion (32) resulting in the production of a hybrid Ice*C*-β-galactosidase protein consisting of approximately three-quarters of the Ice*C* protein (N-terminal part) and expressing high β-galactosidase activity was chosen for the construction of the P*tac* promoter fusions as outlined in Figure 1. The construction steps were designed to remove potential transcriptional terminator elements upstream of the *iceC* promoter and to position the translation initiation codon of *iceC* at the proper distance from the Shine-Dalgarno sequence present in the P*tac* vector, pTB5.

*Bal*31-trimmed *iceC::lacZYA* molecules were ligated to pTB5 vector DNA with appropriately modified ends (Fig. 1) so that the N-terminal part of the fusion protein would be proximal to the *tac* promoter. The ligation products were transformed into *E. coli* MC1061 (a *lacIPOZYA* deletion strain), and the entire population was grown overnight in liquid M9 medium supplemented with 0.2% lactose as a sole carbon source plus leucine to enrich for transformants that expressed high levels of β-galactosidase activity. The enriched population was then plated on Luria-X-gal medium and dark blue colonies were selected for further analysis.

Antibody production and immunochemical analysis. The purified *iceC-lacZ* fusion protein from *E. coli* MC1061(pD124) was used to elicit antibodies. The antigen was purified by affinity chromatography using *p*-amino-phenyl-β-D-thiogalactoside (Ullmann 1984) to recognize β-
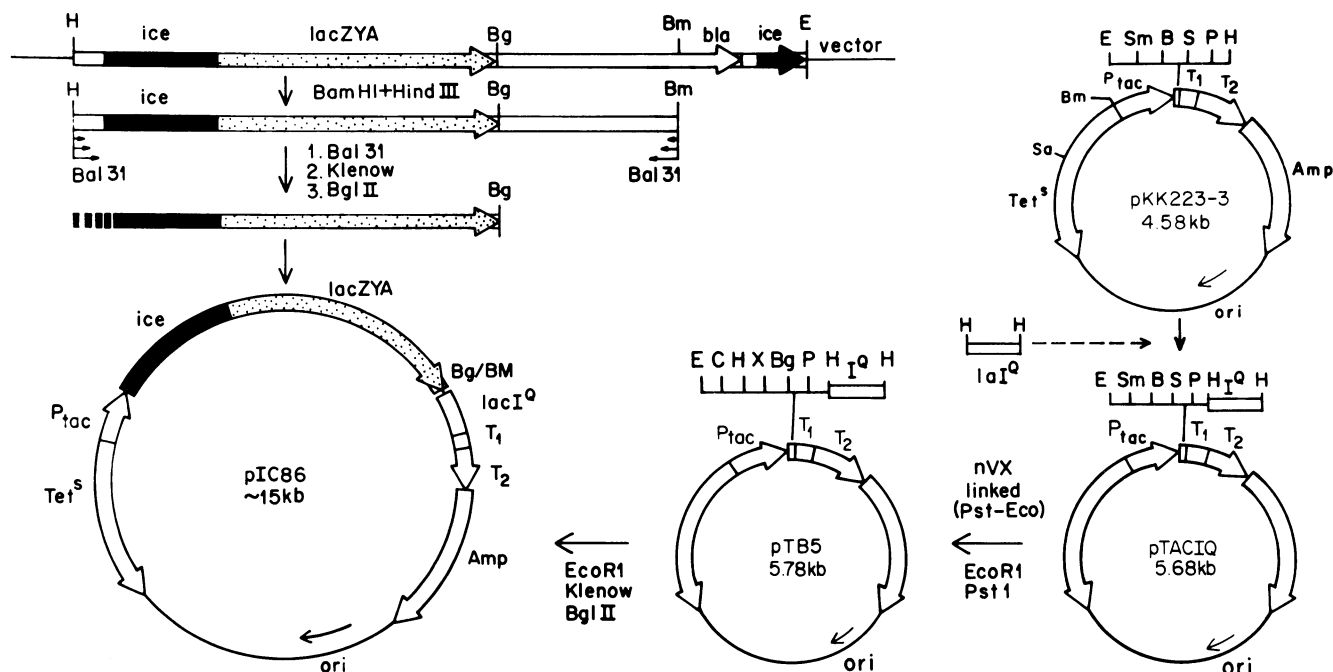
Fig. 1. Construction of *tac* promoter-Ice*C*-β-galactosidase fusion. The source plasmid contained an *iceC::lacZ* fusion (32) and produced an Ice*C*-β-galactosidase fusion protein consisting of the approximately three-quarter N-terminal part of the Ice*C* protein and expressing high β-galactosidase activity. Restriction sites for *Hin*dIII, *Eco*RI, *Sma*I, *Sal*I, *Pst*I, *Xho*I, *Cla*I, and *Bgl*I are indicated by the abbreviations H, E, Sm, S, P, X, C, and Bg, respectively. Restriction sites for *Bam*HI are indicated by B or Bm. T$_1$ and T$_2$ represent ribosomal RNA transcriptional terminators. The *Hin*dIII-*Bam*HI fragment containing this fusion was excised and treated with *Bal*31 to remove sequences lying upstream of *iceC*. The *Bal*31-trimmed molecules were repaired by filling in with the Klenow fragment of DNA polymerase I and further digested with *Bgl*II to remove the non-*lacZYA* portion and to produce ends suitable for directional cloning. The *tac* promoter vector pKK223-3 was modified by cloning the *lacI*^{Q} fragment from pMMB22 at the *Hin*dIII site of the pUC8 multilinker to produce plasmid pTACIQ and by replacing the *Pst*I-*Eco*RI segment of the multilinker with part of the πVX multilinker. The resulting plasmid, pTB5, was converted to a form suitable for directional cloning of the *iceC::lacZYA* molecules, which are described above, by digestion with *Eco*RI, filling in the ends with the Klenow fragment of DNA polymerase, and digestion with *Bgl*II.

galactosidase residues and then by preparative SDS-PAGE. The large fusion protein band was excised from gels, pulverized, and suspended in a small amount of phosphate-buffered saline (Rio et al. 1986). A high-titer antiserum was produced by injecting 80 μg of the aforementioned antigen intramuscularly and subcutaneously into New Zealand white rabbits followed by a 40-μg booster injection 4 wk later.

Immunoblot (western blot) analysis was conducted essentially as described by Burnette (1981). SDS-PAGE (100 μg of protein per lane) of total cell lysates was conducted according to Laemmli (1970) and proteins were electrotransferred to nitrocellulose paper (Burnette 1981). Filters were incubated with fusion protein antibodies at a 1:1000 dilution and probed with affinity-purified alkaline phosphatase-conjugated goat anti-rabbit IgG (Bio-Rad) as a second antibody (Smith and Fisher 1984).

RESULTS

The ice nuclei of P. syringae were located almost exclusively in the cell envelope (Table 1). Ice nuclei active at −9° C from cellular materials less than 0.22 μ in diameter obtained from cell washings or culture filtrates of cells grown in GMB, from osmotically shocked cells, or from cells or membrane vesicles treated with 2 M NaCl were always less than 0.03% of the total ice nuclei contained in a sample of P. syringae cells (data not shown). Little or no loss of ice nucleation activity at −9° C was observed following spheroplast formation. While only 19% of the ice nucleation activity of disrupted P. syringae cells was recovered in cell membranes, only 0.01% of this activity remained after high-speed centrifugation (Table 1). The relatively low recovery of ice nucleation activity of P. syringae was attributed to the tenacious aggregation of the membrane vesicles, because it is possible to quantify only the number of independent and physically separable ice nuclei by the droplet freezing method used here. Extensive sonication of P. syringae membrane vesicles isolated by centrifugation increased the apparent yield of ice nuclei active at −9° C approximately fourfold (data not shown).

Table 1. Ice nucleation activity at −9° C of isolated membranes of Pseudomonas syringae strain 31R1

Cellular fraction	Ice nuclei recovered ($\times 10^{10}$)
Starting cell suspension	10.6
Spheroplasts[a]	14.1
Disrupted spheroplasts[b]	13.6
Membrane vesicles and soluble cell contents[c]	10.1
Soluble cell contents[d]	0.001
Total membranes[e]	1.90

[a]Mixture of intact cells and spheroplasts following lysozyme treatment in 0.01 M Tris-HCl buffer (pH 8.0) containing 0.01 M MgCl₂ and 20% sucrose.

[b]Disrupted spheroplasts and intact cells following sonication for 2 min in 0.01 M potassium phosphate buffer (pH 6.2) containing 0.01 M MgCl₂.

[c]Cellfree membranes and soluble cell contents recovered as the supernatant following centrifugation of disrupted spheroplasts at 1,800 × g for 10 min.

[d]Soluble cell contents recovered from the supernatant following centrifugation of cellfree disrupted spheroplasts at 68,000 × g for 90 min.

[e]Total cell membranes recovered from the material sedimented following centrifugation of cellfree disrupted spheroplasts at 68,000 × g for 90 min.

Ice nucleation activity of isolated P. syringae membranes.

The total number of ice nuclei measured at temperatures of −7° C or colder in samples of isolated membranes of P. syringae was identical to the number of ice nuclei measured at temperatures of −4° C or lower in the original intact cell suspension (Fig. 2). However, the number of ice nuclei active at temperatures warmer than −7° C was much lower in isolated membranes of P. syringae than at corresponding temperatures in intact cells (Fig. 2). Thus, although there is a qualitative change in the temperature at which ice nucleating sites in P. syringae exhibit this phenotype, the number of ice nuclei active at −7° C or lower apparently was conserved during membrane isolation. The ice nucleus content following all procedures was therefore determined at −9° C.

The ice nucleation activity of isolated membranes of P. syringae was rapidly eliminated by both trypsin and proteinase K. More than 90% of the ice nuclei in isolated P. syringae membranes were inactivated when 20 μg of proteinase K per milliliter was added within 20 min at 20° C, and more than 95% were inactivated within 60 min (Fig. 3). Addition of more proteinase K or longer incubation did not further decrease ice nucleation activity, suggesting that a small proportion of the ice nucleating sites were not accessible to the protease. P. syringae membrane fragments suspended in buffer alone showed no reduction in ice nucleation activity during the 160-min period reported (Fig. 3). While no significant inactivation of ice nuclei active at −9° C in whole P. syringae cells treated with proteinase K was observed, more than 96% of the ice nuclei were eliminated from suspensions of intact cells following exposure to either 7-diazonium 1,3-naphthylene disulfonate or P-mercuric benzoate (data not shown).

Membrane separation by differential sedimentation velocity. Membrane vesicles of different sedimentation velocity (presumably due to different particle size) were
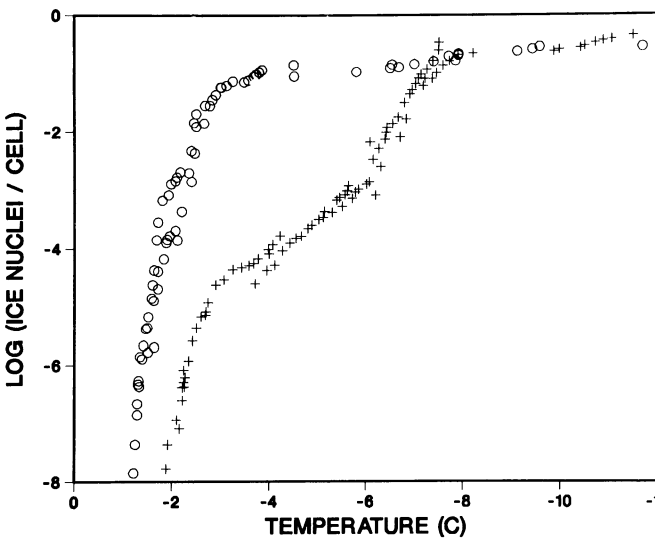

Fig. 2. Ice nucleation activity of intact cells of Pseudomonas syringae strain 31R1 harvested after 3 days growth at 21° C from minimal salts-mannitol agar (○) and outer membrane fragments collected by differential sedimentation velocity after disruption of these cells (+). Ice nucleation activity is expressed as the logarithm of the cumulative ice nucleus content of serial dilutions of samples at the temperatures shown on the abscissa, divided by the concentration of cells in the samples.

obtained following disruption of spheroplasts of *P. syringae* in the presence of 0.01 M MgCl₂. About one-half of the disrupted membranes were sedimented within 1 min at 35,000 × g as indicated by the similar protein content of both slowly and rapidly sedimenting membrane vesicles (Table 2). Ninety percent of the NADH oxidase activity recovered was associated with the rapidly sedimenting membrane fraction (Table 2). Eighty-one percent of the KDO and 82% of the ice nucleation activity recovered were associated with the slowly sedimenting membrane fraction. *E. coli* cells exhibited similar quantitative partitioning of KDO, NADH oxidase activity, and ice nucleation activity between slowly and rapidly sedimenting membrane fractions (data not shown).

Slowly and rapidly sedimenting membrane fractions differed considerably in their protein composition. Many proteins occurred in the total membrane preparation; however, six major protein bands and only about 15 minor

proteins were apparent in the slowly sedimenting membrane fraction (data not shown). In contrast, the rapidly sedimenting fraction was composed of numerous proteins found in low abundance.

Membrane separation by differential detergent solubilization. Treatment of *P. syringae* and *E. coli* membranes with 2% Triton X-100 solubilized 43% of the *P. syringae* membrane proteins and 85% of the *E. coli* membrane proteins within 20 min (Table 3). Triton X-100 treatment greatly reduced the activity of NADH oxidase in both the Triton X-100 soluble and insoluble membrane fractions. However, about 90% of the KDO and 99% of the ice nuclei recovered after Triton X-100 treatment of membranes of both species were associated with the insoluble membrane fractions. Numerous proteins found in similarly low abundance were observed in the Triton X-100 soluble membrane fraction; only seven major membrane proteins and 15 to 20 minor proteins were apparent in the Triton X-100 insoluble membrane fraction examined by SDS-PAGE (data not shown).

Membrane separation by isopycnic sucrose density gradient centrifugation. Three major and two minor translucent bands were observed after sucrose density gradient centrifugation of *P. syringae* strain Cit7 (Fig. 4). Two light bands migrated through approximately 20 and
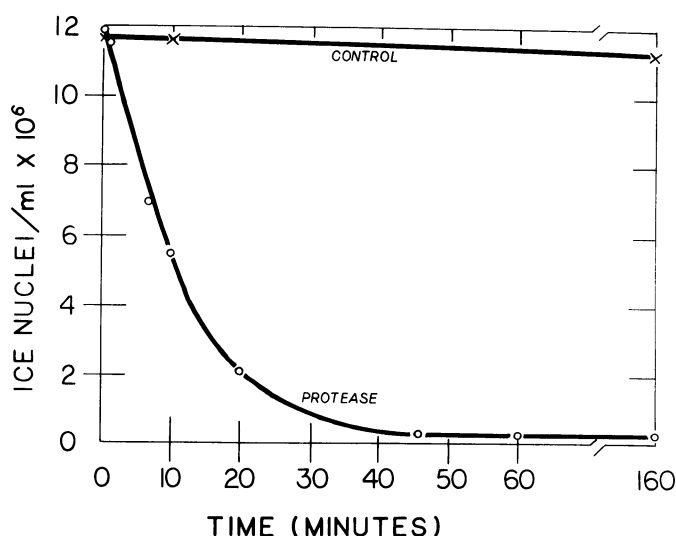
Fig. 3. Sensitivity of ice nucleation activity of isolated *Pseudomonas syringae* strain 31R1 membrane fragments to protease treatment. Total membrane fragments of approximately 1 × 10⁸ *P. syringae* cells grown on mannitol-aspartic acid agar were suspended in 0.01 M Tris-HCl buffer containing 20 μg of proteinase K per milliliter at time 0 (○) or in Tris-HCl buffer alone (×). The ice nucleation activity of samples at −9° C was measured at the times shown on the abscissa.

Table 2. Partitioning of ice nucleation activity and other components in *Pseudomonas syringae* membranes separated by differential sedimentation velocity[a]

Components measured	Slowly sedimenting vesicles[b] (outer membrane)	Rapidly sedimenting vesicles[c] (cytoplasmic membrane)
Protein[d]	9.0	10.8
NADH oxidase[e]	0.10	0.75
KDO[f]	145.	29.
Ice nuclei[g]	9.2 × 10¹⁰	1.7 × 10¹⁰

[a]Approximately 3 × 10¹¹ cells of *P. syringae* strain 31R1 containing 1.1 × 10¹¹ ice nuclei grown on mannitol-aspartic acid agar at 21° C.
[b]Membranes collected by centrifugation of combined supernatants at 43,000 × g and 35,000 × g from total membrane preparations.
[c]Membranes collected by centrifugation at 35,000 × g for 1 min.
[d]Protein expressed as milligrams per sample.
[e]Specific activity expressed as units per milligram of protein.
[f]Concentration of 2-keto-3-deoxyoctonate expressed as micromoles per milligram of protein.
[g]Ice nuclei per milligram of protein in each sample.

Table 3. Partitioning of ice nucleation activity and other components in *Pseudomonas syringae* and *Escherichia coli* membranes separated by Triton X-100 solubilization

Component measured	P. syringae[a]			E. coli[b]		
	Untreated	Soluble[c]	Insoluble[d]	Untreated	Soluble[c]	Insoluble[d]
Protein[e]	42	18	24	18.7	16	3
NADH oxidase[f]	31	0.6	0.6			
KDO[g]	4.6	0.5	4.4	2.4	0.2	1.6
Ice nuclei[h]	4.5	0.03	3.9	9.8	0.07	9.9

[a]Approximately 5 × 10¹¹ cells of *P. syringae* strain Cit7 containing about 5 × 10¹⁰ ice nuclei were disrupted with a French pressure cell before treatment with 2% Triton X-100.
[b]Approximately 10⁸ cells of *E. coli* HB101 (pICE1.2) containing about 10⁷ ice nuclei were disrupted with a French pressure cell before treatment with 2% Triton X-100.
[c]Total membrane material not sedimented after 2% Triton X-100 treatment and centrifugation at 69,000 × g.
[d]Total membrane material sedimented after 2% Triton X-100 treatment and centrifugation at 69,000 × g.
[e]Protein expressed as milligrams per sample.
[f]Specific activity expressed as units per milligram of protein.
[g]Concentration of 2-keto-3-deoxyoctonate expressed as millimoles per milligram of protein.
[h]Ice nuclei × 10¹⁰ for *P. syringae* and × 10⁶ for *E. coli* membrane preparations in each fraction.

35% of the density gradient, while three dense bands migrated through approximately 65, 80, and 95% of the density gradient. The first eight membrane fractions with an average buoyant density of about 1.15 contained the highest specific activity of NADH oxidase (Fig. 4). Membrane fractions 9 to 21 with apparent buoyant densities from 1.18 to 1.30 contained 93 and 87% of the KDO and the ice nuclei, respectively. Many of the ice nuclei were detected in membrane regions of intermediate density ($\rho = 1.17-1.23$). The least dense membrane fractions of *P. syringae* strain Cit7 were not contaminated with significant amounts of outer membrane components, but a significant amount of NADH oxidase was detected in the dense membrane fractions. Similar separations of inner and outer membranes of *P. syringae* strain 31R1 and
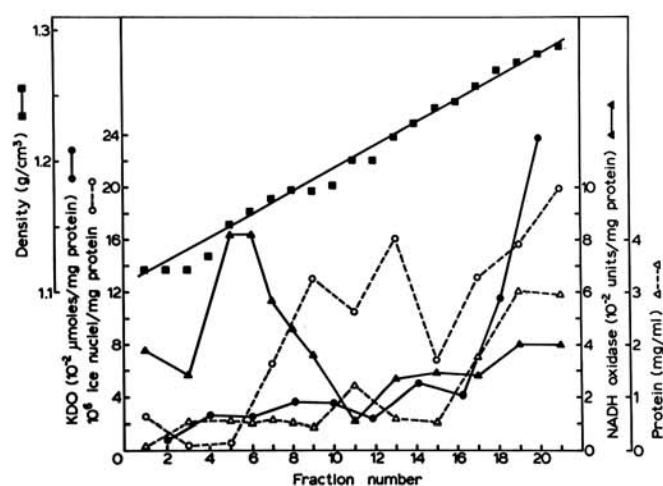
cofractionation of nucleation activity and KDO were observed (data not shown).

The protein composition of high- and low-density *P. syringae* strain Cit7 membrane fractions differed greatly (Fig. 5). Major membrane proteins and relatively few minor proteins characterized the dense membrane fractions; low-density membrane fractions contained more than 50 minor proteins that were poorly separated and had no predominantly major proteins. The lack of prominent bands in low-density (inner membrane) fractions corresponding to the intense bands of the outer membrane fractions confirms chemical analysis showing that little contamination by outer membranes occurred.

Membranes of *E. coli* were more unambiguously separated than *P. syringae* membranes into two major bands by sucrose density gradient centrifugation. As with *P. syringae* membranes, most ice nucleation activity cofractionated with KDO-containing membranes (Fig. 6). More than 90% of the NADH oxidase activity was associated with membrane fractions of low density ($\rho = 1.12-1.19$). More than 90% of the KDO content and more than 80% of the ice nucleation activity of cells were found in dense membrane fractions ($\rho = 1.20-1.23$). As observed in *P. syringae* membranes, some ice nucleation activity was associated with membrane fractions with densities intermediate ($\rho = 1.17-1.20$) to those containing the majority of either NADH oxidase activity or KDO content (Fig. 6).

Specificity of antibodies made toward fusion proteins. Polyclonal antibodies prepared against the *iceC-lacZ* fusion gene product were very specific for this antigen. The amount of the *iceC-lacZ* fusion protein produced, even under the control of the *tac* promoter, was low, as determined by SDS-PAGE (Fig. 7). This 268,000-Da protein, because of its great mass, was easily separable from other *E. coli* proteins by SDS-PAGE, and rather pure antigen

Fig. 4. Separation of membrane fragments of *Pseudomonas syringae* strain Cit7 by isopycnic sucrose density gradient centrifugation. Cells were grown in glycerol mineral broth (approximately 3×10^{11} cells containing 6×10^{10} ice nuclei). Cell membranes were separated according to density (■) and collected in 0.6-ml fractions (abscissa). Fractions were than assayed for NADH oxidase specific activity (▲), protein content (△), 2-keto-3-deoxyoctonate content (●), and ice nucleation activity (○).
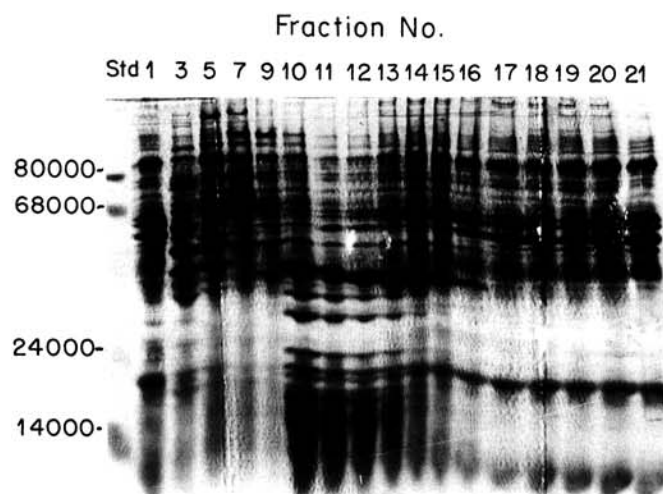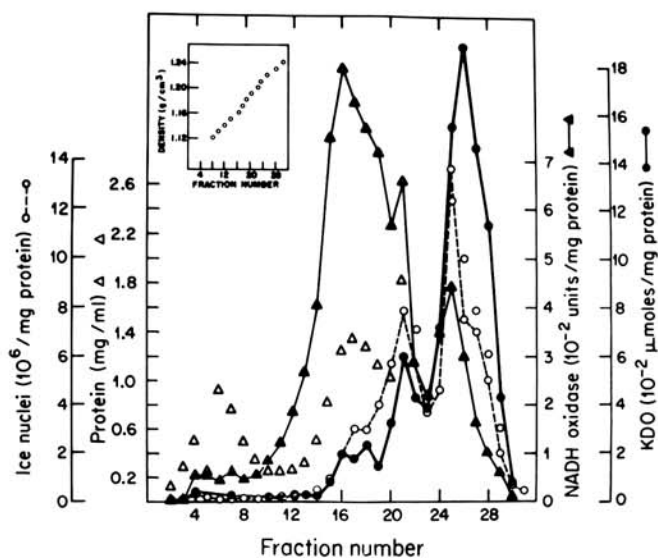

Fig. 5. Ten percent sodium dodecyl sulfate (SDS)-polyacrylamide gel of *Pseudomonas syringae* strain Cit7 membrane fractions (indicated by number) from isopycnic sucrose density gradient centrifugation. Molecular weight standards (Std) are shown by lines on the left. Fraction numbers (least dense on left, most dense on right) correspond to fractions analyzed in Figure 4.

Fig. 6. Separation of membrane fragments of *Escherichia coli* HB101 (pICE1.2) by isopycnic sucrose density gradient centrifugation. Cell membranes were separated according to density (insert) and collected in 0.6-ml fractions (abscissa). Fractions were then assayed for NADH oxidase-specific activity (▲), protein content (△), 2-keto-3-deoxyoctonate content (●), and ice nucleation activity (○).

preparations could be obtained by preparative SDS-PAGE. The antibodies elicited against this fusion protein recognized the fusion protein but not β-galactosidase (Fig. 7). Weak reactive bands of slightly smaller molecular size than the fusion protein, probably representing cleavage products of this protein, were observed in immunoblots (Fig. 7). No other immunologically reactive proteins were observed.
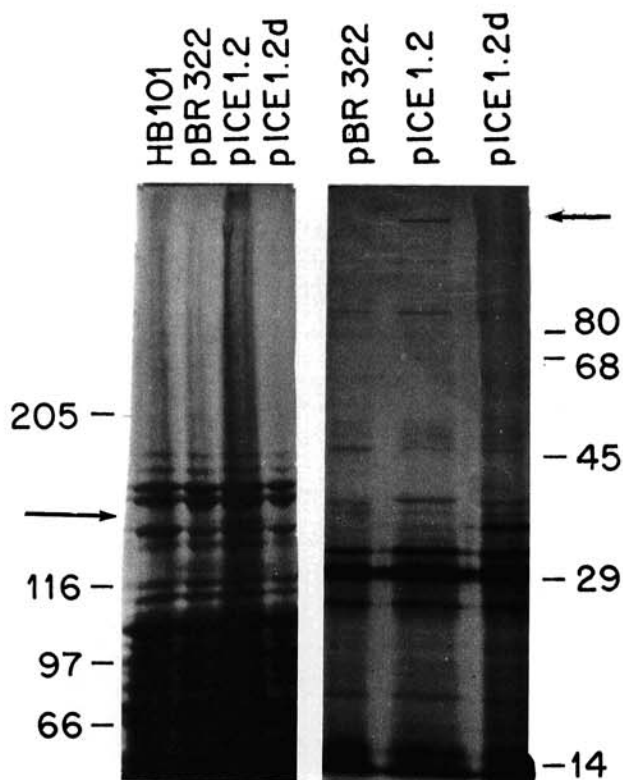
Identification of *iceC* gene product. The product of the *iceC* gene was a single large protein with an apparent mass, based on migration through SDS-polyacrylamide gels, of about 150,000 Da (Fig. 8). Only one protein, occurring in low abundance, with this mass was observed in a ^{35}S-methionine pulse-labeled minicell strain of *E. coli* containing the cloned *iceC* gene, but not in cells containing only the plasmid vector or in cells containing this cloned gene with an internal deletion (Fig. 8). Only one unique type of protein with a mass of about 150,000 Da was observed when the protein profile of this *E. coli* strain containing pICE1.2 was compared with strains containing one of the two other plasmids as controls when examined in two-dimensional SDS-PAGE after ^{35}S-methionine pulse-labeling (data not shown). A single type of protein, also with a mass of 150,000 Da, was detected in Ice$^+$ *E. coli* and *P. syringae* strains by immunoblot analysis using antibodies raised against the *iceC-lacZ* fusion protein (Figs. 9 and 10). The relative abundance of the *iceC* gene product detected in *E. coli* by ^{35}S autoradiography and immunoblot analysis was similarly low (Figs. 8 and 9). The *iceC* gene product was found in lower abundance in the *P. syringae* source strain than in *E. coli* containing this gene on the multicopy plasmid pBR322 (Figs. 9 and 10).

Localization of the *iceC* gene product. The content of the *iceC* gene product varied greatly between different cellular and membrane fractions of the *P. syringae* source strain, Cit7, and in *E. coli* HB101 containing this cloned gene. No *iceC* gene product was detected by immunoblots of proteins either in soluble fractions of *P. syringae* and *E. coli* (Figs. 9 and 10) or in culture filtrates of *E. coli* (Fig. 9). In contrast, the *iceC* gene product was readily detected in whole cell or total membrane preparations of *P. syringae* and *E. coli* (Figs. 9 and 10). Only about 20% as much of the *iceC* gene product was detected in inner membrane fractions of *E. coli* as in outer membrane fractions based on the intensity of bands of immunoblots (Fig. 9). While the quantity of the *iceC* gene product in *P. syringae* was apparently much lower than in *E. coli* HB101 (pICE1.2), this protein was detected only in outer
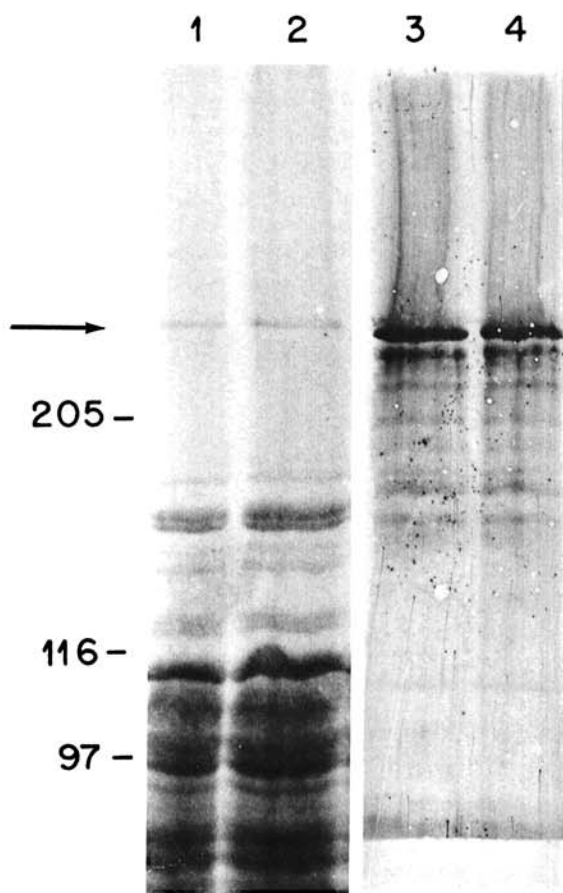
Fig. 7. Visualization of the *iceC-lacZ* fusion gene product in cells of *Escherichia coli* MC1061(pD124) from total cell protein extracts separated by sodium dodecyl sulfate (SDS)-PAGE and stained with Coomassie Brilliant Blue R-250 (lanes 1 and 2) or analyzed by immunoblotting with antibodies directed against the fusion protein (lanes 3 and 4), indicated by the arrow, with an estimated molecular mass of 268,000 Da. The migration of molecular mass standards (in kDa) is indicated on the left.

Fig. 8. Identification of the *iceC* gene product in total cell protein extracts of *Escherichia coli* minicell strain P678-54 (right panel) containing plasmids pBR322 (lane 1) or pICE1.2d (lane 3) conferring an Ice$^-$ phenotype or pICE1.2 (lane 2) conferring an Ice$^+$ phenotype. Total cellular proteins of *E. coli* HB101 containing pICE1.2 (lane 3), pICE1.2d (lane 4), pBR322 (lane 2), or no plasmids (lane 1) were pulse-labeled with ^{35}S-methionine and are also shown (left panel). ^{35}S-methionine pulse-labeled cell proteins of both strains were separated by sodium dodecyl sulfate (SDS)-PAGE and autoradiographed for 5 days. Note the presence of a large protein with a mass of about 150,000 Da (indicated by the arrow) in cells containing pICE1.2 but not pBR322 or pICE1.2d. The migration of molecular mass standards in kDa is indicated on the left and right.

membrane fractions and not in cytoplasmic membranes of the source strain (Fig. 10).

DISCUSSION

Several different methods of separating cytoplasmic and outer membranes of *P. syringae* were used, because the methods developed for other species each did not give optimal separation in this species. Isopycnic sucrose density gradient centrifugation has been used to separate the cytoplasmic and outer membranes of *E. coli*, *Salmonella typhimurium* (Loeffler) Castellani & Chalmers, and many other gram-negative bacteria (Osborn *et al.* 1972; Mizuno and Kageyama 1978). The cytoplasmic and outer membranes of *E. coli*, *S. typhimurium*, *Chromatium vinosum* (Ehrenberg) Winogradsky, *P. aeruginosa* (Schroeter) Migula, and other gram-negative bacteria have also been separated by differential centrifugation after specific solubilization of the cytoplasmic membrane with Triton X-100 in the presence of magnesium ions (Beher *et al.* 1980; Lane and Hurlbert 1980; Schnaitman 1971). Cytoplasmic and outer membranes have also been observed to form vesicles of different sizes when the cell envelope was disrupted (Diedrich and Cota-Robles 1974). The small outer membrane vesicles can be efficiently separated from larger cytoplasmic membrane vesicles in *P. aeruginosa* (Matsushita *et al.* 1978) and *C. vinosum* (Lane and Hurlbert

1980). NADH oxidase has been used in many studies as a marker of the cytoplasmic membrane (Booth and Curtis 1977; Matsushita *et al.* 1978; Osborn *et al.* 1972).

In this study, we found that NADH oxidase activity was greatly enriched in large membrane vesicles (Table 2) obtained after differential sedimentation of membrane vesicles and in low-density membrane fragments of both *P. syringae* and *E. coli* (Figs. 4 and 6). The recovery of only about 19% of the total NADH oxidase activity in small membrane fragments, representing the outer membrane of *P. syringae*, compares favorably with a report of 16% in *P. syringae* strain HS191 (Hurlbert and Gross 1983). Low-density membrane fragments of other bacterial species are greatly enriched for cytoplasmic membrane (Mizuno and Kageyama 1978; Osborn *et al.* 1972). The observed buoyant density of NADH oxidase-enriched low-density membrane fragments of *P. syringae* ($\rho = 1.13$–1.18) was similar to those reported for *S. typhimurium* ($\rho = 1.15$) (Osborn *et al.* 1972) and *P. aeruginosa* ($\rho = 1.16$) (Mizuno and Kageyama 1978) or observed here in *E. coli*. The association of some NADH oxidase activity with dense outer membrane fragments (as indicated by high KDO content) may be the result of hybridization of inner and outer membrane components during cell disruption, as has been observed with other bacteria (Osborn *et al.* 1972).
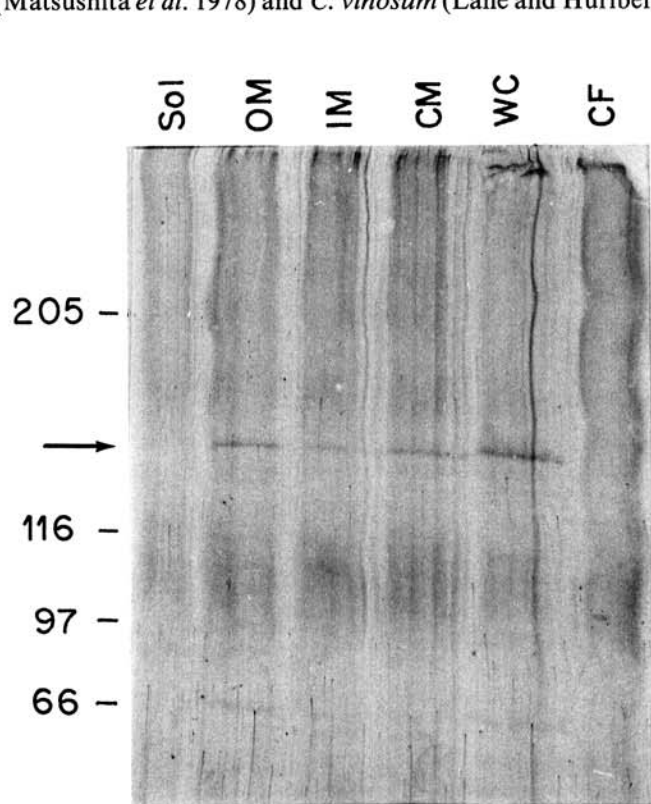
Fig. 9. Identification of the *iceC* gene product in different cellular fractions of *Escherichia coli* HB101 (pICE1.2). Total proteins in filtrates of cells grown in Luria medium (CF) and in whole cells (WC), as well as in whole membrane preparations (CM), soluble cell contents (Sol) and inner membranes (IM), and outer membranes (OM), fractionated by differential sedimentation velocity, were separated by sodium dodecyl sulfate (SDS)-PAGE and analyzed by immunoblotting with antibodies made against the *iceC-lacZ* fusion protein. The migration of the *iceC* gene product is indicated by the arrow. The migration of molecular mass standards (in kDa) is indicated on the left.
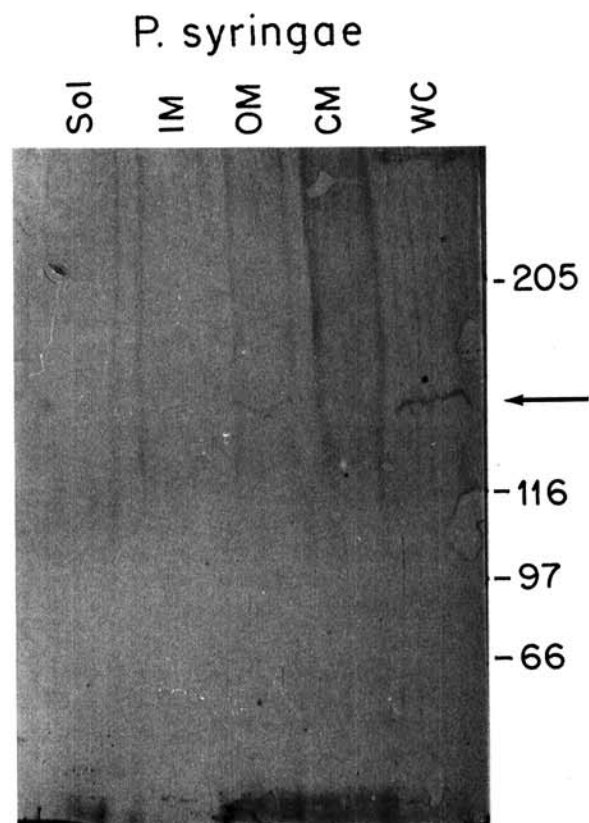
P. syringae

Fig. 10. Identification of the *iceC* gene product (indicated by the arrow) in different cellular locations in the *Pseudomonas syringae* source strain Cit7. Total proteins in whole cells (WC) as well as in whole membrane preparations (CM), soluble cell contents (Sol) and inner membranes (IM), and outer membranes (OM), separated by differential sedimentation velocity, were separated by sodium dodecyl sulfate (SDS)-PAGE and analyzed by immunoblotting with antibodies made against the *iceC-lacZ* fusion protein. The migration of molecular mass standards (in kDa) is indicated on the right.

Treatment of membrane fragments of *P. syringae* with Triton X-100 during Triton solubilization of cytoplasmic membranes interfered with the NADH oxidase activity, as was found in other studies (Baron and Thompson 1975). However, the other parameters could be assayed without Triton removal.

LPSs (as estimated by KDO content) are located preferentially in the outer membrane of gram-negative bacteria (Lane and Hurlbert 1980; Matsushita *et al.* 1978; Mizuno and Kageyama 1978; Osborn *et al.* 1972). The KDO content of Triton X-100 insoluble membranes (Table 3), slowly sedimenting (small) membrane fragments, or dense membrane fractions from sucrose gradient centrifugation of *P. syringae* and *E. coli* was high, indicating that these fractions were substantially enriched in the outer membrane. KDO-enriched membrane fragments of *P. syringae* ($\rho = 1.24–1.28$) were slightly more dense than those reported for *S. typhimurium* (Osborn *et al.* 1972) and *P. aeruginosa* (Mizuno and Kageyama 1978) ($\rho = 1.22$) and observed here in *E. coli* HB101 ($\rho = 1.19–1.23$). *P. syringae* may therefore contain more or a different complement of LPSs or other capsular polysaccharides than other gram-negative bacteria.

The relative purity of isolated cytoplasmic and outer membranes of *P. syringae* and *E. coli* was also indicated by analysis of protein composition of these preparations. The outer membranes of all gram-negative bacteria studied contain one to six prominent proteins (Di Rienzo *et al.* 1978; Lane and Hurlbert 1980; Mizuno and Kageyama 1978), including the murein lipoprotein, the porins, and the heat-modifiable OmpA protein (Di Rienzo *et al.* 1978). The cytoplasmic membranes of these species lack such major proteins (Di Rienzo *et al.* 1978). Only Triton X-100 insoluble membrane fragments, small and slowly sedimenting membrane fragments, and dense membrane fragments of *P. syringae* strain Cit7 isolated by isopycnic sucrose density gradient centrifugation contained these major outer membrane proteins. The small quantity of proteins with mobility similar to these major outer membrane proteins in cytoplasmic membrane fractions is consistent with the finding of little KDO in these fractions and indicates very little outer membrane contamination (probably less than 20%) of cytoplasmic membrane fractions of *P. syringae* (Fig. 5). However, the presence of proteins migrating similarly to many cytoplasmic membrane proteins in outer membrane fractions suggests that these fractions were contaminated with approximately 20% of the cytoplasmic membrane as indicated by their NADH oxidase content (Fig. 5).

Ice nuclei active at $-9°$ C were retained quantitatively when membranes of *P. syringae* were isolated after cell disruption (Table 1, Fig. 2). However, a reduction in the temperature at which bacterial membranes exhibited ice nucleation activity was observed upon extensive disruption of *P. syringae* cells (Fig. 2), as observed in *E. herbicola* (Yankofsky *et al.* 1981b). Whereas the total number of ice nuclei active above temperatures of $-7°$ C in a cell suspension did not change upon cell disruption and membrane isolation, the proportion of the particles that exhibited ice nucleation activity at temperatures above $-4°$ C decreased greatly (Fig. 2). A site exhibiting ice nucleation activity at temperatures of $-7°$ C or lower would be cryptic on a particle that also exhibited ice nucleation

activity at temperatures of $-4°$ C or higher. Intact cells of *P. syringae* typically exhibit very few ice nuclei active at temperatures lower than $-7°$ C that are not also active at temperatures of $-4°$ C or warmer. We cannot exclude the possibility that only those formally cryptic nuclei active as nuclei at temperatures of $-7°$ C or lower are expressed after cell disruption. We hypothesize that these two classes of ice nuclei represent sites of different sizes which are comprised of aggregates of the *iceC* gene product (Govindarajan and Lindow 1988b) and that cell disruption may affect the size or conformation of such aggregates.

This study revealed that ice nucleation activity as well as the *iceC* gene product are membrane-associated and localized principally, if not exclusively, in the outer membrane of *P. syringae* and *E. coli* containing the cloned *iceC* gene. Only very low numbers of ice nuclei were detected in extracellular filtrates, in membrane preparations washed with high salt concentrations, or in membrane-free fractions from disrupted cells. Sites responsible for ice nucleation in *P. syringae* and *E. coli* containing the cloned *iceC* gene appear to be intrinsic components of the membranes, or at least tightly associated with the membranes of these species. Indirect evidence for the location of ice nucleating sites in the outer membrane of *P. syringae* was provided by the demonstration of the sensitivity of this phenotype to 7-diazonium 1,3-naphthylene disulfonate and P-mercuric benzoate. These compounds are unable to penetrate the cytoplasmic membrane of other species but react with proteins that are external to or are attached to the outer surface of the cytoplasmic membrane (Britton and Fridovich 1977). This indirect evidence also indicates that intrinsic cytoplasmic membrane proteins are not responsible for ice nucleation activity but that outer membrane proteins or proteins adhering to the exterior of the cytoplasmic membrane determine ice nucleation activity.

All methods of cytoplasmic and outer membrane separation used in this study indicate an outer membrane location of ice nucleation activity in both *P. syringae* and *E. coli*. Almost no ice nucleation activity was observed in cytoplasmic membrane preparations isolated as Triton X-100 soluble membranes (Table 3) or in very low-density membrane fractions isolated by isopycnic sucrose density gradient centrifugation (Figs. 4 and 6). Nearly all of the ice nucleation activity was recovered in outer membrane-enriched membrane fractions (Tables 1–3, Figs. 4 and 6). While some ice nucleation activity was recovered in cytoplasmic membrane fractions and outer membrane fractions were contaminated with a small quantity of cytoplasmic membrane, there was a direct relationship between the ice nucleus content of membrane preparations and the amount of outer membrane components present (Tables 1–3, Figs. 4 and 6). An inverse relationship between the proportion of inner membrane components (such as NADH oxidase) in membrane preparations and the proportion of the total ice nucleation activity present in these preparations was also observed (Tables 1–3, Figs. 4 and 6).

Inner and outer membranes of *E. coli* were more completely separated than those of *P. syringae* by the methods used here. Ice nucleation activity was even more clearly associated with outer membranes of *E. coli* than of *P. syringae*. Differences in membrane composition between these two species apparently did not affect the

localization of either ice nucleation activity or the *iceC* gene product (Figs. 9 and 10). It is noteworthy that ice nucleation activity of some *E. herbicola* strains is associated with outer membrane-rich vesicles shed from these strains under some cultural conditions (Phelps *et al.* 1986). Much of the ice nucleation activity in *P. syringae* and *E. coli* was associated with membrane fragments of lower density rather than with those containing the bulk of the LPS (Figs. 4 and 6). However, almost no ice nucleation activity was associated with very low-density membrane fragments that contained NADH oxidase.

Because LPS content is largely responsible for the higher buoyant density of outer membranes, ice nucleation activity was apparently often associated with outer membranes that were depleted of LPS. Ice nucleation site sizes in *P. syringae* and *E. coli* are as large as 8,000 kDa (Govindarajan and Lindow 1988b). If such sites are composed of aggregates of the *iceC* protein, as we hypothesize, then LPS may be excluded from these proteinaceous sites over relatively large areas of the outer membrane. It is noteworthy that no ice nucleation activity was observed in unusually dense membrane fractions as observed in an Ice$^+$ *E. coli* strain overexpressing the *inaZ* gene product (Wolber *et al.* 1986). Thus, many differences in the localization of ice nucleating sites between *E. coli* cells with native (low) levels of expression of ice nucleation genes and those overexpressing this gene product are apparent.

Ice nucleation activity and content of the *iceC* gene product were closely related in both *P. syringae* and *E. coli* containing the cloned *iceC* gene. The *iceC* gene product was slightly smaller than the reported *inaZ* gene product from *P. syringae* strain S203 in *E. coli* (Wolber *et al.* 1986; Deininger *et al.* 1988). The overproduction of the *inaZ* protein also caused it to occur in different cellular locations in *E. coli* than observed here, apparently as an artifact of its hyperabundance (Wolber *et al.* 1986).

Theories of ice nucleation, based largely on studies of inorganic nucleating particles, require that a site orient a sufficient number of water molecules into an icelike lattice to enable subsequent ice crystal growth to occur (Hobbs 1974). Such sites probably have strict structural and conformational requirements. Proteinaceous ice nucleation sites may therefore be very dependent on the nature of the chemical and physical environment in which they are located (Govindarajan and Lindow 1988a). It is noteworthy that the *iceC* gene product was not detected in any soluble cell fractions and was located nearly exclusively in the outer membrane. Differences in the amount of ice nuclei present at different cellular locations are not apparently due to differences in activity of proteinaceous nucleating agents.

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