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Components of Ice Nucleation Structures of Bacteria

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Nonprotein components attached to the known protein product of the *inaZ* gene of *Pseudomonas syringae* have been identified and shown to be necessary for the most efficient ice nucleation of supercooled H_2O. Previous studies have shown that cultures of Ina^+ bacteria have cells with three major classes of ice-nucleating structures with readily differentiated activities. Further, some cells in the culture have nucleating activities intermediate between those of the different classes and presumably have structures that are biosynthetic intermediates between those of the different classes. Since these structures cannot be readily isolated and analyzed, their components have been identified by the use of specific enzymes or chemical probes, by direct incorporation of labeled precursors, and by stimulation of the formation of specific classes of freezing structures by selective additions to the growth medium. From these preliminary studies it appears that the most active ice nucleation structure (class A) contains the ice nucleation protein linked to phosphatidylinositol and mannose, probably as a complex mannan, and possibly glucosamine. These nonprotein components are characteristic of those used to anchor external proteins to cell membranes of eucaryotic cells and suggest that a similar but not identical anchoring mechanism is required for efficient ice nucleation structure. The class B structure has been found to contain protein presumably linked to the mannan and glucosamine moieties but definitely not to the phosphatidylinositol. The class C structure, which has the poorest ice nucleation activity, appears to be the ice nucleation protein linked to a few mannose residues and to be partially imbedded in the outer cell membrane.

Numerous studies have shown that the ability of bacteria such as *Pseudomonas syringae*, *Pseudomonas fluorescens*, and *Erwinia herbicola* to nucleate ice formation in supercooled H_2O is due to the products of specific genes called *inaZ* for *P. syringae*, *inaW* for *P. fluorescens*, and *iceE* for *E. herbicola* (3, 8, 20). Plasmids containing known amounts and structures of DNA from these bacteria have been introduced into host *Escherichia coli*, converting the *E. coli* phenotypically from Ina^- (no ice-nucleating activity) to Ina^+ (3, 8, 10, 20). These related bacterial genes all produce membrane-bound proteins with marked similarities to each other; all contain an essential and unique repetitive primary sequence (8, 27, 29). It is thought that the gene product is the template for ice crystal formation (28). The three bacterial genes have all been sequenced, and a consensus sequence has been determined (27). A protein isolated from membranes of transformed *E. coli* cells, which contained the *P. syringae* *inaZ* gene in a plasmid, has been purified, and a number of its properties have been characterized (29, 30). In addition to the central portion, which contains 132 repeats of an octapeptide and about 70% of the amino acids, these proteins have unique amino- and carboxyl-terminal ends. Although the *inaZ* gene specifies a structure containing 1,200 amino acids and a molecular mass of 120 kDa, the isolated protein had an apparent mass on polyacrylamide gel electrophoresis (PAGE) of about 155 kDa (29). This suggested either a unique behavior on PAGE or the posttranslational addition of other components (28). The implications of posttranslational modification (12, 13) are of considerable importance, since they appear to be critical in ice nucleation.

All Ina^+ bacterial cultures examined so far have a population of cells with varied abilities to nucleate ice formation at different temperatures. Only a small fraction of cells are active at −4.4°C or warmer, whereas almost every cell can nucleate at −8°C or lower. Govindarajan and Lindow (7) have proposed that the most nucleation-efficient cells have large highly stable aggregates of the ice-nucleating protein that act cooperatively to nucleate supercooled water. Similarly, Southworth et al. (22) reported that the ice nucleation activity in *P. syringae* cells was correlated with the amount of *inaZ* protein per cell but that the relationship was not linear. In 1989, Wolber and Warren (30) pointed out that chemical heterogeneity could also be involved in varying the nucleation efficiency. For any culture, a plot of freezing nucleus units (FNU) per bacterial cell versus temperature, defined originally by Vali (26), is called a cumulative nucleation spectrum. Several laboratories have suggested that there might be three distinguishable areas of this spectrum; this would imply that there are three types of ice nucleation structures (25). We have recently described properties of ice-nucleating cells indicating that there are three chemically distinct classes, A, B, and C, of structures as well as intermediate or mixed structures on the surfaces of ice nucleation-active cells. These properties included the ability of cell cultures to nucleate supercooled D_2O as compared with the ability to nucleate supercooled H_2O and the distinctive sensitivity of each of the three classes to organic solvents or to various buffers (25). The fact that there are three classes supports the suggestion that the structures of the classes are chemically heterogeneous. The most direct approach to determining any chemical heterogeneity would be the isolation and analysis of the active material. This is extremely difficult for several reasons. The class A and class B structures are quite rare, especially compared with the class C structure, and there are no obvious ways to separate and isolate the class A and class B structures for analysis. Further, the existence of biosynthetic intermediate structures between the three classes complicates the isolation problem. The one report of the isolation and purification of the ice nucleation gene product described a protein with an

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TABLE 1. Bacterial strains used

Bacteria	Properties	Source or reference
Pseudomonas syringae		
C9	Wild type, Ina$^+$	11
S203	Wild type, Ina$^+$	G. Warren (8)
Snomax	Ina$^+$, modified S203	R. DeLuca (13)
Erwinia herbicola	Wild type, Ina$^+$	11
Escherichia coli		
Ina$^-$ K-12 strains		
CR63	Contains no plasmids or ice genes, similar to *E. coli* HB101 used as host for plasmids	13
HB101	Contains no plasmids, host for plasmids	13
JC10291	Contains pUC9 plasmid but no ice genes	4
Ina$^+$ K-12 strains		
C91a	Transformed with pUC8 plasmid containing 15 kDa of *P. syringae* DNA	10
AGS335 (also called MS10)	Transformed with pUC9 plasmid containing 3.8 kDa of *P. syringae* DNA	29

apparent mass larger than that expected from the DNA sequence and with what appears to be class C structure and activity (29).

In this report we present several complementary indirect approaches to identifying the nonprotein components of the three classes of structures in *P. syringae*, in *E. coli* containing *P. syringae* DNA, and to a much lesser extent in *E. herbicola*. Based on earlier results for *E. herbicola* (10, 11, 13, 31), including the demonstration of similar separation of three classes of ice-nucleating structures (25), it is likely that the components found for the different classes of *P. syringae* are also components of the *E. herbicola* nucleating structure. The results reported here include nutritional growth experiments in which one can tentatively identify components of each class or of intermediate structures by the selective increase in the activity of that class. Further, specific enzymes or chemical reagents have been used to destroy certain structures and classes of ice-nucleating activity. And finally, the conclusion that these components do play a role in the structure of ice nucleation sites has been strengthened by showing incorporation of the presumed precursor components, labeled with ^3H, into the structures identified by immunoblotting by using antibody prepared against the purified ice protein (4, 18). In other papers (12, 13), evidence has been presented that phosphatidylinositol (PI) is involved in the formation of the class A structure. Direct evidence is presented in this paper that the class A structure contains PI, mannose, and most likely glucosamine residues. Mannose and glucosamine are also shown to be likely components of the class B nucleating structure. The sites of attachment of these components, and probably galactose, to the ice-nucleating protein and to the cell membrane is considered in an accompanying report (12).

MATERIALS AND METHODS

Bacterial strains and culture methods. Most of the bacterial strains and their origins and relevant properties are given in earlier work (8, 13, 25) and are listed in Table 1. In addition to standard tryptone medium, a modified minimal glycerol-$(NH_4)_2SO_4$-salts medium was used to grow the bacteria. This medium contained the following (per liter): 12 ml of glycerol, 1.1 g of $(NH_4)_2SO_4$, 3.5 g of K_2HPO_4, 1.4 g of KH_2PO_4, 1.0 g of NaCl, 8 g of vitamin-free Casamino Acids (Difco), 2 mg (each) of adenine, guanine, uracil, cytosine, and thymine, 10^{-3} M $MgSO_4$ (151 mg), 10^{-5} M $(NH_4)_6Mo_7O_{24}$ (11.6 mg), 10^{-6} M cobaltous acetate (0.177 mg), and 10^{-7} M (each) ferrous sulfate (152 μg), zinc sulfate (16 μg), and manganous chloride (12.6 μg). This medium, including the molybdate and cobalt compounds, gave the clearest results in both the nutritional and incorporation experiments. Nutritional supplementation experiments were carried out as described earlier (13). Final cell concentrations, which were always quite similar in the unsupplemented and supplemented media, were adjusted to the same turbidity before the ice-nucleating activity was measured. Ice-nucleating activity was measured as originally described by Vali (26).

The incorporation of a radioactive compound such as *m*-inositol, D-mannose, or D-glucosamine into the protein fraction of the various cells was carried out in separate experiments. Usually 50 μCi of the ^3H-labeled compound was added to 10 ml of sterile glycerol-minimal medium containing 2 mM unlabeled glucosamine, mannose, or *m*-inositol, giving a final activity of 2.5 μCi/μmol. The medium was inoculated with bacteria, and the culture was grown overnight with shaking at 23°C. To obtain the protein fraction, the cells were centrifuged in the cold, the supernatant medium was discarded, and the pellets were resuspended in 35% NaCl. The cells were incubated at room temperature for 10 min and then sedimented in the cold. The cells were osmotically shocked by resuspension in 0.05 M Tris buffer (pH 7.5) and treated with 100 μg of DNase per ml (plus 1 mM $MgSO_4$), and then an equal volume of standard sodium dodecyl sulfate (SDS) sample buffer was added. The samples were boiled and loaded onto an SDS–8.5 or 10% polyacrylamide gel. Each lane normally contained material from 5×10^9 cells (25 μg of protein), which is the same amount as that used by Deininger et al. (4, 20). The gel was run overnight at 35 V, and then the voltage was increased until the dye front was about 2 mm from the bottom of the gel. ^{14}C-labeled, prestained molecular weight markers were run simultaneously to determine the sizes. In some experiments the protein bands were transferred to nitrocellulose filters by the method of Towbin et al. (24), treated with an enhancing solution (10% 2,5-diphenyloxazole in toluene), and exposed to X-ray film at −70°C. [*myo*-2-^3H]inositol (23 Ci/mmol), D-[6-^3H]glucosamine hydrochloride (25 Ci/mmol), and D-[2-^3H]mannose (15 Ci/mmol) were all obtained from American Radiolabeled Chemicals, Inc. ^3H-labeled PI (19.9 Ci/mmol), was obtained from Amersham Chemical Co.

In some experiments the nitrocellulose filter was probed with an anti-*inaZ* polyclonal antibody preparation, known as anti-Ina6, which was obtained from Paul Wolber of DNA

Plant Technology (4, 18). It was prepared in rabbits by using a preparation of extremely highly purified ice nucleation protein obtained from transformed *E. coli* cell membranes and was used at a dilution of 1:2,000 or higher. Since no antibody reaction to normal *E. coli* proteins or other bacterial proteins or lipopolysaccharide has been found with this specific antibody preparation, it was not preabsorbed with Ina$^-$ *E. coli*. Labeled bands were immunostained with the goat-antibody horseradish peroxidase system (Bio-Rad), and the band was visualized by the color-producing reaction of the horseradish peroxidase with chloronaphthol (Bio-Rad). We have assumed that this antibody recognized the class C structure, which is the ice nucleation protein plus an unknown but small number of sugar residues, including mannose and possibly glucosamine residues, and would also react with this portion of the class A and class B structures.

In the initial characterization of the specificity of the anti-Ina6 serum, the bacterial extracts were run on a 4 to 15% polyacrylamide gradient–SDS gel from Pharmacia Phast-System, reacted with a 1:5,000 dilution of the serum, and stained in the usual manner (4). The same extracts run on a duplicate gel were silver stained as recommended by Pharmacia.

For treatment with α- or β-mannosidase, alkaline phosphatase (Sigma), α- or β-galactosidase (Sigma), CII phospholipase, or HONO (nitrous acid), the cells were washed off agar tryptone slants and suspended in an appropriate buffer (see below) and the reagent was added at the concentrations indicated to a sample of the cells. After incubation at the appropriate temperature, serial dilutions were carried out; the ice-nucleating activity in the treated sample was compared with that of the untreated sample incubated for the same amount of time with boiled enzyme. The ice-nucleating activity was determined after serial dilutions in Tris buffer (pH 7.5). Nitrous acid was freshly prepared by adding $NaNO_2$ to a final concentration of 0.05 M to 0.1 M acetic acid buffer (pH 5.0). Cells were incubated in this buffer for 30 min at room temperature. In this case the ice-nucleating activity after the nitrous acid treatment was compared with the ice-nucleating activity remaining in the acidic acetate buffer after the same time period.

All other reagents were as described previously (13) or were obtained from commercial sources. Purified phospholipase CII from *Bacillus thuringienisis* cultures was obtained from American Radiochemical Co., or from Dupont, Inc., and a sample of the highly purified enzyme (at least 97%) was a gift from M. Low, who has extensively described its properties (17). All three enzyme preparations gave identical results. In addition, CII phospholipase was purified from supernatant fluids of *Bacillus cereus* cultures (14). The α-mannosidase was obtained from Sigma as a purified suspension from jack beans in 3 M ammonium sulfate plus 0.1 mM zinc acetate; the α-mannosidase was essentially free of other sugar hydrolases (less than 0.05% of the α-mannosidase activity). Only one major protein band representing more than 90% of the protein was detected on PAGE. It was diluted over 50- to 100-fold in the final reaction mixture. The β-mannosidase was obtained from Sigma as a snail acetone powder suspension in 3 M ammonium sulfate plus 10 mM sodium acetate (pH 4.0). It contained about 1% α-mannosidase and β-*N*-acetylglucosaminidase activities and was also diluted about 50-fold in the final reaction medium. Similarly, the alkaline phosphatase was obtained from Sigma as a highly purified preparation.

PI vesicles were prepared as described previously (11) with 10 Triton X-100 molecules per PI molecule. Typically the fresh vesicle preparation used for fusion with the bacteria contained 1 μCi of [^{3}H]PI (0.05 nmol) and 0.4 nmol of carrier soybean PI (Sigma). This vesicle preparation was incubated with 1.0 ml of *E. coli* C91a at 5×10^9 bacteria per ml in 0.01 M potassium phosphate (pH 7.0) plus 8 mM spermine to favor fusion. The mixture was incubated at 30°C for 20 min; usually the change in ice-nucleating activity was determined, the mixture was extracted extensively with cold 5% trichloroacetic acid, the remaining protein was dissolved in SDS-mercaptoethanol, and the sample was subjected to PAGE.

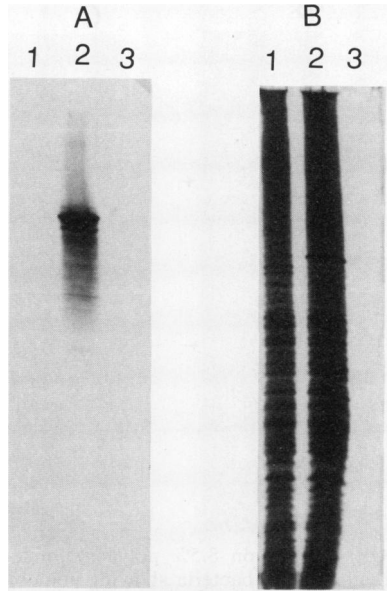

FIG. 1. PAGE (Pharmacia Phast-System) analysis of extracts of Ina$^-$ and Ina$^+$ *E. coli* bacterial extracts. (A) Immunoblot patterns obtained with a 1:5,000 dilution of nonpreadsorbed sera. (B) Patterns obtained after silver staining. No molecular weight standards were included in these runs. This highly sensitive method required an extract from only 1.4×10^6 cells per lane. Lanes: 1, Ina$^-$ *E. coli* (JC10291); 2, Ina$^+$ *E. coli* (JC10291, PMWS10); 3, blank.

Two sets of molecular mass markers were used in the PAGE runs. One set, obtained from Sigma, was prestained and consisted of myosin (200 kDa), phosphorylase *d* (92.5 kDa), bovine serum albumin (69 kDa), ovalbumin (46 kDa), trypsin inhibitor (21.5 kDa), and lysozyme (14.3 kDa). A second set of ^{14}C-radiolabeled standards was obtained from Amersham.

RESULTS

Nature of the ice-nucleating structures. Deininger et al. (4) reported earlier that extracts from Ina$^-$ bacteria do not give positive bands on PAGE gels with the antisera against the highly purified InaZ gene product. A repeat test of the specific antisera used in these and companion studies (12) is shown in Fig. 1. Extracts of an Ina$^+$ *E. coli* strain, containing the pUC9 plasmid plus the *inaZ* gene, and an Ina$^-$ *E. coli* strain, containing the pUC9 plasmid but not the *inaZ* gene, were run on identical gels of Pharmacia Phast-System and either immunoblotted (4) or silver stained. The results in Fig. 1 show a typical immunoblot pattern from the Ina$^+$ strain and no bands at all in the Ina$^-$ strain. The silver stain of an identical run shows that the total proteins in two extracts were identical. We have found that extracts of Ina$^-$ *E. coli*

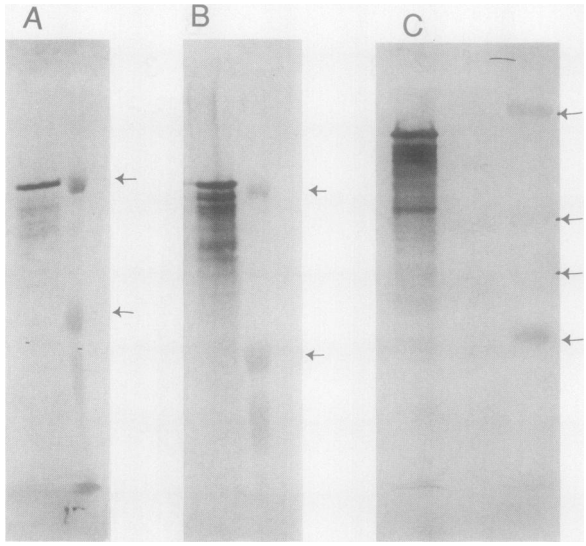

FIG. 2. PAGE analysis on 8.5% polyacrylamide–SDS gels of whole-cell extracts of Ina$^+$ bacteria showing immoblotting against the *inaZ* protein: (A) *P. syringae* C9, (B) *E. coli* C91a, (C) *P. syringae* S203. The material applied to each gel came from 5×10^9 cells. The arrows indicate the following standards run simultaneously (from the top) 200, 92.5, 69, and 43 kDa. For gels A and B, only the 200- and 92.5-kDa standards have arrows.

K-12 strains HB101 and CR63 also give no positive bands when treated with this serum (data not shown). Intact Ina$^+$ bacterial cells such as *P. syringae* C9, Ina$^+$ *E. coli* C91a, and *P. syringae* S203 were extracted and then treated with conventional reagents for PAGE, subjected to PAGE, and transferred to nitrocellulose. Then the ice-nucleating proteins were visualized by immunoblotting with the anti-Ina6 serum (Fig. 2). A major problem in interpreting the Western immunoblots of material extracted from whole cells of *P. syringae* or *E. coli* is the continual intracellular degradation of the ice-nucleating proteins by normal bacterial proteases (15). This was clearly described by Deininger et al. (4) and Mueller et al. (18) in the major papers on antibodies against ice-nucleating proteins. *P. syringae* C9 in this experiment yielded a broad major band, possibly containing several different components, with an apparent molecular mass of 210 kDa (Fig. 2A) as well as additional bands of lower molecular mass. Similarly, the extract from Ina$^+$ *E. coli* C91a cells gave a number of immunopositive bands, including a very strong broad band, again at about 210 kDa, and a number of additional bands at 175 kDa and smaller (Fig. 2B). The major band at 210 kDa is similar to that found by Mueller et al. (18) for material from a transformed Ina$^+$ *E. coli* strain (17). The almost identical immunoblot patterns for extracts from *P. syringae* C9 and *E. coli* C91 provide additional evidence that the antiserum is reacting only with the ice protein or its degradation products. *P. syringae* and *E. coli* cells have only about 2% homology in their DNAs, and the similarity of the immunoblots from these bacteria argues strongly against the possibility that the positive bands, especially the strong bands at 210 and 170 kDa, are due to the reaction of the antisera with other bacterial proteins or bacterial lipopolysaccharide.

A major question is whether all the smaller bands are due solely to proteolytic breakdown or whether they represent intermediates in the conversion of the original 120-kDa ice gene transcript to the final active nucleation structure. In these experiments the extracts were not exposed to octylthio-glucoside, which was used by Deininger et al. (4) to improve the reaction with the antibody preparation. The largest band extracted from *P. syringae* S203 had an apparent size of about 170 kDa with many additional smaller components (Fig. 2C). This was close but not identical to the main immunopositive band reported earlier for extracts of *P. syringae* S203 (and for *P. fluorescens*) of close to 155 kDa (4, 18, 28). However, these bacteria were grown in different and richer medias. Given that both *P. syringae* strains, C9 and S203, in these experiments were grown in identical synthetic medium and treated identically, it seems likely that there is a genuine difference in the ice nucleation structure of the two as revealed by their mobilities on PAGE. Since the sequence of the *inaZ* gene in these two bacteria is thought to be quite similar, it seems likely that in the medium used, the protein product is modified similarly in *P. syringae* C9 and in *E. coli* C91a but differently in *P. syringae* S203.

Since our earlier results (25) indicated that there were three major classes of ice-nucleating structures, we attempted to identify the different immunologically positive bands on PAGE with these structures. Based on cumulative freezing spectra, the quantitative amounts of structures in three classes (A, B, and C) is roughly 1.0:100:1,000. Class A structures are difficult to detect, especially relative to the two other classes. The presence of biosynthetic intermediate structures also complicates the separation of the structures on gels. Tentatively, it appears for *P. syringae* C9 and *E. coli* C91a that the highest broad-molecular-weight band(s) at 210 kDa includes the class A structure and the class B structure. This proposal is based on the incorporation of radioactive inositol and mannose (see below). The top band, in addition to the immunoreacting protein, definitely contains inositol and mannose, whereas the lower-molecular-weight bands do not contain inositol but do appear to contain mannose and glucosamine in addition to the protein. As shown below, there are reasons to suspect that there are many mannose residues linked in both α and β linkages to the ice-nucleating protein. The ice protein appears to be linked to this complex mannan moiety and appears to separate into these two bands. The addition of PI would only add an additional 1,000 Da, and a structure with PI could not be distinguished on PAGE. The binding of the glucosamine residues is discussed in the accompanying report (12). We believe with Deininger et al. (4) and Mueller et al. (18) that the lower-molecular-mass compounds, especially all those below 120 kDa but possibly larger components, are proteolytic breakdown products.

Presence of PI in the class A structure. In the earlier papers on phospholipids of the ice-nucleating bacteria (11, 13), evidence was presented correlating the appearance and the amount of PI in the cell and class A nucleation activity. We have now examined the effect of additional enzymes that hydrolyze PI on the three different classes of ice nucleation structure. The effect of a highly purified phospholipase enzyme from *B. thuringienisis* cultures on *P. syringae* nucleating activity is shown in Fig. 3, and the effect of highly purified alkaline phosphatase is shown in Fig. 4. Both enzymes had to be used at relatively high concentrations, but this is expected in dealing with proteins attached to bacterial surfaces. Both enzymes were active on *P. syringae* but less active on the Ina$^+$ *E. coli* strains and hardly active at all on *E. herbicola*. Alkaline phosphatase cleaves the phosphodiester bond between the glycerol and inositol of PI on the inositol side of the bond, whereas the *B. thuringienisis*

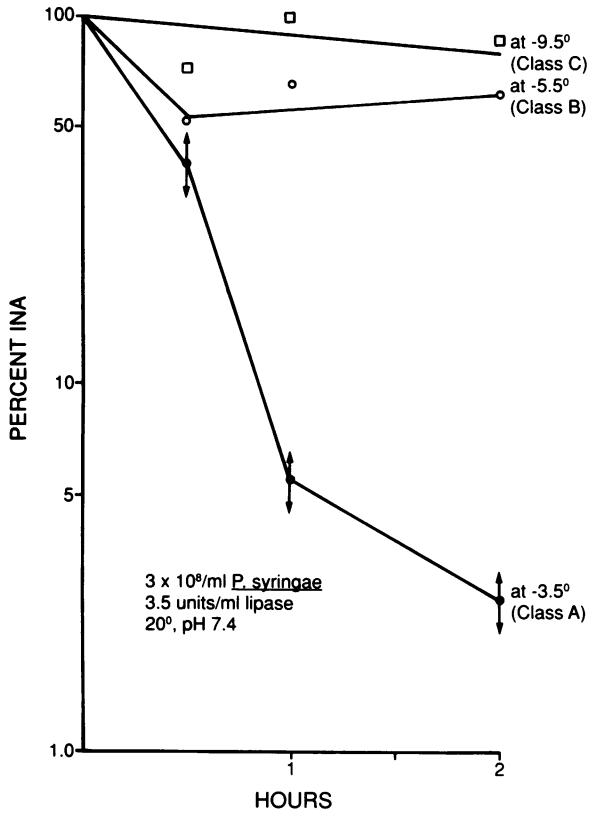

FIG. 3. Effect of highly purified *B. thuringienisis* phospholipase CII specific for protein bound PI on the ice-nucleating activity (INA) of *P. syringae* C9.

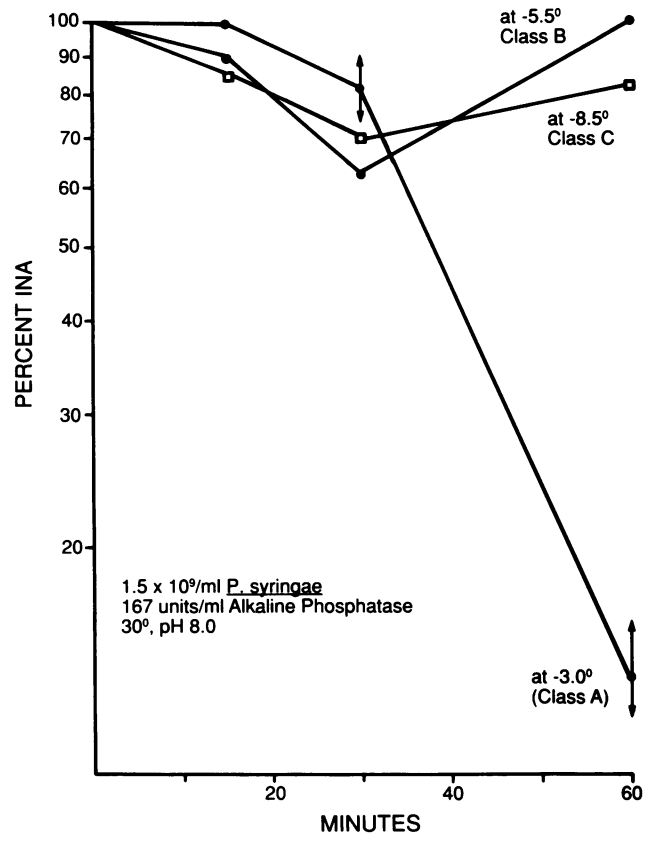

FIG. 4. Effect of highly purified alkaline phosphatase on the ice nucleation activity (INA) of *P. syringae* C9.

enzyme cleaves the same bond on the glycerol side of the bond. Both enzymes destroyed the class A nucleation structure but had only a small effect on the activity of class B and class C structures. It should be emphasized that the *B. thuringienisis* enzyme is a phospholipase CII, which is highly active on PI covalently attached to proteins (6, 17). Although this enzyme also has activity on free PI, we have not detected any release of radioactivity from cells grown with [^{3}H]inositol (12, 13), which are known to have PI in their cell membranes. The *B. thuringienisis* enzyme used in the experiment shown in Fig. 2 was obtained from Martin Low (17), but identical results were obtained with purified enzymes from ARC Corp. or with a highly purified preparation obtained from Dupont, Inc., all at similar enzyme concentrations. Finally, a similar phospholipase CII enzyme purified from *Bacillus cereus* culture medium (14) was found to reduce class A activity but to have only a minimal effect on class B or class C activity.

A major question in the interpretation of the effects of these and other enzymes (12) on ice nucleation of whole bacterial cells is whether the effect is directly on the ice nucleation structure or is caused by an attack on some other substrate on the cell. One test is whether the cells maintain their viability, but the more critical requirement is that the ice nucleation structure must contain the presumed substrate and that this substrate must change during the treatment. Treatment with phospholipase CII carried out as described in the legend to Fig. 3 destroyed only the class A nucleation activity of four different bacteria and did not affect bacterial viability (Table 2). A variety of other treatments and their effects on the different classes of ice nucleation activity and on cell viability are shown and are discussed below. It is apparent that there is no correlation between treatments affecting class A or class B activity and cell viability. The effect of UV irradiation (5 min of UV from a germicidal lamp at 30 cm) shows also that there is no necessary coupling between ice-nucleating activity and cell viability, since the UV light essentially killed all of the cells without affecting nucleation activity (Table 2).

The Ina$^+$ *E. coli* K-12 strain C91a and the Ina$^-$ *E. coli* K-12 strain CR63 were grown in medium containing [*m*-^{3}H] inositol and then extracted and analyzed on PAGE, the proteins were transferred to nitrocellulose (Fig. 1), and then a radioautogram was prepared (Fig. 5). A separate experiment was carried out with Ice$^+$ *E. coli* AGS335, and similar results were obtained. No labeled inositol experiments were carried out with *P. syringae*, since these cells metabolize inositol and the *E. coli* cells do not (Table 3). The amount of radioactivity incorporated into the Ina$^+$ *E. coli* cells was extremely low, and the extract on PAGE gave only very faint bands on X-ray film even after over 3 months of exposure. Similar faint bands from Ina$^+$ *E. coli* AGS335 extracts could be observed by careful examination of the film but could not be reproduced adequately for publication and are not shown. *E. coli* C91a had two radioactive bands near the top of the gel (Fig. 4). No radioactivity was seen elsewhere on the gel; the apparent molecular mass of the topmost band near the loading lanes is about 240 kDa, and the slightly lower radioactive band was at about 210 kDa. The Ina$^-$ *E. coli* K-12 extract in Fig. 4 produced no

TABLE 2. Effect of various treatments on ice nucleation activity and cell viability

Treatment[a] and bacterial strain	% Decrease in ice nucleation activity[b]			% Decrease in cell viability
	Class A	Class B	Class C	
Phospholipase CII				
P. syringae C9	50	0	0	14
E. herbicola	38	0	0	4
E. coli C91a	90	0	0	10
E. coli AGS335	78	0	0	0
α-Mannosidase				
P. syringae C9	99	90	30	0
E. coli C91a	99	?	?	50
E. herbicola	60	0	?	0
β-Mannosidase				
P. syringae C9	95	95	15	10
E. coli C91a	99	99	10	40
E. herbicola	70	15	0	0
β-Galactosidase				
E. coli C91a	60	0	?	0
P. syringae	80	20	?	0
25 mM nitrous acid				
P. syringae C9	100	100	30	9
E. coli C91a	100	100	?	0
E. coli AGS335	100	100	30	0
30 mM phenylboronate				
E. coli C91a	100	99	0	0
UV light				
P. syringae C9	0	0	0	100
E. coli C91a	0	0	0	100

[a] See the text.
[b] Class A, class B, and class C activity changes were measured at −4, −5.5, and −8°C, respectively.

radioactive bands. It is possible that the radioactivity just below the loading lanes is due to bacterial PI nonspecifically bound to SDS-protein complexes, but the unique 210-kDa band appears to be directly related to ice nucleation activity.

No immunoblot was done in this experiment, but the results shown in Fig. 2 indicate that the inositol-containing protein at 210 kDa cannot be distinguished from the 210-kDa ice protein. The extremely low amount of activity incorporated appears to be in part the result of the relatively high concentration of unlabeled inositol in the culture medium. *E. coli* has no active transport system for inositol, and the inositol is taken up by passive diffusion. Any endogenous synthesis of inositol would further reduce the amount of [^{3}H]inositol incorporation. However, the low amount of radioactivity would also be compatible with one or a few PI moieties per nucleating element (see below), especially as compared with the large amount of radioactivity from D-[^{3}H]mannose shown below.

The experiments showing stimulation of class A nucleation activity after fusion of bacterial cells with vesicles containing PI (13) and those reported above for inositol incorporation into a high-molecular-weight ice protein suggested that PI itself was being attached to the ice protein. However, an alternative possibility was that upon fusion the PI changed the bacterial cell surface to favor aggregation of the ice proteins in the membrane and thus increase the nucleation efficiency. This latter viewpoint was put forth by Govindarajan and Lindow (7) for the stimulation of class C ice nucleation they observed when PI was added to bacterial membrane preparations. Many experiments with ^{3}H-labeled PI–Triton X-100 vesicles were carried out to directly demonstrate the coupling of PI to proteins after vesicle fusion. In these experiments only very small amounts of radioactivity were taken up by the cell, usually 0.5 to 1% of the added activity. When the cell protein after fusion was subjected to PAGE and then exposed to X-ray film, no bands were observed. To improve the sensitivity of the fusion experiment (Fig. 6) carried out as described in Materials and Methods, the protein extract was subjected to PAGE; then each lane of the gel was sliced into 50 equal segments, and each slice was assayed for radioactivity in a scintillation counter. It is apparent that significant amounts of [^{3}H]PI are bound to a variety of different-sized proteins. Although no radioactive protein component was found at 210 kDa, the distribution pattern of the radioactive protein is similar to that reported by Deininger et al. (4) and that found for mannose and glucosamine in this work. Deininger et al. (4) pointed out that the ice nucleation protein is subject to a

TABLE 3. Use of compounds as sole carbon sources for growth[a]

Bacterial strain	Net optical density increase after 24 h[b] of cells grown on:			
	Glucose	Mannose	Glucosamine	Inositol
P. syringae C9	1.7	0.0	0.0	0.4
E. herbicola	1.7	0.02	1.7	ND[c]
Ina$^+$ *E. coli* K-12 strains				
C91a	0.57	0.43	0.24	0
AGS335	0.77	0.26	0.04	0
Ina$^-$ *E. coli* K-12 strain CR63	1.5	0.02	1.5	0

[a] Minimal M-9 synthetic medium was used with the indicated compound as the main or sole carbon source. The medium for all of the Ina$^+$ *E. coli* strains was supplemented with 10 μg each of proline, arginine, leucine, and isoleucine per ml.
[b] A net optical density of 1.0 is equal to about 10^9 cells per ml.
[c] ND, not determined.

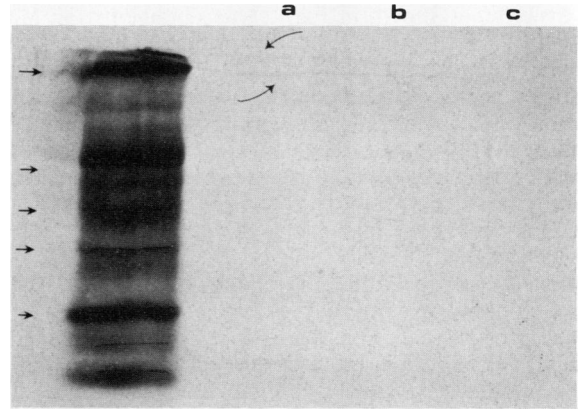

FIG. 5. Radioautogram (110 days) of PAGE analysis on 10% polyacrylamide–SDS gel of extracts of *E. coli* K-12 strain C91a (lanes a and b) and *E. coli* K-12 strain CR63 (lane c) grown in the presence of [^{3}H]inositol. The standards indicated by the arrows are as follows (top to bottom): 200, 92.5, 69, 46, and 30 kDa.

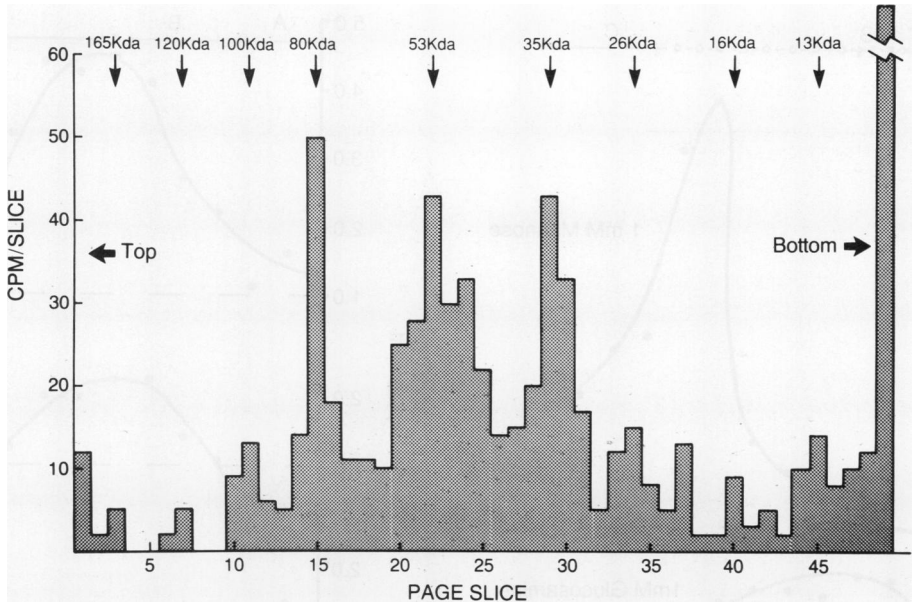

FIG. 6. Distribution of radioactivity on PAGE after fusion of [^{3}H]PI vesicles with *E. coli* C91a cells. The fusion was carried out as described earlier (13) without additional processing. The bacteria were treated with 5% trichloroacetic acid and washed three times in 5% trichloroacetic acid with centrifugation. The protein pellet was dissolved in SDS-mercaptoethanol in the usual fashion and then applied to a 10% polyacrylamide–SDS gel. After the electrophoresis run, the gel was sliced into 50 equal slices; each slice was put into a separate scintillation vial, and the vial was filled with scintillation fluid and counted for 100 min. The counts above background for each slice are plotted against the molecular size determined from the molecular size standards.

periodic cell protease degradation (15) and that there appears to be a site sensitive to protease attach every 3 kDa. For the lowest-molecular-mass bands in Fig. 6, these results show a ca. 3-kDa periodicity. Based on the radioactivity at 165 and 120 kDa and the periodicity of degradation, it appears that the PI is coupled to high-molecular-weight proteins with the size and properties of the ice proteins. Apparently during the 30 min for fusion, not only is the PI coupled but the PI-protein complex is also degraded. In identical fusion experiments with the Ina$^-$ *E. coli* K-12 strain CR63, no labeled proteins were ever detected, either with radioautograms or with the slice technique.

Presence of mannose in the ice nucleation structures. In addition to PI, one of the major components of the structure anchoring proteins to membranes in eucaryotic systems is a complex mannan (5, 6, 17). Several approaches to determining the possible presence of a mannan in the nucleating structures of Ina$^+$ bacteria were carried out. The abilities of *E. coli* C91a and *P. syringae* C9 to metabolize and use mannose and other relevant compounds as the sole carbon source is shown in Table 3. Neither *P. syringae* nor *E. herbicola* could use mannose as the sole carbon source for growth. The transformed Ina$^+$ *E. coli* strains could use mannose but only poorly for growth, and it is of interest that the nontransformed *E. coli* K-12 strains could not use mannose at all as the sole carbon source. We conclude that all of these strains have a limited ability to convert mannose to other compounds. The first experiments involved the effect of adding mannose to the glycerol-synthetic medium on the formation of the ice-nucleating activities of *P. syringae* and *E. coli* C91a (Fig. 7 and 8). The addition of mannose to the glycerol-synthetic medium stimulated five- to sevenfold the formation of the structures responsible for the intermediate nucleation activity from class C to class B but not the class A, class B, or class C activity for either bacterium. The addition of galactose had no effect on the formation of ice nucleation activity.

When ^{3}H-labeled mannose was added to the standard growth medium for *P. syringae*, radioactivity was incorporated into the high-molecular-mass proteins shown in Fig. 9. No protein-associated radioactivity was found in PAGE analysis of extracts of the Ina$^-$ *E. coli* K-12 strain grown in the identical medium. The incorporation of relatively large amounts of radioactivity added as labeled mannose into proteins gives the clearest picture of the different-sized ice nucleation structures. It is clear that the ^{3}H from mannose was covalently bound to proteins at about 210 and 200 kDa and that these labeled proteins are related to those detected immunologically (Fig. 2). Other bands notably include less labeled material at 92, 69, and 50 kDa. The question of whether the protein-bound radioactivity is due to an intact mannose residue or to some metabolite of mannose is of some importance. The labeled mannose contained ^{3}H only at the number 2 carbon atom. If mannose were converted to glucose, glucosamine, galactose, pentose, any amino acid, or any triose, the ^{3}H would be lost. The site of the label, then, supports the conclusion that the mannose itself, or a simple derivative, is linked to the ice protein.

The effects of incubation with α- or β-mannosidase preparations on the cell viabilities and the ice-nucleating activities of *P. syringae* C9, *E. coli* C91a, and *E. herbicola* are shown in Table 3 and in Fig. 10 through 12. Both of these glycosidase preparations rapidly and irreversibly destroyed class A and class B activities and the intermediate class C-class B activity of *P. syringae* C9 and had a small effect on the class C activity (Fig. 10). This result agrees with the nutritional effect of mannose, since sequential formation of first class B structures and then class A structures incorporating mannose would make them both susceptible to these enzymes. It is important to note that neither enzyme prep-

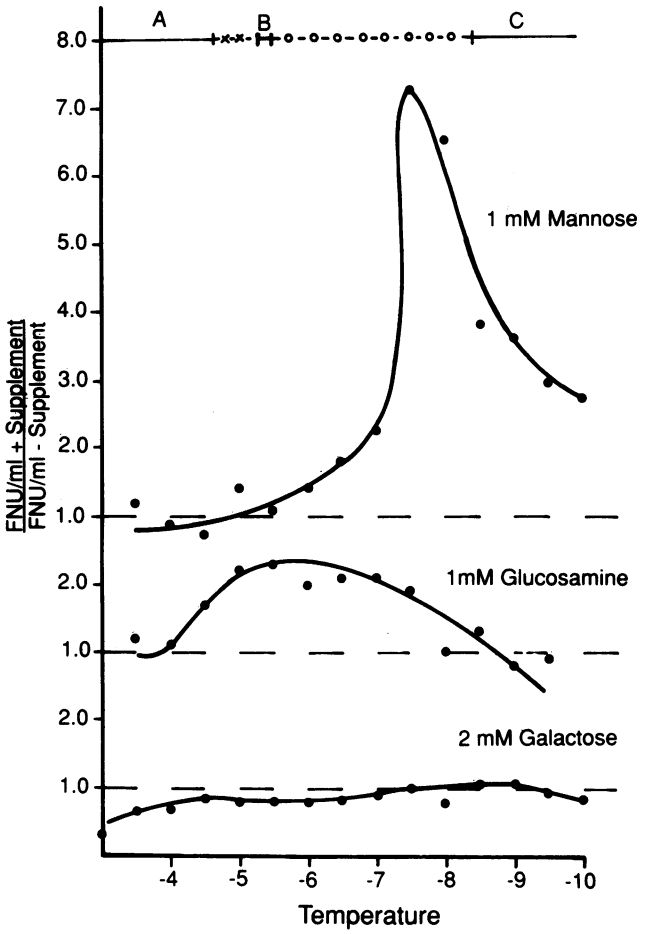

FIG. 7. Effect of various supplements in the minimal-glycerol growth medium on the ice nucleation activity of *P. syringae* C9. The ratio of FNU per milliliter of cells grown with the supplement to the FNU per milliliter of cells (at the same concentration) grown without the supplement is plotted against the freezing temperature.

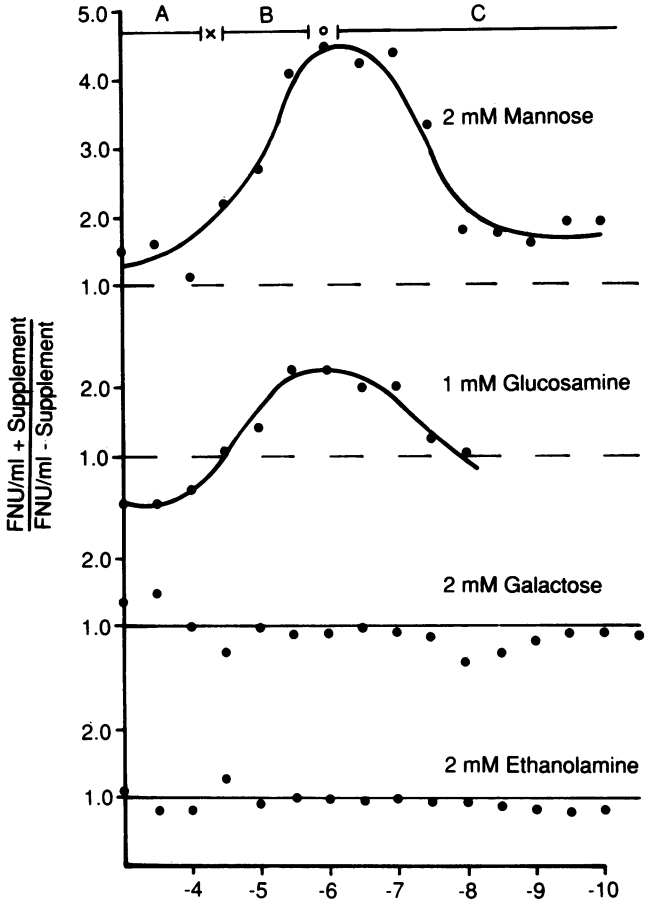

FIG. 8. Effect of adding various supplements to glycerol-minimal medium on the ice nucleation activity of *E. coli* C91a.

aration affected cell viability. The action of these two enzyme preparations supports the view that the ice-nucleating structure of *P. syringae* could contain a complex branched mannan with α- and β-linked sugars. For *E. coli* C91a (Fig. 11), the effects of these two enzyme preparations were somewhat different. Although the β-mannosidase preparation destroyed the class A, class B, and intermediate class C-class B activities, the α-mannosidase treatment gave an unexpected result. Class A activity was destroyed, but the class B and class C activities were greatly increased; the class C activity reached a FNU/ml value that was over 10^3-fold higher than the number of cells. This increase in FNU to values larger than the number of cells is typically ascribed to the shedding of subcellular vesicles, each with nucleation activity, from the cell (21). The effect of these two enzymes on the ice-nucleating activity of *E. herbicola* (Fig. 12) resembles that found with *E. coli*. The β-mannosidase preparation was the most effective, destroying class A, class B, and class C-class B structures, whereas the α-mannosidase was less effective and caused apparent shedding of ice-nucleating material from the cell. The possibility that the mannosidase preparations contained a protease that was the major component in the attack on the ice nucleation structure is considered in the accompanying report (12), in which additional evidence is presented supporting the conclusion that the effects were due to the mannosidases themselves.

These differences between the effects of the mannosidase preparations on these three bacteria suggest that there is a complex mannan that is bacterial strain specific. Since the mannan probably makes up the bulk of the additional mass above the DNA-specified 120 kDa, it is to be expected that the mannan would be bacterial strain specific, since mannan formation would require the action of host enzymes. As additional controls, various ice-nucleating bacteria were treated with α-galactosidase (12) and α- and β-glucosidases. None of these enzyme treatments specifically destroyed any class of ice-nucleating activity, and some of these enzyme treatments actually increased the ice-nucleating activity. The effect of β-galactosidase, which did decrease class A activity, is considered in the companion paper (12).

The inhibition of ice nucleation by compounds such as borate or phenylboronate was reported earlier (14) as indicating that a sugarlike compound with *cis*-hydroxyl groups was part of the nucleation structure. This observation was made before the existence of the three classes of nucleating structures was known. A similar but new experiment is shown in Fig. 13. It is apparent that these compounds inhibited class A, and class B, and intermediate class C-class B nucleating activities of *E. coli* C91a, and it seems likely that mannose residues, and possibly (but much less likely)

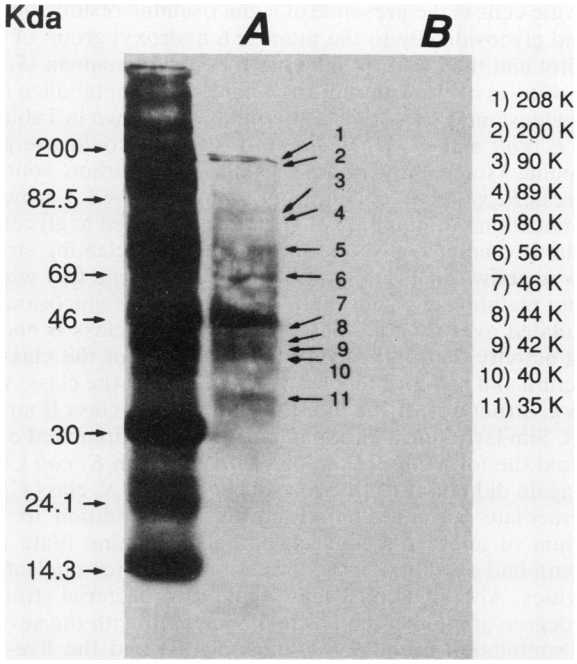

FIG. 9. Radiogram (62 days) of PAGE analysis on an 8.5% polyacrylamide–SDS gel of extracts of *P. syringae* C9 (lane A) and Ina$^-$ *E. coli* CR63 (lane B) grown in the presence of D-[^{3}H]mannose.

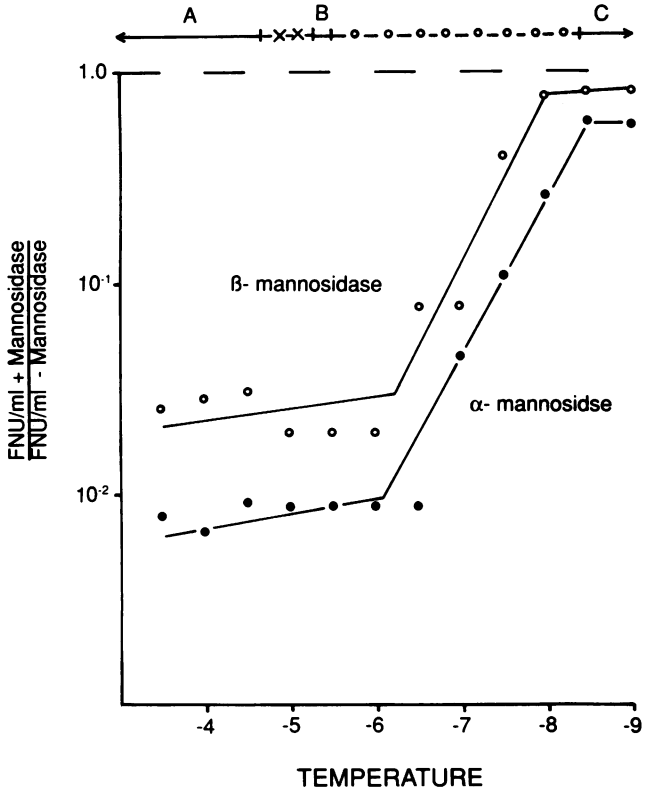

FIG. 10. Effect of α- and β-mannosidases on the ice-nucleating activity of *P. syringae* C9.

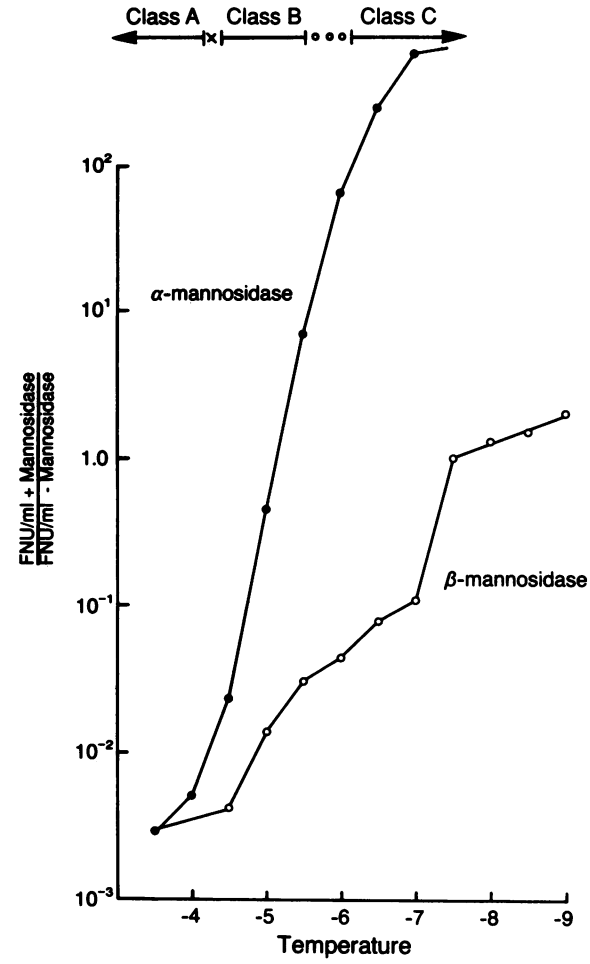

FIG. 11. Effect of α- and β-mannosidases on the ice-nucleating activity of *E. coli* C91a.

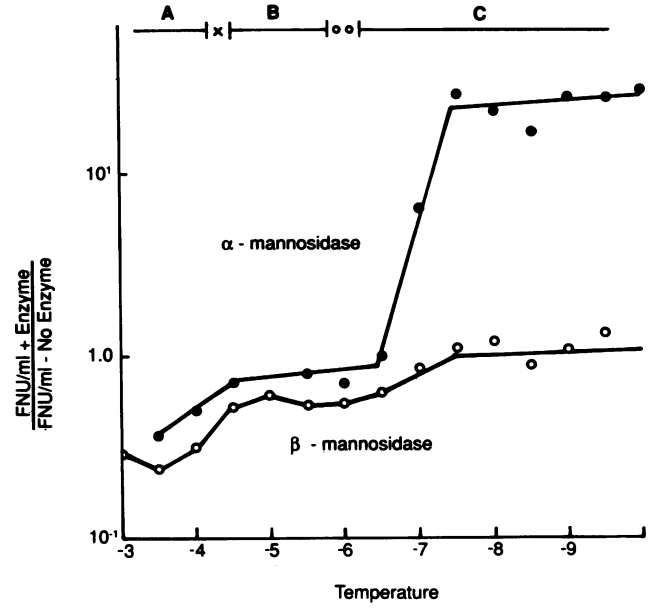

FIG. 12. Effect of α- and β-mannosidases on the ice-nucleating activity of *E. herbicola*.

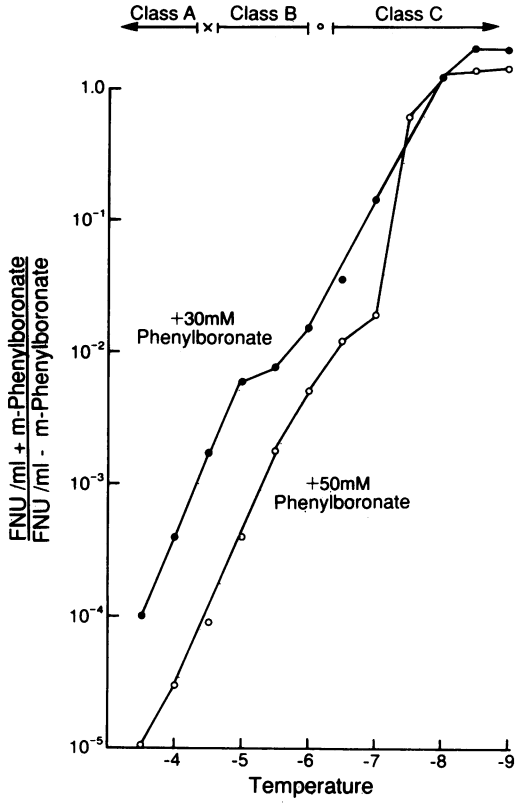

FIG. 13. Effect of *m*-phenylboronate on the ice nucleation activity of Ina$^+$ *E. coli* C91a.

inositol and glucosamine residues, are the major sites of borate binding.

Presence of glucosamine in the ice-nucleating structure. One constant feature of the protein-anchoring structures in eucaryotic cells is the presence of a glucosamine residue that is bound glycosidically to the number 6 hydroxyl group of the inositol and then in turn linked to a complex mannan (5, 6). The abilities of the different Ina$^+$ bacteria to metabolize and use glucosamine and other compounds are shown in Table 3. The *E. coli* and *E. herbicola* strains tested could use glucosamine (some only poorly) as the sole carbon source, whereas *P. syringae* could not use glucosamine for growth. The nutritional stimulation of glucosamine added to glycerol-synthetic medium on the formation of ice-nucleating structures is shown in Fig. 7 and 8. For *P. syringae* C9, which cannot metabolize glucosamine, the addition of glucosamine stimulated over twofold the formation of the class B nucleating activity and presumably the formation of the class B structure and had no effect on the formation of the class A or class C structures or the intermediate class C-class B structures. Similarly, the addition of glucosamine stimulated over twofold the formation of the class B activity in *E. coli* C91a and again did not affect the formation of class A, class C, or intermediate activities or structures. The addition to the medium of another amino sugar, galactosamine (data not shown), had no effect on the formation of any ice-nucleating activities. Although consistent for the two bacterial strains, this degree of stimulation is small compared with the sevenfold stimulation found for *m*-inositol (11) and the five- to sevenfold stimulation found for mannose.

One reagent used to identify the glucosamine residues in the eucaryotic anchoring structure is nitrous acid, and Ferguson (6) has summarized various studies showing its high specificity for degrading PI-anchored proteins. The action of 25 to 50 mM nitrous acid on two *P. syringae* strains and two Ina$^+$ *E. coli* strains is shown in Fig. 14 and Table 2. Class A, class B, and class C-class B activities were completely inactivated, whereas the class C activities were much less affected. The fact that nitrous acid treatment had no affect on cell viability supports the conclusion that the main attack was directly on the external nucleation structure. Along with the nutritional stimulation of the formation of class B struc-

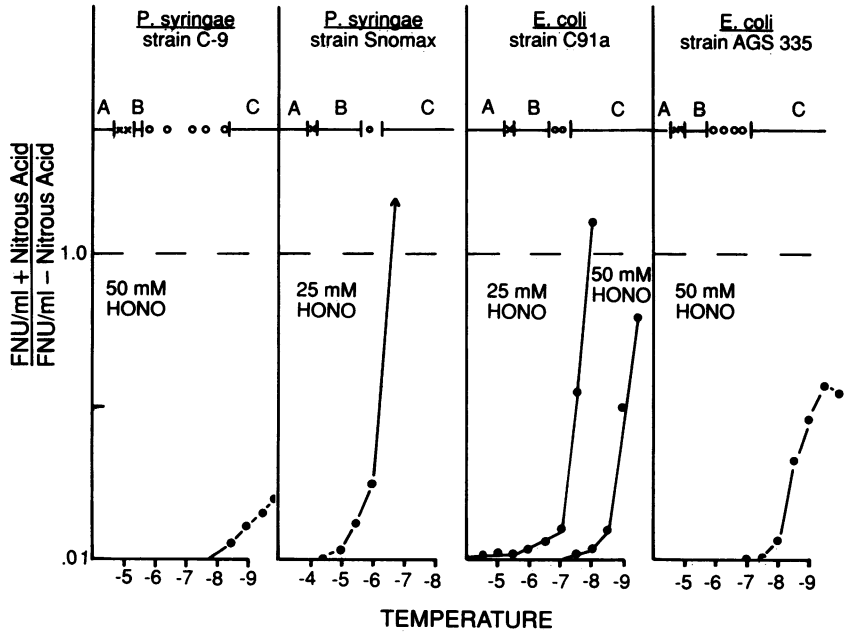

FIG. 14. Action of nitrous acid on the ice nucleation activity of various Ina$^+$ bacteria.

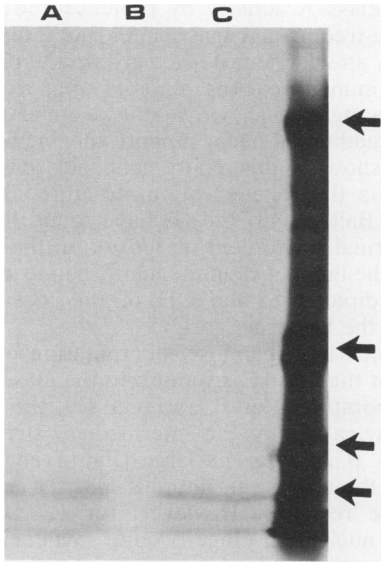

FIG. 15. Radioautogram (73 days) of PAGE analysis on an 8.5% polyacrylamide–SDS gel of cell extracts of *P. syringae* C9 (lanes A and C) and *E. coli* K-12 strain CR63 (lane B) after growth in the presence of [^{3}H]glucosamine. Standards are as in the legends to Fig. 2 and 5 (top to bottom): 200, 92.5, 69, and 46 kDa.

tures shown in Fig. 7 and 8, the nitrous acid destruction supports the conclusion that glucosamine is a component of class B and class A structures.

The distribution of [^{3}H]glucosamine in the proteins of *P. syringae* C9, *E. coli* C91a, and Ice$^-$ *E. coli* K-12 after the addition of this compound to the glycerol medium is shown in Fig. 15 and 16. The labeled glucosamine used contained ^{3}H at the number 6 carbon atom, and this label would not be altered by the conversion of glucosamine to a number of other compounds such as glucose or galactose. This fact must be kept in mind in evaluating the evidence that glucosamine as such is attached to the bacterial protein. Figure 15 is a radioautogram of extracts of *P. syringae* C9 (lanes A and C) and Ina$^-$ *E. coli* K-12 (lane B). The *P. syringae* extracts (lanes A and C) contained labeled proteins with apparent molecular sizes of 93, 46, and 42 kDa as well as lower-molecular-mass components. Although these bands do not correspond to the immunopositive bands shown in Fig. 2, lane A, for this bacterium, they do correspond to immunopositive bands seen in other extracts of Ina$^+$ *E. coli* and to the labeled bands found for [^{3}H]mannose in Fig. 9. On the other hand, absolutely no labeled proteins were seen in the Ina$^-$ *E. coli* K-12 extract. It appears that the labeled bands in the Ina$^+$ strains represent either proteolytic degradation products of the ice-nucleating structures of intermediates in the formation of the ice-nucleating structures.

When an incorporation experiment was carried out with [^{3}H]glucosamine to label proteins in Ina$^+$ *E. coli* C91a, a clearer relationship was evident (Fig. 16). In this experiment the material from the same gel was transferred to nitrocellulose and used to obtain first a Western blot and then a radioautogram. It is apparent that, in contrast to the Ina$^-$ *E. coli* shown in Fig. 15, this Ina$^+$ *E. coli* C91a strain took up the labeled amino sugar and attached it to many proteins. This was indicated by a faint radioactive band at 180 kDa, strong bands at 103, 88, 81, 76, and 70 kDa, and very strong bands at 55 kDa as well as a number of smaller bands. The *E. coli* C91a extract contained many immunopositive bands, including those at 210 and 195 kDa and smaller bands (Fig. 2B). The correspondence between the many radioactive and immunopositive bands for the C91a extracts, including those at 160 and 155 kDa, is marked on Fig. 16. These two gel patterns were printed with slightly different photographic enlargements, so the correspondence is not perfect. There were no radioactive bands corresponding to the largest strongly immunopositive bands. It should be noted that the major glucosamine-labeled bands found for *P. syringae* in Fig. 15 are also on the gels for *E. coli* (Fig. 16). Although the incorporation data are greatly complicated by the proteolytic degradation occurring in these bacteria, the partial agreement between the pattern of immunopositive and radioactive component, the nutritional stimulation, and the nitrous acid sensitivity support the conclusion that glucosamine is a likely component of the class A and class B nucleating structures.

Absence of ethanolamine in the ice-nucleating structure. In eucaryotic systems the mannan is attached to the anchored protein via an ethanolamine residue (5, 6, 17). The addition of ethanolamine to synthetic medium for *E. coli* C91a or any other Ina$^+$ bacterium did not stimulate the formation of any class of nucleating structure (Fig. 7), and there is no evidence that ethanolamine is a component of the bacterial nucleating structure.

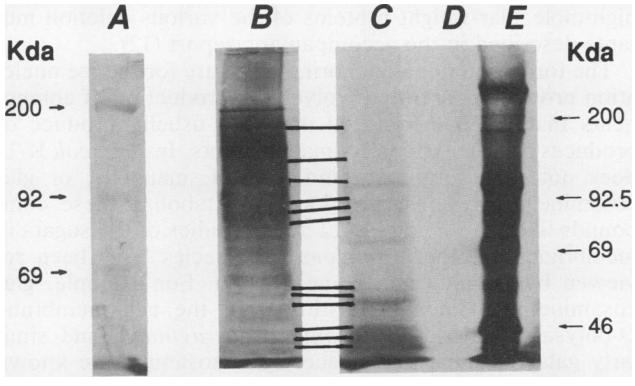

FIG. 16. Radioautogram (120 days) and Western blot of extracts of *E. coli* C91a after growth in glycerol-synthetic medium containing [^{3}H]glucosamine. Lanes: A, standards for Western blot; B, Western blot of extract of *E. coli* C91a; C, radioautogram of extract of *E. coli* C91a; D, blank; E, radioautogram standards.

DISCUSSION

Since the isolation and analysis of the class A and class B ice-nucleating structures and intermediate biosynthetic structures are not yet feasible, the evidence for assigning different nonprotein components to different classes is indirect. The selective search for the components found was based on a suspected analogy to the anchoring system in eucaryotic cells, in which the hydrophobic fatty acids of PI are imbedded in the cell membrane and the inositol is bound to a glucosamine residue, which in turn is linked to a complex mannan that in turn is linked to the protein via an ethanolamine residue (6, 17). It should be apparent that additional and as yet unsuspected components could be part of the nucleating structures, since the bacterial anchoring system is not identical to those on eucaryotic cells. For example, ethanolamine is not a metabolite as such in bacterial systems, although it can be formed from phosphatidyl-

serine, and glucosamine (see below) cannot play the same role in bacterial cells as it does in eucaryotic cells. The pathways of formation of the anchored structure are also quite different. In ice-nucleating bacteria the anchoring elements appear to be added sequentially to the protein, whereas in eucaryotic cells the anchoring system is built on the PI in the membrane and the protein is attached last. Further, it appears that there are structural differences between bacterial strains. For example, the apparent molecular size of the largest ice-nucleating structure from *P. syringae* C9 is 210 kDa, whereas the largest structure from *P. syringae* S203 is 170 kDa.

The validity of the nutritional studies indicating the involvement of specific components in the formation of the different structures deserves comment. The data are presented as the ratios of nucleation activities of the cells grown with the supplement to those of cells grown without the supplement. The precision of these ratios is about ±20% (Fig. 6 and 7); the addition of either galactose or ethanolamine had no stimulating effect and gave activity ratios of 1.0 ± 0.2. The ability of some compounds to stimulate the formation of one class of freezing structure for two different bacterial strains and not others lends reliability to assigning the addition of different components to different steps in the formation of the final structure. For example, the fact that these measurements are from a cumulative freezing spectrum, in which the total number of freezing nucleation units goes up as the temperature is reduced, means that the true stimulation in forming class B structures in the nutritional experiments is probably higher, possibly by as much as 50%.

The conclusion that inositol as PI is involved in the formation of the class A structure is based on the detailed nutritional, analytical, and enzymatic evidence given in the accompanying report (12) and in earlier work (13) and the enzyme degradation and incorporation results in this report. There is no reason to believe that the inositol is metabolized or converted into anything other than PI, and the presence of labeled inositol migrating at 210 kDa is strong direct evidence for a PI-anchored ice-nucleating protein. Similarly, selective destruction of class A nucleating activity without changing cell viability by highly purified CII phospholipase confirms the view that the ice-nucleating protein is anchored to the cell membrane.

The identification of a complex mannan as part of the anchoring structure is almost as certain. The nutritional and incorporation studies show that mannose, or a closely related compound derived from mannose, does play an important role in the conversion of the class C structure to the more hydrophilic class B structure.

In previous work (13), the addition of Mn^{2+}, a required cofactor for the activity of phosphatidylinositol synthase, was found to greatly stimulate (sevenfold) the formation of class A nucleation structures of *P. syringae* and *E. coli* C91a cells grown in synthetic medium. Surprisingly, class B nucleation activity and, most importantly, the intermediate class C-class B activity were also increased by the addition of Mn^{2+}, although to a lesser extent. The formation of class C activity was unaffected. In nonbacterial systems, Mn^{2+} has been shown to stimulate the attachment of sugars such as mannose to proteins (23). This Mn^{2+} stimulation would be in accord with the data from the nutritional experiments in Fig. 6 and 7.

The effects of various enzyme probes support the view that mannose as a mannan is part of the class A structure. Besides the CII phospholipase, which is known to attack preferentially mannan-containing proteins, there is the destruction of class A activity by protease-free *N*-glycanase and protease-free *O*-glycanase (13), both of which also preferentially attack mannose-rich glycoproteins. The disappearance of immunoreacting material after α- and β-mannosidase treatment is shown in the accompanying report (12). These additional data support the view that in the experiments shown in this report mannosidase was attacking the mannan in the ice nucleation structure. It is of some interest that Ballou and Yee (1) have found that mannose itself is a normal substituent on inositol in the tuberculosis bacterium. The link between the many mannose residues on the protein directly to the OH of the inositol has this precedent in the bacterial world.

The evidence that mannose incorporation occurs in the conversion of the class C structure to the class B structure requires elaboration. For *P. syringae* C9, the temperature range for nucleation by the intermediate structures from class C-class B activities is large (Fig. 7 and 10), and it appears that the class C structure itself may contain only a few mannose residues. However, for *E. coli* C91a the intermediate nucleation range is quite small (Fig. 8 and 11) and the mannose stimulation extends into the activity area associated with the class C structure. It seems likely that the class C structure of the transformed *E. coli* also contains mannose residues but probably more than the number in the class C structure of *P. syringae* C9. It might be noted that the addition of mannose and glucosamine residues to the protein would make the structure less hydrophobic and would be in accord with earlier evidence that the class B structure is much more hydrophilic than the class C or class A structure (25).

In anchored structures in eucaryotic cells, glucosamine plays an important and essential role linking the inositol moiety to the mannose. These present results do not support the same essential function for glucosamine in this bacterial system. The nutritional data suggest that mannose residues are added first to the protein (11) and that glucosamine residues are then added, perhaps by glycosidic bonds, to the various mannose residues or to serine or threonine hydroxyls of the ice protein. The addition of glucosamine does improve the ice nucleation activity but not to a large extent. The absence of significant [^{3}H]glucosamine from the distinct 210-kDa component of *E. coli* C91a (Fig. 16) suggests either that glucosamine cannot be a critical and essential component of the structure or that the ^{3}H on carbon 6 of the glucosamine is lost when the final structure is formed. However, significant [^{3}H]glucosamine was found in the high-molecular-weight proteins of the various deletion mutants described in the accompanying report (12).

The formation of an anchoring structure for the ice nucleation protein apparently involves the production of components that the bacterial cell does not usually produce or produces only in extremely small amounts. Ina$^-$ *E. coli* K-12 does not incorporate or bind inositol, mannose, or glucosamine to proteins, not does it metabolize these compounds like Ina$^+$ *E. coli* K-12 does. Studies of the sugars in membranes of other *Pseudomonas* species have been reviewed by Nikaido and Hancock (19). For example, glucosamine is a known constituent of the cell membrane *O*-polysaccharide of *Pseudomonas aeruginosa*, and similarly galactosamine and *N*-acetylgalactosamine are known components of the lipopolysaccharide of the same bacteria. Each step in the formation of an anchoring structure requires a separate enzyme, and the genetic information for these enzymes, especially in *E. coli* containing plasmids with only limited amounts of DNA, cannot be specified by the *ice* gene

itself. Like the formation of PI discussed earlier (13), the information for these enzymes must be in the bacterial genes; one suspects that it is the presence of the *ice* gene product that stimulates the expression of these cryptic genes (2, 9, 16).

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The control Western blots were carried out by Paul Wolber and Caroline Vance of DNA Plant Technology.

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