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# Formation of Bacterial Membrane Ice-Nucleating Lipoglycoprotein Complexes

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**The preliminary finding that nonprotein additions to the protein product of the ice-nucleating gene of *Pseudomonas syringae* or *Erwinia herbicola* are essential for ice nucleation at the warmest temperatures has led to experiments aimed at identifying possible linkages between the ice protein and the other components. It appears that the protein is coupled to various sugars through *N*- and *O*-glycan linkages. Mannose residues are apparently bound via an *N*-glycan bond to the amide nitrogen of one or more of the three essential asparagine residues in the unique amino-terminal portion of the protein. In turn, these mannose residues are involved in the subsequent attachment of phosphatidylinositol to the nucleation structure. This phosphatidylinositol-mannose-protein structure is the critical element in the class A nucleating structure. In addition to sugars attached to the asparagine residues, additional sugar residues appear to be attached by *O*-glycan linkages to serine and threonine residues in the primary repeating octapeptide, which makes up 70% of the total ice protein. These additional sugar residues include galactose and glucosamine and most likely additional mannose residues. These conclusions were based on (i) the changes in ice-nucleating activity due to the action of *N*- and *O*-glycanases, α- and β-mannosidoses, and β-galactosidase; (ii) immunoblot analyses of ice proteins in cell extracts after enzyme treatments; and (iii) the properties of transformed Ice$^+$ *Escherichia coli* cells containing plasmids with defined amino-terminal and carboxyl-terminal deletions in the ice gene. Finally, evidence is presented that these sugar residues may play a role in aggregating the ice gene lipoglycoprotein compound into larger aggregates, which are the most effective ice nucleation structures.**

The tentative identification of phosphatidylinositol (PI), mannose, and probably glucosamine residues as components of the different classes of bacterial ice-nucleating structures (13, 21) leads directly to the proposal that the ice-nucleating protein is extruded out from the cell surface and anchored to the outer cell membrane via PI in a manner similar but not identical to the anchoring of many proteins to cell membranes in eucaryotic cells (5, 7, 15). This report is concerned with defining the biosynthetic steps in which the protein product is posttranslationally modified in a series of reactions leading to the final anchored structure. We have attempted to construct a reaction sequence leading to various biosynthetic intermediates and to correlate these intermediates with the three classes of ice-nucleating structures previously identified (20) and called class A, class B, and class C. In general and with some differences between strains, class C structures are the least active and nucleate at −8.0°C or colder, class B structures nucleate between −6.0 and −7.0°C, and class A structures, which are the most active, nucleate at −4.5°C or warmer. Finally, roles for all of the components of the ice-nucleating structure, the main protein core, the sugar substituents, and the PI, are suggested. These roles relate the exposure of the ice-nucleating region to the supercooled water of the medium, to firm attachment to the cell membrane, to maximum mobility in the cell membrane, and to regions of this structure that are involved in cross-linking and aggregation to produce the most efficient ice-nucleating structure (8, 16, 19).

This work has been largely concerned with the structures responsible for ice nucleation by *Pseudomonas syringae* and transformed *Escherichia coli* containing various amounts or fragments of the *inaZ* gene from *P. syringae* and to a lesser extent with the ice nucleation structures in *Erwinia herbicola* and transformed *E. coli* carrying the *iceE* gene from *E. herbicola*. No studies have been carried out on the nucleating structures on *Pseudomonas fluorescens*, containing the *inaW* gene, but the protein products of *P. syringae*, *P. fluorescens* and *E. herbicola* (22) *ice* genes all cross-react immunologically. More important, all three bacterial ice genes have the same type of unique structure specifying a protein with an internal repeating octapeptide with 132 repeats and unique carboxyl-terminal (48 codons in *P. syringae*) and amino-terminal ends (175 codons in *P. syringae*). The consensus sequence that has been determined for all three genes (22) helps identify critical conserved residues. The obvious candidates for binding sites for sugars or phospholipids are the asparagine and OH-containing residues in the unique amino- and carboxyl-terminal sequences and the one serine and two threonine residues in each of the repeating octapeptides. The consensus sequence contains three strictly conserved Asn residues in the N-terminal region and no conserved Asn residues in the carboxyl-terminal region or in the repeating octapeptide. The strictly conserved Asn residues are 14, 15, and 43 residues from the amino terminus. There are no strictly conserved serine or threonine residues in the amino-terminal region, but there is one conserved threonine residue in the carboxyl-terminal region, 33 residues from the C-terminal end. The repeating consensus octapeptide Ala-Gly-Tyr-Gly-Ser-Thr-Gly-Thr, which makes up 70% of the protein, contains two threonine residues and one serine residue. The OH groups of these amino acids in the main core of the ice protein could also serve as attachment sites for galactose, mannose, glucosamine, or other unidentified sugars. Green et al. (9) constructed deletion mutants and showed that deletions

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from the amino terminus of the first 46 codons or of larger sections, all of which deleted the first three highly conserved Asn residues, greatly decreased ice nucleation at the warmest temperatures. Our interpretation of these results is that these deletions abolished most of the class A and class B activities (both down by about $10^3$-fold) and the intermediate class C-class B activity (also down by about $10^3$-fold) and left some class C activity. On the other hand, the mutants containing deletions in the carboxyl-terminal region still had measurable class A and class B activities, although they were noticeably decreased especially as compared with the remaining class C activities. It is apparent that both the amino- and carboxyl-terminal ends are necessary for maximum ice nucleation activity at the warmest temperatures, but that the amino-terminal structure is absolutely essential for class A and class B activities.

## MATERIALS AND METHODS

Most of the bacterial strains and methods are described in the accompanying paper (21) or a previous report (13). Six additional bacterial strains were kindly furnished by G. Warren of DNA Plant Technology. These were transformed *E. coli* strains containing plasmids with deletion mutants of the *inaZ* gene from *P. syringae* S203 (9). The deletion mutants all contained deletions from either the 175 amino acids at the unique amino-terminal end or from the 48 amino acids from the unique carboxyl-terminal end. The two mutants with carboxyl-end deletions are mutant Δ12.1, with 19 codons deleted from the carboxyl-terminal end, and mutant Δ10.1, with 21 codons deleted also from the carboxyl-terminal end. The four mutants with N-terminal deletions include the following: Δ937, with the initiator codon and the first 46 codons deleted; Δ931, with the first 144 codons deleted; Δ934, with the first 222 codons deleted; and Δ928, with an internal deletion of 25 codons and the addition of 4 codons of a synthetic sequence in the N-terminal region approximately from codons 118 to 145. The nucleation properties of these mutants have been described (9).

Three enzyme probes in addition to those described earlier (13, 21) were used. β-Galactosidase was a highly purified enzyme from an *E. coli* overproducer and was obtained from Sigma Co. Two enzymes that break down glycoproteins were obtained from Genzyme Corp. One, an *O*-glycanase from *Streptococcus pneumoniae*, was highly purified and contained no detectable proteases (<0.001%) or other sugar hydrolases. This enzyme is highly specific for mannan-rich glycoproteins and hydrolyzes Gal-β(1,3)-GalNAc disaccharides attached to protein serine or threonine residues to give free oligosaccharides and unsubstituted serine or threonine groups. It does not attack GlcNAc-β-(1,3)-GalNAc. This enzyme was obtained as a suspension in 50% glycerol–2.5 mM EDTA and was diluted over 20-fold in the reaction mixture.

The second enzyme from Genzyme was an *N*-glycanase from *Flavobacterium meningosepticum*. It is also highly effective on mannan-rich glycoproteins and hydrolyzes all common Asn-linked oligosaccharides from glycoproteins to give the free sugar and the free protein. It, too, was free of all other detectable sugar hydrolases, including mannosidases. It was also free of proteases. This enzyme, like the *O*-glycanase, works most effectively on denatured proteins and only poorly on native proteins. It also was supplied in 50% glycerol–2.5 mM EDTA, which was also diluted over 20-fold when used.

The other chemicals and the lectins were obtained as described earlier (12, 13, 21). The methods for cell growth, extraction, analysis on polyacrylamide gel electrophoresis (PAGE), and immunoblotting were as described in the accompanying report (21). The silver stain for proteins was that of Wray et al. (25).

## RESULTS

**Effect of *N*-glycanase on the ice nucleation properties of various bacteria.** The linkage of the various non-amino acid components (21) to the ice-nucleating protein was examined by using various enzyme treatments. The effect of a highly purified and specific *N*-glycanase on the nucleation properties of six whole-cell preparations of $Ina^+$ bacteria was examined. The class A nucleation activities of the two *P. syringae* strains, the three $Ina^+$ *E. coli* strains, and the *E. herbicola* strain were consistently reduced 60 to 90% upon treatment with this enzyme (Fig. 1). The effect on other classes of nucleation structures was more varied. In general the class B structures were resistant, and the enzyme treatment even increased this activity. One possibility for the increase in class B activity is that some of the class A structure may have been converted by the *N*-glycanase to the class B structure. In some strains the activity of the class C structure was also reduced, suggesting some heterogeneity in the substitutions on the proteins in the different strains. It should be emphasized that the *N*-glycanase only poorly attacks native proteins such as those on the cell surface and that the consistent 60 to 90% destruction across six strains is highly significant. The effect of this enzyme on the ice protein itself is presented below (see Fig. 4 through 6) and confirms that this enzyme attacks the ice protein itself and does not cause these changes by attacking other components in the cell surface surrounding the ice-nucleating structure. Although changes in cell viability were not measured in these experiments, there is no reason to suspect that these changes in class A activity were linked to cell survival, since all other enzyme treatments that reduced class A activity did not affect cell viability (21).

**Effect of *O*-glycanase treatment on the ice nucleation activity of various bacteria.** The effect of *O*-glycanase treatment on three *P. syringae* strains and $Ina^+$ *E. coli* C91a is shown in Fig. 2. Again, the results suggest multiple changes, but in all cases the class A nucleation activity was reduced by 50 to 80%. The intermediate class B-class A and class B activities were less sensitive and were even slightly increased, and for three of the strains (all except for *P. syringae* S203) the class C activity was not effected by the enzyme treatment. These results show that class A activity does involve sugar linkages to the serine or threonine residues that are most likely in the main core portion of the protein.

**Effect of β-galactosidase on the ice nucleation activities of various bacteria.** Since *O*-glycanase is known to attack specifically the disaccharide Gal-β-(1-3)-GalNAc, the effect of highly purified β-galactosidase on the nucleation activities of four bacterial strains was examined (Fig. 3). The results were similar in considerable detail to those found after the *O*-glycanase treatment: 50 to 80% of the class A activity was destroyed, whereas class B activity was either not affected or slightly increased and class C activity was slightly decreased. As a control, these bacterial strains were treated with α-galactosidase, which had either a slight increase or no effect on any of their nucleation activities.

The fact that treatment with either *O*-glycanase or β-ga-

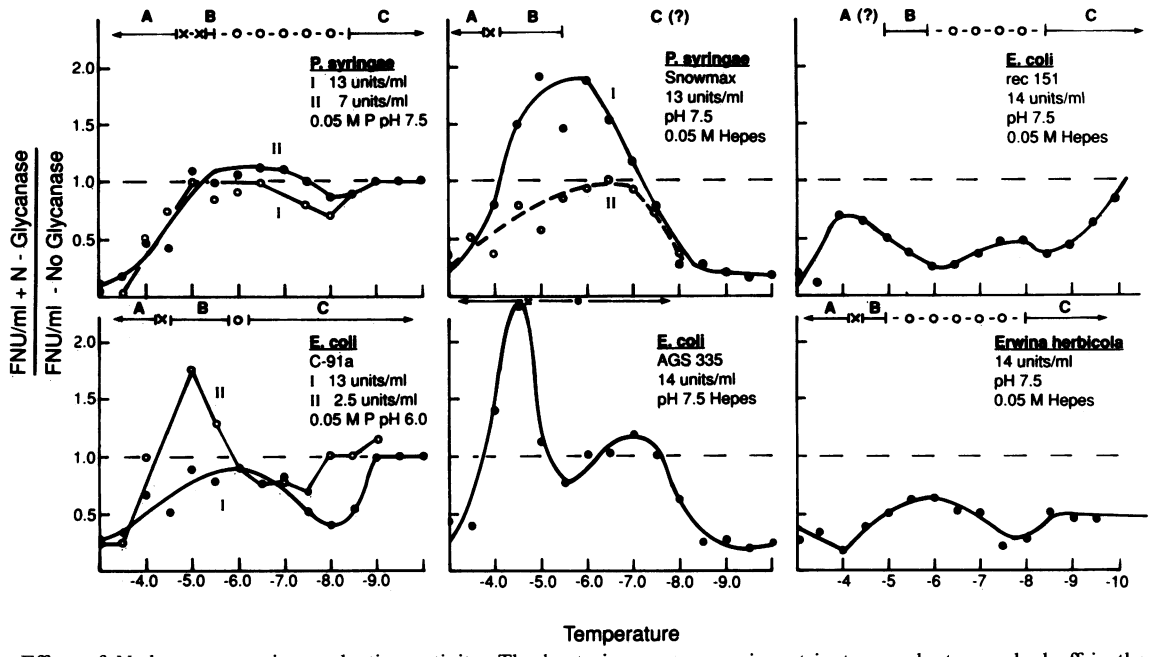


FIG. 1. Effect of *N*-glycanase on ice-nucleating activity. The bacteria were grown in nutrient agar slants, washed off in the buffer as indicated, and usually filtered through a 10-cm-long Sephadex G-10 column to remove most cations and free polysaccharides. The bacteria were divided into samples of about $10^{10}$/ml and incubated with or without *N*-glycanase for 1 h at 25°C. *P. syringae* Snowmax, which is supplied as a powder, was suspended in buffer at a concentration of 10 mg/ml and filtered like the other strains. FNU, freezing nucleus units.

lactosidase produced the same effect indicates that a galactose residue linked by a β linkage (and possibly linked to *N*-acetylgalactosamine) is a likely component of the class A freezing structure. However, it should be noted that both galactose and galactosamine, in contrast to mannose and glucosamine, added to synthetic medium (21) did not stimulate any increase in nucleation activity. These results suggest that only a few galactose residues are involved in the

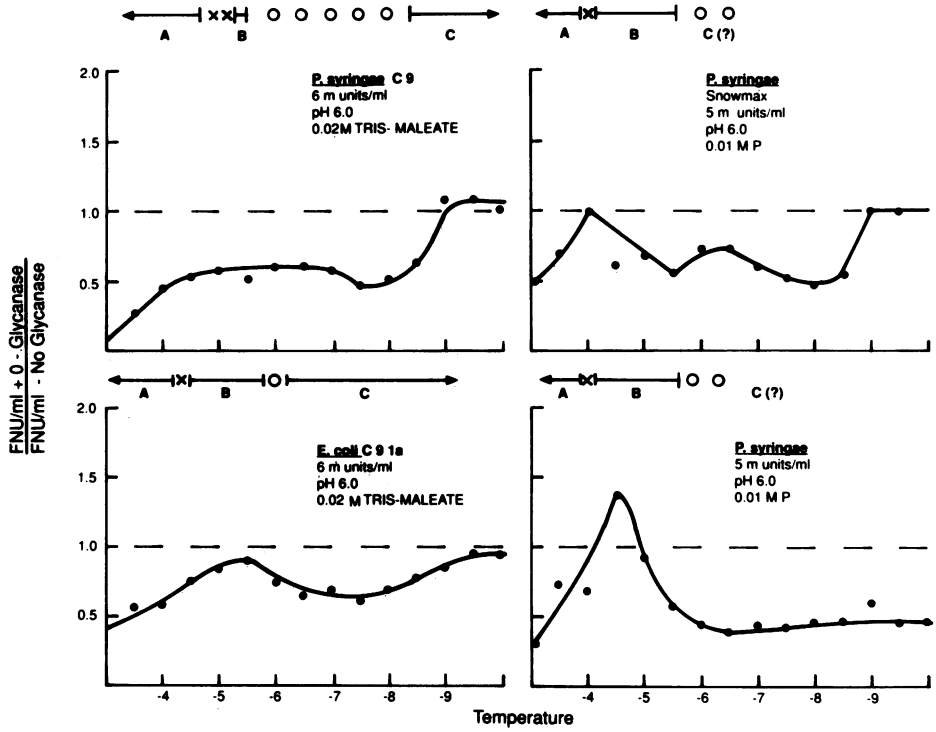


FIG. 2. Effect of *O*-glycanase on ice-nucleating activity. The procedure was as described in the legend to Fig. 1. The *P. syringae* strain in the lower right was S203.

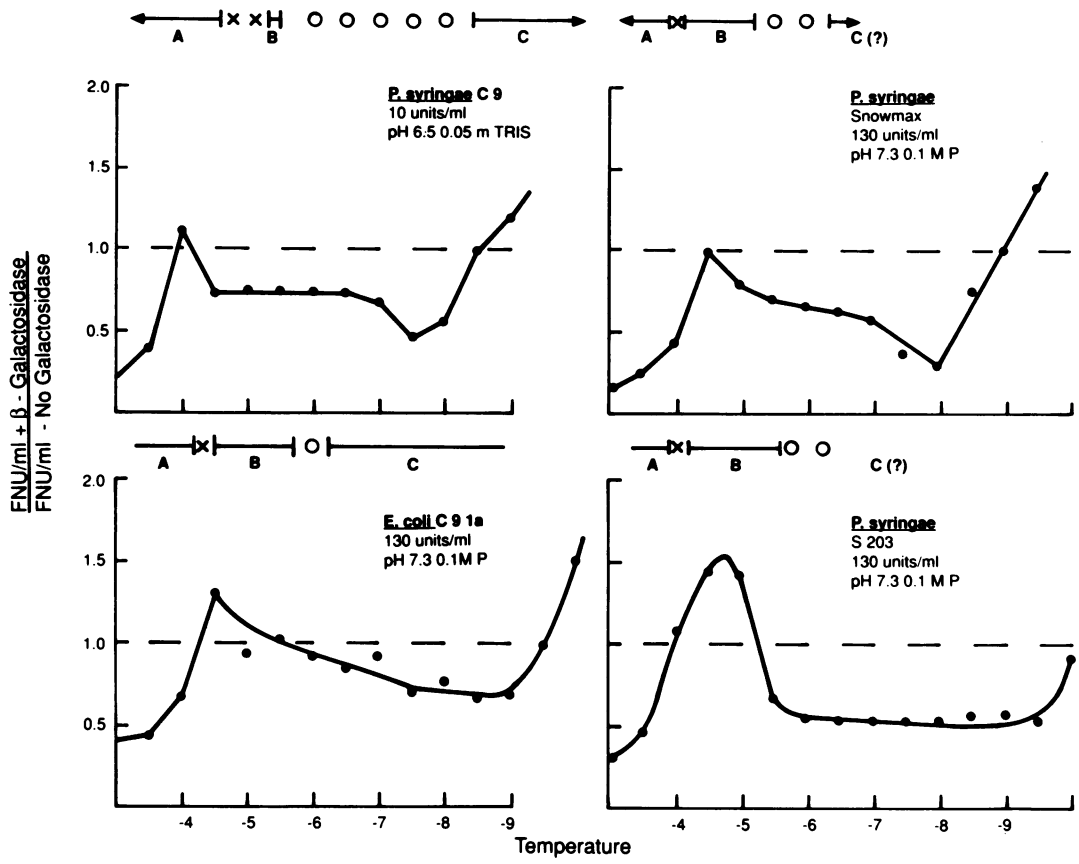


FIG. 3. Effect of β-galactosidase on ice-nucleating activity. The bacteria were grown on nutrient agar slants, washed off in the buffers as indicated, and usually filtered through a 10-cm-long Sephadex G-10 column to remove most cations and free polysaccharides. The bacteria were divided into samples of about $5 \times 10^9$/ml and incubated with or without β-galactosidase for 1 h at 25°C. Snomax powder was suspended in buffer at a concentration of 10 mg/ml.

structure and that the synthetic ability of the bacterial cell (some strains are known to synthesize galactose [17]) is enough to provide the sugars needed for this structure.

**Effect of various enzymes on denatured ice-nucleating structures.** The effects of *N*-glycanase, *O*-glycanase, α- and β-galactosidases, α- and β-mannosidases, and phospholipase CII on broken-cell extracts of *P. syringae* C9 and *E. coli* C91a under various conditions are shown in Fig. 4 and 5. The glycanases are quite active on denatured glycoproteins in the presence of high concentrations of sodium dodecyl sulfate (SDS) and detergent and only minimally active on the native glycoproteins on intact cells. Figure 4a shows that various enzyme treatments of boiled extracts of *P. syringae* C9 changed the antigenic blots exhibited by the ice proteins in these extracts. In this series of treatments with no extra detergent (see below) and a relatively low SDS concentration, neither *N*-glycanase, *O*-glycanase, nor α- or β-galactosidase appeared to have any effect on the antigenic reactivity of the ice proteins in the *P. syringae* extracts. However, α-mannosidase and, to a lesser extent, β-mannosidase caused the disappearance of the major band at 210 kDa. Although there is an air bubble in lane E, which contains the extract after the α-mannosidase treatment, it is clear that the glycoprotein complex has been degraded and that there is no apparent single product, only a smear of antigenic material at lower molecular weights. With an effect similar to that on the nucleation activity (21), β-mannosidase treatment decreased the intensity of the highest-molecular-weight bands. The disappearance of the main large-molecular-weight bands and the absence of definable products suggests that these enzymes could be removing the antigenic determinants on the ice protein.

Figure 4b shows the same action and effect of these enzymes with low SDS concentrations on boiled extracts of *E. coli* C91a. Only the two mannosidases, especially α-mannosidase, caused disruption of the ice protein with the accumulation of many bands of lower-molecular-weight material.

Figure 5 shows a similar experiment with a higher SDS concentration and additional detergent in the reaction mixture to aid the action of the glycanases. In this experiment both the *N*- and *O*-glycanase treatments (Fig. 5, lanes C and D) destroyed the antigenicity of the ice proteins of *E. coli* C91a, as did the β-galactosidase treatment. These results support the conclusion that the effects on cellular ice nucleation shown in Fig. 1 through 3 are due to the action of these enzymes on the ice nucleation structure. However, the α- and β-mannosidase treatments did not destroy the ice protein (Fig. 5), probably because of the increased concentrations of detergent and SDS. Similarly, phospholipase CII did not appear to be active, but this is difficult to judge since the removal of a diacyl-glycerol moiety would only change the apparent size from 210 kDa to about 209 kDa.

The results shown in Fig. 4 and 5 bear on the question of

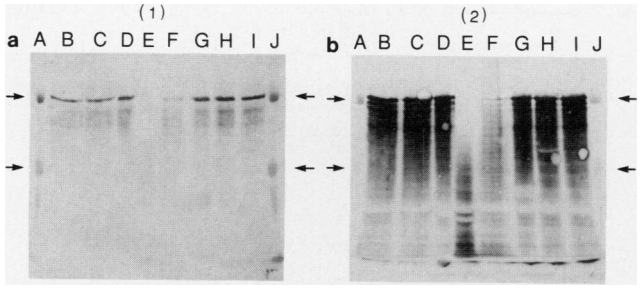


FIG. 4. (a) Western blot analysis of the effect of various enzyme treatments on the antigenicity and size of the ice protein in *P. syringae* C9 extracts containing a low concentration of SDS. Lanes: A and J, molecular mass standards of 200 kDa (top band) and 92.5 kDa; B and I, untreated extract; C, treatment with 7 U of *N*-glycanase (Fig. 1); D, treatment with 6 mU of *O*-glycanase (Fig. 2); E, treatment with 2 U of α-mannosidase (21); F, treatment with 0.2 U of β-mannosidase; G, treatment with 130 U of α-galactosidase; H, treatment with 130 U β-galactosidase. The bacteria were washed off L-broth (nutrient broth plus 0.5% yeast extract) slants with cold 0.025 M *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid buffer (pH 6.8) and adjusted to a concentration of about $2 \times 10^{10}$/ml. SDS was added to a final concentration of 0.38%, mercaptoethanol was added to a final concentration of 0.027 M, and the mixture was boiled. The extract was treated with RNase (final concentration, 70 μg/ml) and DNase (70 μg/ml)–10 mM $MgSO_4$, bovine serum albumin was added to a final concentration of 800 μg/ml, and then the SDS was increased to 0.48% and the mercaptoethanol was increased to 0.05 M. The mixture was boiled again, and samples of the extract were distributed into tubes with appropriate buffers and enzymes and incubated overnight at 37°C. The final volume of the reaction was about 0.3 ml. SDS and mercaptoethanol were then increased to the concentration usually used to prepare samples for PAGE on a 10% polyacrylamide gel. The samples were distributed, and the ice protein was visualized as described elsewhere (21). (b) Western blot analysis of the effect of various enzymes on the antigenicity and size of the ice proteins in *E. coli* C91a extract. The procedure was identical to that described above for panel a.

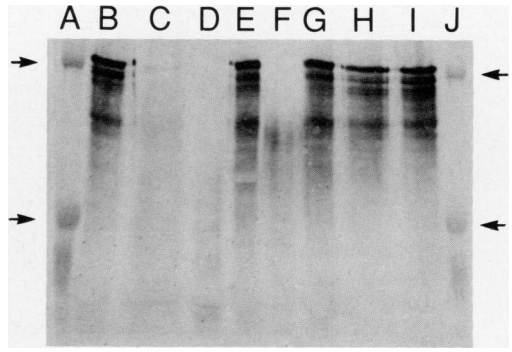


FIG. 5. Western blot analysis of the effect of various enzyme treatment on the antigenicity and size of the ice protein in *E. coli* C91a extracts containing a relatively high concentration of SDS. Lanes: A and J, molecular mass standards of 200 kDa (top) and 92.5 kDa; B and I, untreated extract; C, treatment with 13 U of *N*-glycanase per ml plus 1.2% Triton X-100; D, treatment with 6 mU of *O*-glycanase per ml plus 1.2% Triton X-100; E, treatment with 0.58 U of *Bacillus thuringiensis* phospholipase CII; F, treatment with 100 U of β-galactosidase; G, treatment with 2 U of α-mannosidase; H, treatment with 0.2 U of β-mannosidase. In this experiment, compared with that described in the legend to Fig. 4a, the concentrations of SDS and mercaptoethanol were 0.28% and 0.08 M, respectively. Bovine serum albumin was added to a final concentration of 1.2 mg/ml only to tubes D (*O*-glycanase), E (CII lipase), F (α-galactosidase), and G (β-galactosidase). The effect of these additions was that there was more Triton X-100 detergent and a higher SDS concentration with the glycanases compared with those in the experiments shown in Fig. 4. The rest of the procedure was as described in the legend to Fig. 4.

the purity and specificity of the enzyme probes. This is especially relevant to the preparation containing the α-mannosidase. Although the α-mannosidase preparation gave only one major band upon PAGE analysis (data not shown), the possibility that it contained a small amount of protease and that the protease caused the changes has to be considered. There is direct evidence from the nutritional and labeled mannose incorporation experiments that the ice protein contains mannose. Further, the results of the glycanase treatments support the presence and antigenicity of mannose attached to the ice protein. Although some action of a contaminant protease cannot be ruled out, the major effects on ice nucleation and the proven mannosidase substrate in the cell lead us to conclude that the nucleation structure does contain a mannan. Further evidence of this was found in the experiment on the deletion mutants (see below).

**Properties of various deletion mutants.** The role of asparagine residues in the unique amino-terminal region in binding sugars and the role of serine and threonine in the repeating core octapeptide were examined in experiments with *E. coli* cells transformed with plasmids containing specific deletions in the *inaZ* gene.

Western blot analysis of the proteins in some of these mutants is shown in Fig. 6a. In these extracts the appearance of many positive bands is clearly due to the action in the cell of many internal proteases (4). Extracts of *E. coli* AGS335 and, more successfully, *E. coli* C91a, which both contain the complete *inaZ* genome, showed many bands (lanes B and C), including a faint band at about 210 kDa for strain C91a (lane C) (21). The deletion mutants were all derived from the gene

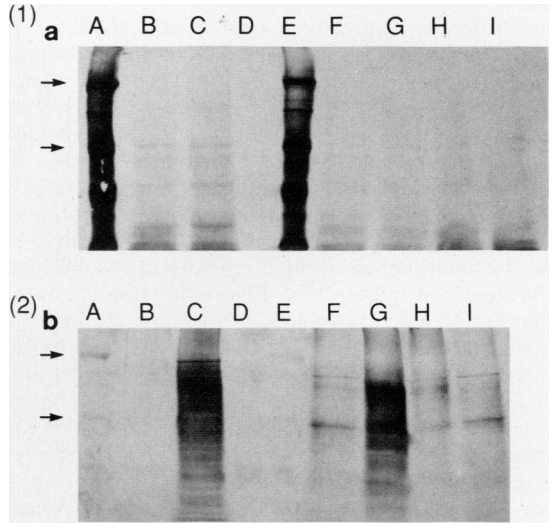


FIG. 6. (a) Western blot analysis of ice-nucleating proteins in extracts from various strains of *E. coli*. Lanes: A and E, molecular mass standards; B, strain AGS335; C, C91a; D, blank; F, Δ928; G, Δ934; H, Δ937; I, Δ10.1. (a) Radioautogram of the gel in panel a. All bacteria were grown in medium containing [$^3$H]glucosamine. The print of the Western blot was photographically enlarged somewhat compared with the radioautogram.

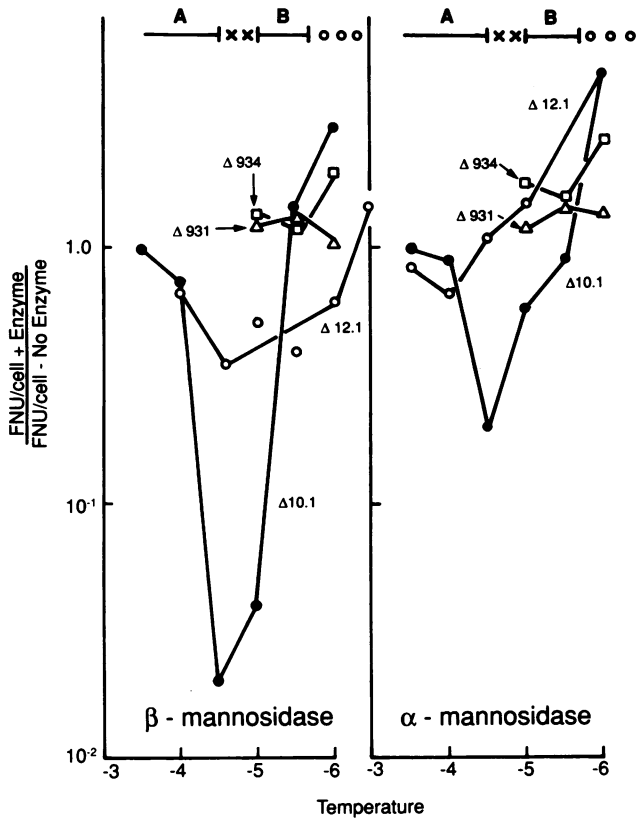


FIG. 7. Effect of α- and β-mannosidases on the ice nucleation activity of *E. coli* containing plasmids with different deletions in the *inaZ* genes. Bacteria Δ10.1 and Δ12.1 have deletions in the nonunique C-terminal end of *inaZ*, whereas Δ931 and Δ934 have deletions in the nonunique N-terminal end of *inaZ*. Detailed descriptions of these mutants are given in Materials and Methods, and the procedures for the enzymatic treatment are described in the accompanying report (21).

from *P. syringae* S203, for which the highest-molecular-mass band appeared at about 170 kDa (21, 23, 24). Mutant Δ928 (lane F) contains a small internal deletion in the N-terminal region and acts biologically like the wild-type S203, and mutant Δ10.1 (lane I) is missing 22 codons from the carboxy terminus. Extracts from both gave patterns of ice proteins that included a band at 170 kDa and were essentially like the pattern in the wild-type S203. Mutant Δ934 (lane G), which is missing 222 codons from the N terminus, does not give an extract with this largest (170-kDa) component. Mutant Δ937 (lane H) still has a large band at about 170 kDa, even though the first 46 deleted codons include the conserved three Asn residues. However, the *P. syringae inaZ* gene has two other N-terminal codons for Asn at residues 163 and 172; although not conserved among all ice-nucleating strains, these Asn groups are potential but probably much less favored sites for binding sugars.

Mueller et al. (16) have described the mobilities on PAGE of the ice proteins from various *E. coli* deletion mutants. They found that the molecular size varied with the deletion size, but their analysis was not directed at ice proteins with specific C- or N-terminal deletions. They interpreted their results to conclude that the ice nucleation protein was processed similarly in *E. coli* and in *Pseudomonas* species or was not processed at all. Our results do show processing and

TABLE 1. Stimulation of ice-nucleating activity by fusion with vesicles containing PI

| Bacterial strain | Deletion | Effect of PI vesicle fusion on class A nucleation activity |
|---|---|---|
| *P. syringae* S203 | | Small distinct two- to threefold stimulation |
| Ina$^+$ *E. coli* | | |
| Δ10.1 | 22 codons from C terminus | Two- to sixfold stimulation |
| Δ12.1 | 19 codons from C terminus | Two- to sixfold stimulation |
| Δ928 | 25 internal codons from N terminus | Identical to results with *P. syringae* S203 |
| Δ937 | First 46 codons of N terminus | No stimulation |
| Δ931 | First 144 codons of N terminus | No stimulation |
| Δ934 | First 222 codons of N terminus | No stimulation |

indicate similar processing in *E. coli* and *P. syringae* C9 but different processing in *P. syringae* S203.

Two other properties that relate to the attachment of mannose and/or PI were examined. Figure 7 shows the effect of α- and β-mannosidases on these deletion mutants. The nucleation activities of the mutants with normal N-terminal structures but with carboxyl-terminal deletions were susceptible to these enzymes, although the effect at different nucleating temperatures was unexpected. For example, at −3.5°C the nucleation activity was resistant, but at −4.0°C the activity was highly sensitive to these enzymes. One possible explanation for this difference may be the increased aggregate size at the warmer temperature (8). On the other hand, these enzymes had no effect on the small remaining class A nucleation activity of the N-terminal deletion mutants. One can tentatively conclude that critical mannose residues are attached to the N-terminal Asn residues. These experiments also support the conclusion that the mannosidases are acting as sugar hydrolases and not as proteases.

The effect of PI vesicle fusion with these cells is summarized in Table 1. Previous work (13, 21) had shown that fusion with liposome vesicles containing PI resulted in covalent binding of the PI to protein and marked specific increases in only the class A nucleation activity. Wild-type *P. syringae* S203, both C-terminal deletion mutants, and the internal N-terminal mutant Δ928 (which still has the conserved N-terminal Asn residues) all showed stimulation of class A nucleation activity upon fusion. However, the three N-terminal deletion mutants lacking these Asn residues, Δ937, Δ931, and Δ934, failed to respond at all to fusion with PI vesicles.

Although these experiments indicate that mannose residues and subsequently PI are joined via *N*-glycan bonds to the amino-terminal portion of the ice protein, the binding of sugars to the protein via *O*-glycan linkages, particularly the possible binding of glucosamine and galactose or galactosamine, was not easily resolved. Figure 6b shows the radioautogram produced by the same gel as that analyzed by Western blotting in Fig. 6a. The cells in this experiment (Fig. 6a and b) were grown in synthetic medium containing [$^3$H]glucosamine as described earlier (21). The radioactivity

in extracts of Ina$^+$ *E. coli* AGS335 and *E. coli* C91a (lanes B and C) was distributed among the various sizes of ice protein as described earlier. Ina$^-$ *E. coli* did not take up any radioactivity (21). Interestingly, all four deletion mutants, including Δ934 (missing 222 amino-terminus codons) and Δ10.1 (missing 22 carboxy-terminus codons), took up large amounts of glucosamine radioactivity. The prominent radioactive band at about 90 kDa in all mutants is especially noticeable, as is a similar immunogenic band of the same mobility. Since neither the entire N terminus nor the last 22 codons of the C terminus can be the site of glucosamine binding, the only possible O linkage site is the one Thr residue (no. 1167) (10) in the nonunique C terminus or the approximately 396 serine and threonine residues in the repeating core part of the *P. syringae* ice protein. Based on the amount of radioactivity incorporated, the serine and the threonine residues in the core of the protein seem to be highly likely sites.

**Release of the ice protein from the bacterial cell surface.** One of the major properties of anchored proteins on eucaryotic cells is their sensitivity to phospholipase CII treatment, which usually results in their release into the medium (5, 7, 15). In fact, this was the major observation that led to the concept of PI-protein anchoring. Similar experiments were carried out with all of the reagents described herein in an attempt to release the ice protein from the bacterial cell surface (21). Every attempt to release the protein failed. Cells were incubated with the reagent, the cells were sedimented, and the supernatant medium was concentrated and analyzed by PAGE with either Western immunoblotting or a highly sensitive silver stain (25). Reagents attacking the *O*-glycan linkage, including *O*-glycanase and β-galactosidase, did not release detectable ice protein or other proteins. Similar treatment with *N*-glycanase with or without subsequent *O*-glycanase treatment also failed. This is perhaps expected for the removal of side groups that play no direct role in holding the protein to the cell. However, other reagents, such as phospholipase CII, α- and/or β-mannosidase, and nitrous acid, also did not release detectable material, although all of these reagents had marked effects on ice nucleation (21). It has been shown that the ice protein complex is aggregated on the cell surface to form large stable active structures (8, 16, 19). It seems likely that the failure to release material is due to cross-linking away from the bonds cleaved by these enzymes or chemical treatments.

**Cross-linking of ice-nucleating structures by lectins.** The inhibition of cellular ice nucleation by certain mannose- and/or glucose-specific lectins was described previously (12). Treatment of cells with lectins specific for galactose or other sugars had no effect on ice nucleation activity. In these earlier experiments, the number of lectin molecules per cell was about $8 \times 10^6$, and the inhibitory lectins were presumed to inhibit, like borate (21), by binding to sugarlike residues on the nucleation protein. If these lectins were binding to each individual ice-nucleating structure or site, aggregation could be inhibited or reversed and ice nucleation efficiency, especially at the warmest temperatures, would be decreased. In the experiment shown in Fig. 8, two of these mannose- and/or glucose-specific lectins, concanavalin A and lentil lectin, were mixed with *E. coli* C91a at a much lower lectin molecule/cell ratio. For concanavalin A and lentil lectin the ratios were decreased 100- and 70-fold, respectively, both to about $10^5$. The lectin preparations were added to the bacterial cells suspended in buffer at room temperature for 1 to 2 min and then diluted in buffer containing the lectin. Under these conditions, both lectins greatly stimulated the appearance of class A nucleation activity, so that almost every cell exhibited class A activity. As measured at −4.0°C, the stimulation was $10^3$- to $10^4$-fold. Since there is no new synthesis under these conditions and no evidence for the shedding of vesicles, there must be a rearrangement of the nucleation structures. One possible explanation of this stimulation is that the lectins, both of which are divalent and have a relatively small mass of 20 to 30 kDa, were able to bind to the sugars on different ice nucleation molecules on the cell surface and link them together to form a stable, larger, and more efficient ice nucleation structure.

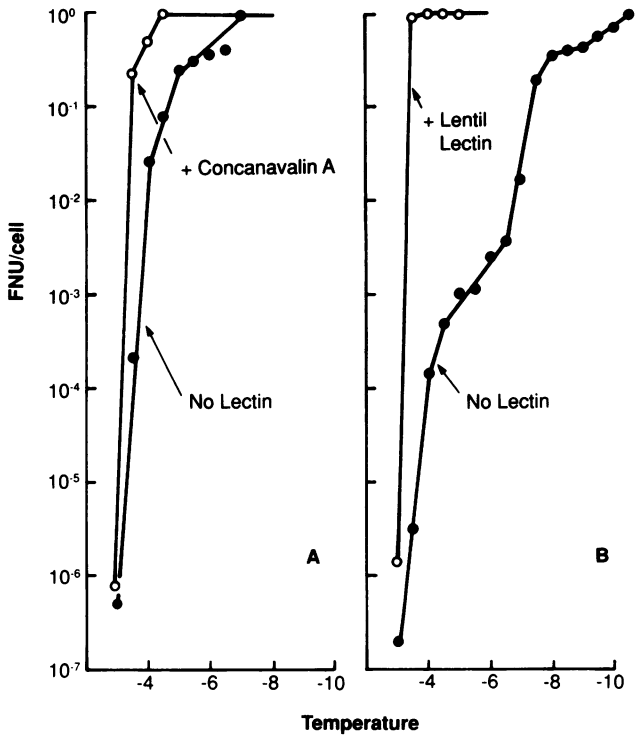


FIG. 8. Effect of lectins on the ice-nucleating activity of *E. coli* C91a. Concanavalin A and lentil lectin were added to separate bacterial suspensions in Tris buffer (pH 7.0). (A) The final concentration of concanavalin A was 4 μM, and the final bacterial concentration was $3.5 \times 10^{10}$/ml. (B) The lentil lectin concentration was 4 μM, and the final bacterial concentration was $2.1 \times 10^{10}$/ml. The cells were mixed with the lectin, kept for 1 to 2 min at room temperature, and diluted in Tris buffer (pH 7.0) in the presence of the lectin. The ice-nucleating activity was then measured.

## DISCUSSION

The evidence presented here and elsewhere (13, 21), although preliminary, supports the conclusion that the product of the functioning ice gene eventually forms aggregates of lipoglycoprotein complexes that are the most active and effective ice nucleators. The formation of a complex lipoglycoprotein itself requires a number of enzyme-catalyzed reactions and raises the question of the source of information for the formation of these enzymes. These enzymes must originate and be controlled by the bacterial genes (3). The ice gene itself, especially when present in plasmids in transformed cells, cannot provide the information for a large number of new enzymes. We are left with the possibility that the protein product of the *ice* gene activates other bacterial

genes that are normally cryptic (2, 11, 14) to form the machinery for the production of the ice-nucleating complex.

Without the isolation and chemical analysis of the nucleation complexes, these experiments provide only a model for integrating the current work in this field. This model depends on the work of Lindow and his colleagues (8, 18) and that of Warren, Wolber, and their colleagues (4, 9, 10, 16, 19, 22–24). These latter workers have considered the possibility that the *ice* gene product might be posttranslationally modified but have not put forth any experimental evidence on this point.

Based on earlier work and these studies, we propose that the carboxyl end of the protein, which is critical for the most efficient nucleation (9), extends into the medium; it might lie closer to the body of the protein, but there is no evidence that this primary terminal structure is modified. The repetitive portion is the likely site for the addition of sugars, such as mannose, glucosamine (or *N*-acetylglucosamine), and very probably galactose and possibly galactosamine. Given one serine and two threonine residues for the 132 apparent repeating octapeptides, there are about 396 potential OH bonding sites. The molecular mass discrepancy from the DNA sequence to that on PAGE for *P. syringae* S203 is 120 kDa versus the observed 170 or 50 kDa. There are enough bonding sites for single sugars as well as for oligosaccharides to add the additional mass. The role of these sugars in aggregation is discussed below (see Fig. 10). Finally, there is attachment of the inositol of the PI via a complex mannan containing both α- and β-linked mannose residues (and possibly a glucosamine residue) to the asparagine on the unique N-terminal end. From the degree of radioactivity incorporated (21), one would expect only a few PI molecules per ice protein molecule. In this formulation the PI plays an anchoring role functionally like that in eucaryotic systems but with a significantly different chemical structure for the anchor.

Extruding the ice protein into the medium and anchoring it to the cell membrane by the hydrophobic diacylglycerol portion of the PI offers considerable advantage to an ice-nucleating system. The main nucleating element, presumably the C-terminal end plus the repetitive portion of the protein, is away from the cell, but its hydrophobic tether to the cell permits energetically easy migration through the hydrophobic cell membrane. Burke and Lindow (1) have calculated that the energy required for the movement of unmodified ice protein molecules through the membrane is readily available, but a hydrophobic PI anchor would be even more efficient in allowing movement and the subsequent formation of large complexes.

The presence of the sugars on the different nucleating structures suggests that they play a significant role in ice nucleation. It should also be noted that many, but not all, fish antifreeze proteins contain galactose and *N*-acetylgalactosamine residues linked to a threonine residue on a repeating tripeptide consisting of Ala-Ala-Thr (3, 6). These sugars could be involved in binding to the hydrogen and oxygen of water molecules and could either favor or inhibit the bond angle changes occurring during ice formation. For ice nucleation, one additional structural role for the sugar residues could be to form the bridge from the protein to the PI. The critical aggregation of individual nucleating structures to improve nucleation efficiency also could involve the sugar residues. The ability of lectins with affinities for mannose and/or glucose to bring together scattered ice protein molecules on the outer cell surface offers a model involving these sugars for the binding and cross-linking that must occur

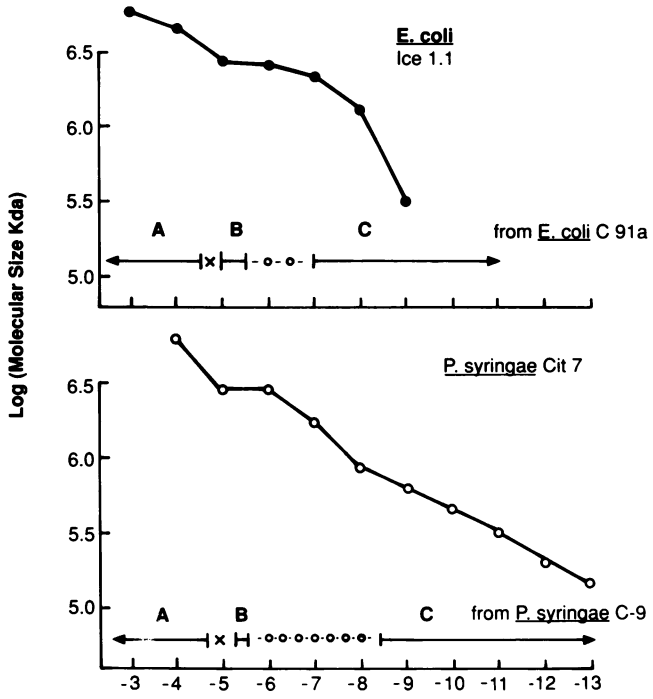


FIG. 9. Relationship of the molecular mass values from gamma ray inactivation measurements, nucleating activity (8), and the three classes of nucleating structures (20).

when large, extremely stable, efficient aggregates form. Whether the glycoprotein acts as its own lectin and self-aggregates or, indeed, whether there is some additional bacterial surface lectinlike molecule is unclear. In a different connection and more speculatively, one might wonder whether similar plant lectin-induced aggregations of nucleation complexes occur on the outer membranes of bacterial cells on the plant leaf surfaces when plant lectins are present or are liberated when a plant is supercooled.

The question of effective nucleating size was first addressed by Govindarajan and Lindow (8), who used gamma ray inactivation to relate nucleation efficiency and target size. They showed that very large and sturdy complexes were formed; the largest, with an apparent molecular mass of almost 8.7 MDa, was an efficient nucleator at −3°C. Only a little later Southworth et al. (19) also showed that nucleation efficiency was related to the size of the molecular aggregate.

In considering the aggregation process in the formation of the nucleating structure complexes, we have reexamined the experiment of Govindarajan and Lindow and their interpretation of their data (8). The data in the log plots of the molecular size from target theory against the temperature of ice nucleation were somewhat scattered, and these workers drew a reasonable straight line through their data points. With their permission, we took their data for two bacteria for which we defined the different classes of ice-nucleating structures and redrew the line (Fig. 9), relating mass and nucleating efficiency at the different temperatures. There are apparent inflection points indicating three separate portions of the curve, and these portions correspond closely to the three classes of nucleating activity. The molecular mass for the class B structure for both bacteria does not appreciably increase as its nucleating efficiency increases.

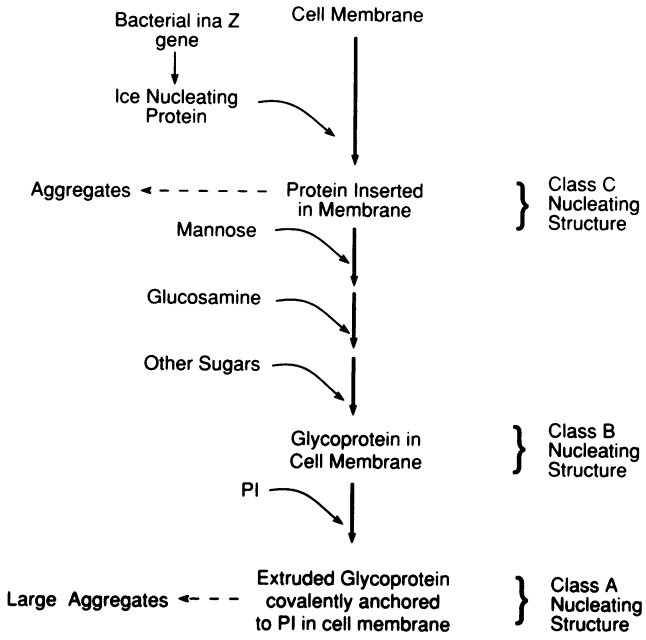


FIG. 10. Suggested sequential formation of the most effective ice-nucleating structure from class C to class B to class A. Side-to-side aggregation is thought to involve the substituted sugars, and PI provides a mobile anchor to the cell membrane.

In Fig. 10 a tentative flow path is outlined for the conversion of the ice gene product to the final modified lipoglycoprotein and its subsequent aggregation. Burke and Lindow (1) have proposed that the most likely nucleation structure consists of a disk ice nucleator on top of a cylinder that is 7.5 nm high. Presumably the cylinder is the extruded ice nucleation protein. Our tentative model, in which side-to-side aggregation involves the sugar residues on the protein core and the extrusion from the cell surface via a mannan-PI anchor, would be in accord with their model.

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