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Identification and purification of a bacterial ice-nucleation protein

(*Pseudomonas syringae/Pseudomonas fluorescens/tac* promoter/heterologous expression/bacterial membrane)

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ABSTRACT **The protein product of a gene (*inaZ*) responsible for ice nucleation by *Pseudomonas syringae* S203 has been identified and purified after overexpression in *Escherichia coli*. The amino acid composition and the N-terminal sequence of the purified, denatured protein corresponded well with that predicted from the sequence of the *inaZ* gene. The product of *inaZ* was also found to be the major component in preparations of ice-nucleating, proteinaceous particles, obtained after extraction with and gel filtration in a mixture of urea and the nondenaturing detergent octyl β-D-thioglucopyranoside. The activity of these preparations in the absence of added lipid implies that the protein participates directly in the nucleation process.**

Some bacteria of the genera *Pseudomonas, Erwinia*, and *Xanthomonas* can nucleate the crystallization of ice from supercooled water (1–3). Genes encoding the ice-nucleation active (Ina$^+$) phenotype have been cloned from *Erwinia herbicola, Pseudomonas syringae*, and *Pseudomonas fluorescens* (4–7). Theoretical considerations (8), as well as the actual sequence of the *inaZ* gene from *P. syringae* (6), suggest that the bacteria synthesize a template for ice-crystal formation, rather than an enzyme. The translation product predicted from the *inaZ* sequence is a protein with repetitive primary structure; its tertiary structure, which might also be repetitive, could provide considerable insight into the mechanism of ice nucleation. The ice nuclei of *P. syringae* are associated with its outer membrane (9) and are believed to contain both protein (9–11) and lipid (11) components. One report has suggested that the *inaZ* product is a phosphatidylinositol synthase, and that the lipid phosphatidylinositol is a key component of the water-binding template (12).

Here, we argue that the active component of bacterial ice nuclei is a protein, the product of *inaZ*. We have reached this conclusion after purifying the InaZ protein. To facilitate its isolation, we have attempted to maximize its expression by placing *inaZ* downstream of the *tac* promoter (13). This construct confers an Ina$^+$ phenotype on *Escherichia coli* and causes the accumulation of large quantities of a membrane-associated protein, which we have purified in both denatured and active forms.

MATERIALS AND METHODS

Materials. All chemicals were reagent grade, obtained from Sigma, except where stated otherwise. DNA restriction and ligation enzymes were obtained from New England Biolabs, and electrophoresis supplies, from Bio-Rad.

Bacterial Strains and Culture Conditions. The host used for all plasmids was *E. coli* K-12 strain JC10291 [*ara galK his lacY leuB mtl proA rpsL supE thi thr tsx* Δ(*recA–srl*)*303*] (14). For ice-nucleation, protein, and membrane studies, *E. coli* were cultured with aeration at 37°C in Luria broth [10 g of tryptone (Difco), 5 g of yeast extract (Difco), 5 g of NaCl, and 1 g of glucose per liter] containing 100 μg of ampicillin per ml and 250 μM isopropyl β-D-thiogalactopyranoside, to an OD_{600} between 0.4 and 0.5. Cells were then grown for an additional hour at 23°C (final $OD_{600} < 0.8$), chilled 15 min on ice, and harvested by centrifugation. Cell concentrations were inferred from OD_{600} values, using a standard turbidity of 7.1×10^8 ml^{-1}·OD_{600}^{-1} (15).

Ice-Nucleation Spectra. Ice-nucleation frequencies were measured by a drop-freezing method with an instrument constructed as described by Vali (16). For analysis of column fractions, 20 drops of 10 μl per dilution were examined at a dilution interval of 10^{-2}; in all other cases, 40 drops of 10 μl per dilution were tested, with a dilution interval of 10^{-1}. All spectra of subcellular fractions were normalized to the frequency per cell, by dividing the frequency per ml value for a given sample by the ratio (volume of sample)/(number of cells used to prepare sample).

Construction of pMWS10. The region of DNA encoding the *P. syringae* S203 *inaZ* gene (6) was digested with restriction enzymes *Aha* III (cutting at nucleotide 775) and *Eco*RI (cutting at nucleotide 4453), resulting in a fragment beginning 23 base pairs 5′ of the initiator codon. The *Eco*RI end was converted to a *Hin*dIII end by addition of a linker, and the fragment was inserted into pKK223.3 (17), so that *inaZ* was placed downstream of the *tac* promoter. The construct retained the original ribosome binding site of *inaZ*. The plasmid pMWS10 was then used to transform *E. coli* to the Ina$^+$ and ampicillin-resistant phenotypes.

Bacterial Membrane Preparations. Inner and outer membrane fractions were prepared from *E. coli* harboring pMWS10 by the method of Osborn and Munson (15) or Ito *et al.* (18) as described, except that 1 mM phenylmethylsulfonyl fluoride was included during lysozyme treatment, and sonication was performed in the presence of 10 mM EDTA. These additional treatments inhibited proteolytic degradation of the overproduced protein (19). No differences were observed between membranes prepared by the two methods.

Protein Fractionation. Denatured proteins were separated by hydroxylapatite (Bio-Rad, DNA grade) chromatography in the presence of $NaDodSO_4$, by the method of Moss and Rosenblum (20) as described, except that the sample was boiled 10 min before loading. The pooled hydroxylapatite fractions were dialyzed against 0.1% (wt/vol) $NaDodSO_4$ and then concentrated by vacuum centrifugation (Savant Instruments) to a volume of 0.5 ml, boiled 10 min, and loaded onto a Sephacryl S-400 (Pharmacia) column. The 30 cm × 1 cm i.d. column was developed with 0.1% $NaDodSO_4$/0.1 M NaP_i, pH 6.4/1 mM 1,4-dithio-L-threitol at 6.0 ml/hr. The fractions from gel filtration were pooled, concentrated by vacuum

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Abbreviation: OSG, octyl β-D-thioglucopyranoside.

centrifugation, dialyzed against 0.1% (wt/vol) $NaDodSO_4$, and stored frozen until use.

Membrane proteins were solubilized with 40 mM octyl β-D-thioglucopyranoside (OSG, from Calbiochem–Behring) essentially as described for the *E. coli* H^+-ATPase (21). The solubilizer (80 mM OSG/10 mM Tris Cl, pH 7.8/0.7 M sucrose/13 mM dithiothreitol) was mixed 1:1 (vol/vol) with the total membrane suspension (≈5 mg of protein per ml), incubated for 30 min at 37°C, and then ultracentrifuged 1.5 hr at 4°C, 215,000 × *g*, to remove undissolved material.

Protein Quantitation and Amino Acid Analysis. Protein concentrations of complex mixtures were estimated by the A_{280}/A_{260} method (22), after dissolving the samples with 1% (wt/vol) $NaDodSO_4$. For amino acid analyses, protein aliquots were hydrolyzed, dansylated, and analyzed by HPLC (23). Amounts of proteins separated by $NaDodSO_4$/polyacrylamide gel electrophoresis ($NaDodSO_4$/PAGE), using the discontinuous system of Laemmli (24), and stained with Coomassie Blue were measured *in situ* by gel scanning (LKB 2202 laser densitometer). Samples of the $NaDodSO_4$-purified protein used for amino acid analysis were subjected to electrophoresis; after the gel was stained and dried, the bands were scanned with the laser densitometer, and the measured band optical densities were used to calculate the concentration of InaZ protein in bands in gels prepared, run, and stained in the same way as the reference gel.

Finally, the purified InaZ protein could be quantitated by its absorbance at 275 nm. The amino acid sequence predicted from the *inaZ* gene contains 68 tyrosine and 7 tryptophan residues per molecule. Using extinction coefficients at 275 nm of 1340 for Tyr and 4000 for Trp (25), we estimate the extinction coefficient at 275 nm of the InaZ protein to be $1.2 \times 10^5\ M^{-1}\cdot cm^{-1}$.

N-Terminal Sequence Analysis. Residual $NaDodSO_4$ was extracted from purified, denatured protein samples with a mixture of acetone, triethylamine, acetic acid, and water (17:1:1:1, by volume). The protein was then dissolved in trifluoroacetic acid and applied to the reaction chamber of a gas-phase sequenator (Applied Biosystems, Foster City, CA) assembled and operated as described by Hewick *et al.* (26). The stepwise-liberated phenylthiohydantoin amino acid derivatives were analyzed using a Cyano-HPLC analytic column (IBM Instruments) and the gradient elution system described by Hunkapiller and Hood (27).

RESULTS AND DISCUSSION

A cumulative ice-nucleation spectrum (16) of *E. coli* harboring pMWS10, a plasmid that places *inaZ* under control of the *tac* promoter, is presented in Fig. 1. The equivalent spectrum of *P. syringae* S203 (the source of the *inaZ* gene) is shown for comparison. It may be seen that the recombinant organism displays a spectrum with a shape indistinguishable from that of the source organism, but shifted to slightly colder temperatures. The frequency asymptotes, approached at lower temperatures, indicate that every bacterial cell possesses at least one ice nucleus.

Membrane fractions were prepared from Ina^+ cells harboring pMWS10, and, as a control, from Ina^- cells. Two characteristics distinguished the membrane fractions from Ina^+ cells: their significant content of active ice nuclei (Fig. 1) and the presence of a protein, identified after $NaDodSO_4$/PAGE, whose apparent molecular weight was 153,000. This protein (p153) was present in large quantities in membrane fractions (Fig. 2*A*, lane 7), whereas fractions containing periplasm plus cytoplasm (lane 2), cytoplasm alone (lane 3), or control membranes from Ina^- cells (lane 4) lacked p153 and also lacked ice nuclei. Three membrane fractions were examined in detail: inner membranes, outer membranes, and

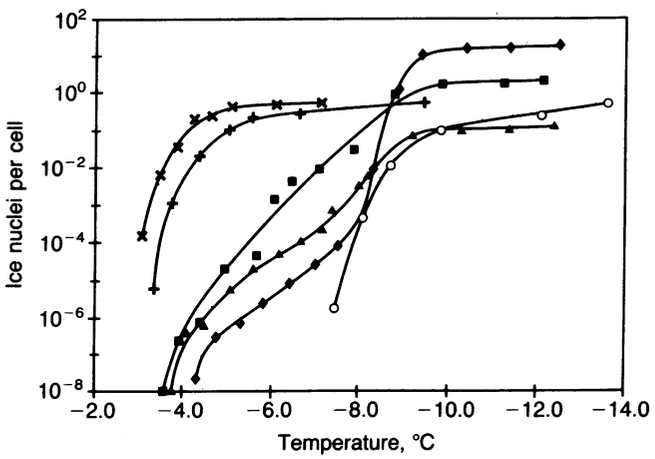

FIG. 1. Ice-nucleation spectra of *P. syringae* S203 (×), *E. coli* harboring pMWS10 (+), and cell fractions of *E. coli* harboring pMWS10: inner membranes (■), outer membranes (▲), dense fraction (◆), and OSG/2 M urea extract of dense fraction (○). Normalization to number of cells was carried out on the basis of the OD_{600} of the original cell suspension. Ina^- *E. coli* and membranes derived from them contained $<10^{-8}$ nuclei per bacterial cell at −10°C.

an unusually dense fraction, not observed in control cells, that pelleted through 55% (wt/wt) sucrose (1.26 g/cm³) but not 65% (wt/wt) sucrose (1.32 g/cm³). Ice-nucleation spectra of the three fractions (Fig. 1) revealed that nuclei active at warmer temperatures were concentrated in the inner and outer membrane fractions but that 90% of the total nuclei

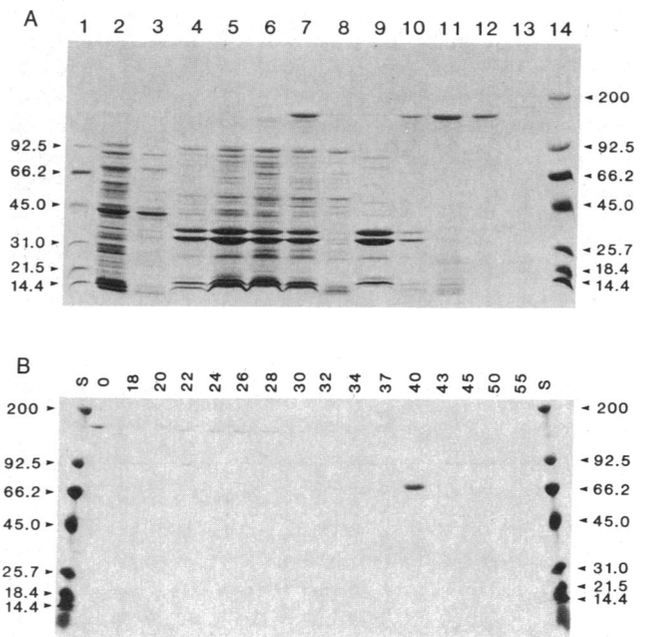

FIG. 2. (*A*) $NaDodSO_4$/PAGE of *E. coli* cell fractions. Cells harboring pMWS10: cytoplasm plus periplasm (lane 2), cytoplasm (lane 3), total membranes (lane 7), inner membranes (lane 8), outer membranes (lane 9), dense fraction (lane 10), pooled hydroxylapatite fractions (lane 11), purified p153 (lane 12). Cells harboring the *inaW* plasmid pLVC41 (7): total membranes (lane 6) and purified p180 (lane 13). Lanes 4 and 5 show total membranes from Ina^- *E. coli* harboring plasmids pUC9 (28) and pACYC184 (29), respectively. Lanes 1 and 14 contain standards; molecular weights are shown ($M_r \times 10^{-3}$). (*B*) $NaDodSO_4$/PAGE analysis of fractions eluted from Sephacryl S-400 with OSG/urea. Lanes are marked with fraction numbers; fraction 0 is an aliquot of the original sample loaded on the column. Lanes S contain standards; molecular weights are given at sides of the gel ($M_r \times 10^{-3}$). Both gels were 5–15% gradient gels, with 3% stacking gels.

were concentrated in the dense fraction. Examination of the membrane fractions by $NaDodSO_4$/PAGE (Fig. 2*A*, lanes 8–10) revealed that all three fractions contained p153 but that 83% of this protein was concentrated in the dense fraction (protein measured by gel scanning). The dense fraction may be an artifact of overexpression (30, 31); perhaps its accumulation results from overloading of the membrane insertion and transport systems. The high density of the fraction indicates that it is lipid-poor; the presence of two outer membrane protein bands (OmpA and OmpF proteins, running between M_r 31,000 and 45,000) indicates that outer membrane components associate with the dense bodies, either during growth or during the fractionation process.

Because p153 appeared to be associated with the Ina^+ phenotype, it was purified from the dense fraction in denatured form, by a combination of hydroxylapatite and Sephacryl S-400 chromatography. The $NaDodSO_4$/PAGE profile of the pooled, enriched fractions from the hydroxylapatite column is shown in Fig. 2*A*, lane 11. The $NaDodSO_4$/PAGE profile of purified p153 after Sephacryl S-400 gel filtration is shown in Fig. 2*A*, lane 12. The A_{275} of the material eluted from each column was monitored (data not shown), and the fractions containing p153 were in each case coincident with a major A_{275} peak. The purified, denatured protein showed no ice-nucleation activity.

To see if p153 might be the product of the *inaZ* gene, we determined its amino acid composition. Table 1 presents the averaged results of three determinations and, for comparison, the prediction for InaZ protein. Correspondence was close; significantly, p153 was observed, and InaZ protein predicted (6), to be unusually rich in serine, threonine, and glycine [the contents for an "average" protein (32) are 7.0% serine, 6.0% threonine, and 8.4% glycine]. The largest deviation from prediction was observed for tyrosine; however, tyrosine levels observed in the analysis of standards were particularly variable. Tyrosine can form three different dansyl derivatives; only the didansyl derivative was counted for this analysis.

A more definitive proof of a protein's identity is N-terminal Edman sequencing. We therefore sequenced the first nine residues from a sample of p153 purified from the dense cell fraction. The sequence observed was Met-Asn-Leu-Asp-Lys-Ala-Leu-Val-Leu-, in perfect agreement with the sequence predicted from *inaZ* (6). The initial coupling yield was 25%; given the observation that the sequencing method used typically gives initial coupling yields between 20% and 40%, we conclude that this sequence is representative of the majority of the protein loaded onto the sequenator. Interestingly, the sequence begins at the start of the open reading frame; this may indicate that the InaZ protein is not posttranslationally processed. However, if the dense cell fraction is an artifact of overproduction, then its lack of processing may have a trivial explanation: the protein may have never been exposed to the posttranslational processing machinery. The composition of p153, its N-terminal sequence, and its correlation with the Ina^+ phenotype together lead us to conclude that p153 is the InaZ protein.

Table 1. Amino acid analysis of p153

Amino acid	Mole %	
	Measured	Predicted
Ala	12.84 ± 0.46	11.92
Arg	2.16 ± 0.16	2.60
Asx	6.10 ± 0.25	5.95
Cys	1.22 ± 0.69	0.33
Glx	6.99 ± 0.69	7.62
Gly	16.55 ± 0.50	16.42
His	0.89 ± 0.09	1.09
Ile	3.11 ± 0.67	3.69
Leu	6.68 ± 0.47	7.12
Lys	1.02 ± 0.05	1.18
Met	0.58 ± 0.08	0.67
Phe	0.81 ± 0.05	0.83
Pro	1.24 ± 0.19	1.26
Ser	18.31 ± 2.28	16.33
Thr	14.83 ± 1.08	13.83
Tyr	3.69 ± 0.35	5.70
Val	2.96 ± 0.22	3.10

The values reported are averages of three determinations. Standardization was performed by analysis of a hydrolysate of hen egg white lysozyme. Methionine was determined as methionine sulfone.

The apparent molecular weight of p153 is 34,000 higher than the molecular weight of 119,000 predicted by translation of *inaZ*. This discrepancy might have any of several causes (33). First, the InaZ protein may be posttranslationally modified by covalent attachment of lipid and/or oligosaccharide. Second, the large amounts of serine, threonine, and glycine in the sequence may lead to an anomalously low affinity for $NaDodSO_4$. Finally, the protein may be highly nonglobular and not fully denatured under the conditions of electrophoresis. It should be noted that another protein containing large amounts of hydrophilic, repeating structure, the malarial circumsporozoite protein, also migrates at an anomolously high apparent molecular weight when examined by $NaDodSO_4$/PAGE (34). The apparent molecular weight of the InaZ protein does not change markedly upon addition of 4 M urea to the $NaDodSO_4$/PAGE system but is dependent on the acrylamide percentage used (data not shown); this property is probably indicative of insufficient $NaDodSO_4$ binding (33).

Another bacterial protein, p180, the product of the *inaW* gene of *P. fluorescens* MS1650, is also known to be associated with an ice-nucleation effect (7). This protein was purified in smaller quantities, by a similar procedure. In this case, the *inaW* gene was not present in an overexpression vector, and a dense cell fraction was not observed. However, we utilized the observation that treatment with the detergent OSG (21) preferentially solubilizes membrane components other than p180 (this is also true for p153). Therefore, total membranes from the transformed *E. coli* strain were extracted with OSG. The OSG-insoluble material, which was enriched in the *P. fluorescens* gene product, was then solubilized for hydroxylapatite chromatography. The membranes of control and transformed *E. coli* are shown in Fig. 2*A*, lanes 5 and 6, respectively, and the purified p180 is shown in lane 13. Sizes, solubility properties, and affinities for hydroxylapatite are thus similar for p180 and p153; this suggests that they possess similar sequences and structures.

To analyze the role of InaZ protein in ice nucleation, we attempted to reconstitute the purified, $NaDodSO_4$-solubilized p153 into *E. coli* lipids (Avanti Polar Lipids). These experiments resulted in low, irreproducible levels of ice-nucleation activity. We therefore developed a second purification scheme, based upon solubilization with a mixture of urea and OSG, which optimized the recovery of activity at some expense to the recovery of protein. The dense cell fraction from cells harboring pMWS10 was treated with OSG as in the purification of p180; the insoluble material was then treated for 1 hr at 37°C with OSG solubilizer containing 2 M urea, and here the supernatant was retained after ultracentrifugation. The supernatant contained ≈10% of the total InaZ protein present in the original dense cell fraction and was active in ice nucleation after 1:100 dilution into 10 mM KP_i buffer (pH 7.0, 4°C). An ice-nucleation spectrum of the supernatant is shown in Fig. 1; the asymptotic activity was 1.2×10^{10} nuclei/ml. Finally, the supernatant was passed over a Sephacryl S-400 column. The gel electrophoretic patterns of the resulting fractions are shown in Fig. 2*B*.

Clearly, the InaZ protein is the major protein constituent of the early fractions. The recovery of ice-nucleation activity from this column was quantitative; its elution profile is shown in Fig. 3, along with the A_{275} of the eluant stream and the quantity of InaZ protein in each fraction. The eluted activity could be completely abolished by Pronase treatment (data not shown). The recovered activity follows the protein recovery measured by A_{275} (before fraction 35) and densitometry. However, the specific activity (i.e., number of nuclei per protein molecule) is clearly higher for the fractions at or near the column void volume. The apparent molecular weight of nuclei in these fractions is in excess of 400,000. Thus, the specific activity peaks at a molecular weight significantly larger than that deduced from the gene sequence (119,000), or inferred from $NaDodSO_4$/PAGE (153,000). This suggests that aggregation may be required for the formation of effective ice nuclei.

A mean specific activity (at −13.2°C) can be calculated for the InaZ protein eluted between fractions 18 and 34, either from the amounts of InaZ protein estimated by gel scanning or the integrated A_{275} of the eluant stream. The calculated activities are 1 nucleus per 3.0×10^4 molecules (A_{275}) or 1 nucleus per 3.6×10^4 molecules (gel scanning). These estimates are nearly equal and are not much less than the specific activity of 1 nucleus per 1.2×10^4 molecules estimated for the dense cell fraction (gel scanning). Given such large numbers of molecules per nucleus, we cannot eliminate the possibility that a minor contaminant, which copurifies with the InaZ protein, is responsible for ice nucleation. This possibility will be difficult to refute until preparations with higher specific activity can be obtained.

The active preparation of the purified InaZ protein has three noteworthy properties. First, the bulk of the activity is associated with particles considerably larger than monomers of InaZ protein. This suggests that the active nucleus is a homomeric aggregate of InaZ protein, in agreement with the interpretation of γ-ray inactivation studies of bacterial ice nuclei (35). Second, the number of InaZ protein monomers required per nucleus is much larger than the number (≈1 at −13°C) that may be inferred from γ-ray inactivation studies (35). If the latter inference is correct, then either most of the purified protein is inactive, or else it aggregates further upon dilution (it is only possible to measure the number of independently sorting nuclei). Third, the active InaZ protein preparation contains no added lipids (other than residual OSG) and no substrates for lipid synthesis and has been subjected to conditions that are sufficient to remove all but the most tightly bound lipids from membrane proteins. If the InaZ protein is a phosphatidylinositol synthase (12), the phosphatidylinositol so produced must be tenaciously bound to the protein. In light of our results, we feel that the most reasonable model of ice-nucleus structure places the ice-nucleation gene product in the central role of forming an ice-crystal template. In this role, it could be aided by the planar supporting structure of the bacterial membrane.

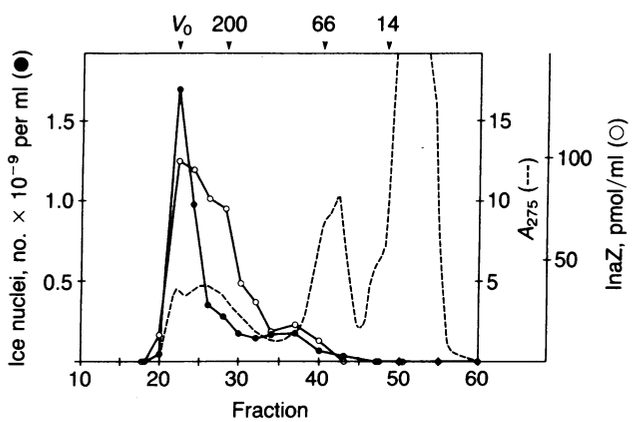

FIG. 3. Purification of active ice nuclei. A Sephacryl S-400 column (60 cm × 1 cm i.d.) was loaded with 0.65 ml of OSG/urea-solubilized ice nuclei and developed with 2 M urea/100 mM NaP_i, pH 6.4/12 mM OSG/1 mM dithiothreitol at 6.0 ml/hr and 4°C. Fractions of 1.0 ml were collected. Shown are the asymptotic ice-nucleation activity (at −13.2°C) (●), the continuously monitored A_{275} of the eluant stream (----), and the amount of InaZ protein eluted (○), estimated by densitometry of the gel in Fig. 2*B*. The elution positions of the void volume (V_0, measured with Blue dextran) and three molecular weight markers ($M_r \times 10^{-3}$) are shown at the top of the figure.

Note Added in Proof. Recently, a physical and partial chemical characterization of cell-free ice nuclei shed by *Erwinia herbicola in vivo* has been published (36).

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