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Bacterial Ice Nucleation: Molecular Biology and Applications

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Introduction

Water can be cooled below 0°C and still remain liquid: this is the phenomenon of 'supercooling'. The introduction of an 'ice nucleus' into supercooled water results in a chain reaction of freezing, which is spectacularly rapid at sufficiently low temperatures. Besides the rather electric effect this can have on people witnessing it for the first time, there are more serious uses for ice nuclei. They are currently used in snow-making, and they have potential applications in the production of ice cream and other frozen foods, in immunoassays, and as a replacement for silver iodide in cloud seeding. In addition, the prevention of ice nucleation would protect some crop plants from frost damage. Of the various types of biological ice nucleators, bacteria have been the subject of most research and also appear most relevant to the anticipated practical uses. The identification and ecology of ice-nucleating bacteria have been the subjects of excellent reviews by Lindow (1982, 1983a), and the phenomenon of nucleation has been covered in the hard-hitting mini-review by Franks (1987). The intent of this article is to explain the basis of the existing and intended applications for bacterial ice nuclei, and to discuss the aspects of molecular biology which are relevant to their practical development.

POTENTIAL APPLICATIONS AND THEIR REQUIREMENTS

Although many substances are ice nucleators at lower temperatures, relatively few are active above −10°C, and these are not abundant as impurities in water. Hence, any process which requires the independent freezing of

Abbreviations: CDP, cytidine 5'-diphosphate; Ina, ice nucleation activity; PAGE, polyacrylamide gel electrophoresis; PI, phosphatidylinositol; SDS, sodium dodecyl sulphate.

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large numbers of small water droplets, at temperatures between −10°C and 0°C, stands to benefit from the presence of ice nuclei. In snow-making applications, the droplets are suspended in air: water is sprayed upwards at high pressure, and its heat loss to the air is maximized if droplets are small and supercooling is prevented by ice nucleation. (Snow-making is practised both by recreational ski resorts (Woerpel, 1980), and by engineers conducting oil exploration in the Arctic (Duthweiler and Utt, 1985), who have uses for ice as a construction material.) Ice nuclei also find use during the initial freezing process in some types of food preparation, where independent water droplets are present as emulsions with other fluids. Arai and Watanabe (1986) give an example in the preparation of bean curd; ice-cream preparation is another promising area of application. The outdoor use of ice nuclei requires that they be robust and environmentally safe: such criteria are met by some of the naturally occurring ice-nucleating bacteria. On the other hand, for use in food it is most important that the ice nuclei be non-toxic, non-pathogenic and palatable. These criteria are likely to be met best by preparations in which the ice nuclei have been extracted and purified away from their bacterial source. Cell-free preparations are also the most likely to be acceptable by national regulatory agencies in the current climate of uncertainty about regulation. (Because use of ice nuclei in food is still in the experimental stage, intact bacteria can be used at present in order to determine the effects of nucleation on texture: the bacteria are harmless but unacceptable to regulatory agencies as a commercial food additive; moreover, they taste bad.)

A different and, at present, hypothetical use of ice nuclei is related to their property of signal transduction: that is, their ability to convert one type of signal into another. The presence of a single submicroscopic body (the ice nucleus itself) may be transduced to a macroscopic physical change (the phase transition of a volume of water) in less than a second. This property may find use in detection systems of high sensitivity, for example, immuno-assays. It would probably require that the ice nuclei be small and uniform. Thus, their extraction from bacteria would again be necessary.

The protection of certain plants from frost damage could be achieved by *reducing* their colonization by ice nucleation-active bacteria. The tissues of some tender plants contain no nucleators active at warm temperatures (Marcellos and Single, 1979), and on these plants, bacterial epiphytes are the sole source of ice nuclei and thus the causative agents of any freezing damage that results (Lindow, 1982, 1983a,b). One approach to reducing colonization by bacterial ice nucleators is the use of bacteriophages (Kozloff and Schnell, 1983), although the author is unaware of any instance of practical use for bacteriophages in biological control. A second possibility for biocontrol is in the use of competitor bacteria to replace or displace the natural populations (Arny and Lindow, 1977, 1979). This requires that the competitors be as ecologically fit as the strains which they are to displace, and that they be non-ice-nucleating themselves. A straightforward way of obtaining such strains has been to derive them from the strains that are normally epiphytic residents, by genetic manipulation to inactivate their ice-

nucleation genes (Orser *et al.*, 1984b; Lindemann and Suslow, 1987; Warren *et al.*, 1987).

THE PHENOMENON OF ICE NUCLEATION

What makes it possible for water to supercool? It is well known that ice melts at 0°C; this is the equilibrium temperature for the solid/liquid phase transition of water, but an equilibrium is not necessarily attained by liquid water unless it is already in contact with its solid phase. The difficulty of achieving equilibrium is explained by the thermodynamic prediction that solid/liquid equilibria are shifted in favour of the liquid when only a very small body of the solid is present. This is because the loss of entropy by molecules joining the solid state becomes more significant when a body of the solid has a high surface:volume ratio (Fletcher, 1970). Although ice lattices form spontaneously by the random motion of molecules in liquid water, such lattices occur only on so small a scale that the equilibrium temperature at the lattice is approximately −40°C: thus the spontaneously forming ice lattices melt again in water above −40°C.

Because the equilibrium temperature depends on the scale of an ice lattice, at each temperature there is a critical surface:volume ratio, and hence a critical size, for incipient ice lattices (Fletcher, 1958). The lattices above this size will serve as templates for the further deposition of ice from liquid water; therefore the appearance of an ice crystal above critical size is a *nucleation* event for the freezing that ensues. Nucleation seems similar to catalysis, but there are some distinctions. Both processes involve reducing the free energy of an unstable intermediate. However, unlike a catalyst, a nucleating agent need act only once, and it is not necessarily regenerated by the process that it evokes. 'Heterogeneous' ice nucleation occurs in supercooled water when an added substance, or a discrete impurity, causes the formation of an ice crystal above the critical size. Heterogeneous ice nucleators must impose order on molecules of the liquid phase, presumably by providing a more favourable lattice match with the crystalline structure of ice than with liquid water (Hallett, 1968; Fletcher, 1970)*. It also follows that, depending on the size of ice lattice which can be organized, a heterogeneous ice nucleus will have a threshold temperature, above which it will be inactive. 'Homogeneous' ice nucleation describes the formation of an ice lattice above critical size without the influence of a macromolecular organizing factor. It is thus a process resulting from the strictly spontaneous assembly of ice lattices from random motion of water molecules and, as implied above, homogeneous nucleation is statistically improbable in pure water above −40°C. Physical agitation, such as the scratching of a solid surface immersed in supercooled water, can also nucleate crystallization: this is viewed as a distinct mechanism and is termed 'mechanical' ice nucleation, although in

*It may seem ironic that antifreeze proteins, found in some polar fish species, achieve the converse effect in a similar way. By possessing a higher affinity for the ice–water interface than for water, they can stall ice formation by binding to, and effectively poisoning, the sites of crystal growth (Knight, DeVries and Oolman, 1984). Antifreeze proteins are, however, too small to *organize* ice lattices of significant size.

some cases it may reflect the transient formation of heterogeneous ice nuclei. The remainder of this article will be concerned solely with heterogeneous ice nucleation.

In the atmospheric sciences, there has long been an interest in ice nucleation. It was in this connection that plants were discovered to be sources of airborne ice nuclei (Schnell and Vali, 1972, 1973), and the nuclei were found to be of bacterial origin (Maki *et al.*, 1974). It should be mentioned that the significance of bacterial ice nuclei to atmospheric phenomena is not yet resolved, although it has been more usual to hypothesize that they have an effect, than to suggest that they do not. Bacterial ice nucleation became of interest to plant pathologists with the discovery that it increased the frost sensitivity of some plants (Lindow, Arny and Upper, 1978). The ice-nucleating bacteria were found to belong to the species *Pseudomonas syringae* (Arny, Lindow and Upper, 1976), *Pseudomonas fluorescens* (Maki and Willoughby, 1978), *Pseudomonas viridiflava* (Anderson and Ashworth, 1986), *Erwinia herbicola* (Lindow, Arny and Upper, 1978), and *Xanthomonas campestris* (D. C. Sands, S. E. Lindow and C. S. Orser, unpublished work, reported by Orser *et al.*, 1985). Although all these reports concerned strains associated with terrestrial plants, ice-nucleating strains of *P. fluorescens* have also been isolated from the marine environment (Fall and Schnell, 1985).

Some insects (Duman and Horwath, 1983) and at least one angiosperm (Krog *et al.*, 1979) have evolved the ability to nucleate ice crystallization at relatively warm temperatures; the evolution probably occurred independently, although this will become clearer with the characterization of the genes controlling the phenomenon in each case. Both the angiosperm and the insect ice-nucleators appear to derive a cryoprotective benefit from the activity: for these organisms, the slow growth of intercellular ice at temperatures just below 0°C is preferable to the rapid and more disruptive intracellular crystallization which can occur in water that has supercooled to lower temperatures. (This contrasts with the situation in tender plants, the tissues of which cannot tolerate freezing at any temperature.) The selective advantage to bacteria probably derives from the ability to cause plant tissue damage, but there are other possibilities which will be discussed.

From an applied point of view, the bacterial ice nuclei are of particular interest because they may be readily obtained in large numbers, and they are more amenable to genetic manipulation than ice nuclei from other organisms. They also display the highest threshold temperatures among insoluble nucleating agents; the crystals of some amino acids display still higher thresholds (Parungo and Lodge, 1967) but are useless in practice because they dissolve.

Properties of ice nuclei

THE PARAMETERS OF ICE NUCLEATION

The threshold temperature of an ice nucleus should ideally be defined as the temperature at which the probability of ice nucleation in a given time interval

exceeds a given value: that is, as the temperature at which the reaction exceeds a given rate. Vali and Stansbury (1966) examined the relationship between rate and temperature, and found that the nucleation rate increases extremely rapidly as temperature decreases. Therefore, within the bounds of interval lengths that are experimentally practicable, it makes little difference what time interval is used to estimate threshold temperature, and the interval is not always standardized.

For an individual nucleus, threshold temperature is the most meaningful parameter of ice nucleation, and it may be measured by slow or stepwise cooling of a water droplet containing the nucleus, and recording of the temperature at which the droplet freezes. Two parameters are required to describe a population of ice nuclei: threshold and frequency. Vali (1971) described the determination of a cumulative nucleation frequency spectrum (hereafter abbreviated to cumulative spectrum), in which a graph represents the population frequency of ice nuclei with thresholds at or above each temperature in a temperature range (*see Figure 1*). Experimentally, slow or stepwise cooling is used to test the nucleus content of a number of independent droplets of bacterial dilutions over a range of temperatures; for each temperature, Poisson statistics are employed to infer the frequency of ice nuclei from the proportion of droplets which are seen to contain exactly zero ice nuclei. Differentiation of a cumulative spectrum yields a histogram representing the frequencies of nuclei having thresholds within a number of narrow temperature ranges (*Figure 1*). This additional manipulation of the

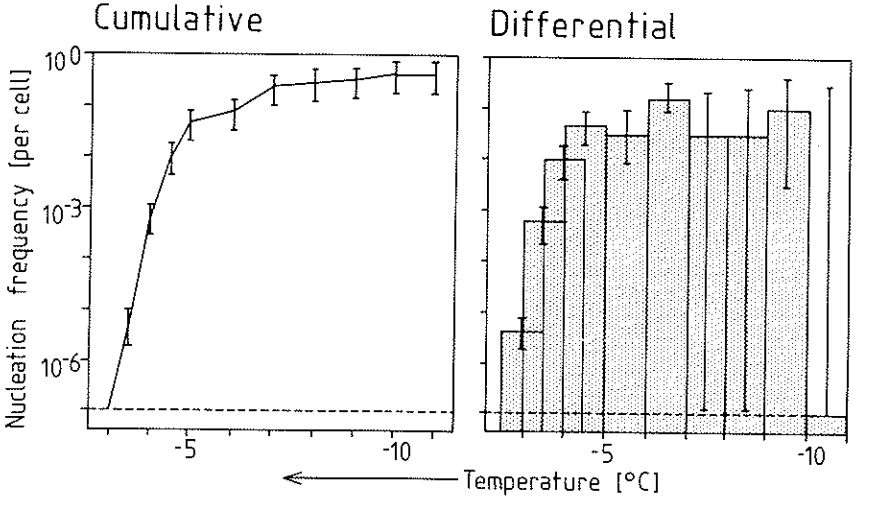

Figure 1. Comparison of cumulative and differential spectra of ice nucleation frequency. Both spectra are derived from the same set of data (in this case, from clones of *E. coli* expressing the *inaZ* gene at a particular level: Southworth, Wolber and Warren, 1987). Error bars demonstrate the much greater difficulty of obtaining an accurate differential spectrum. Nucleation frequency (per cell) was obtained by dividing the concentration of nuclei by the concentration of cells. Dashed lines represent the detection limit for frequency in the particular experiment which generated these data.

data is not usually helpful to its interpretation, although in some instances it is valuable. Methods have been published for making the observation of cumulative spectra less tedious (Parody-Morreale *et al.*, 1986) or more accurate (Makino, 1982).

The measurement of warmest nucleation temperatures offers a much quicker and cruder method of assessing population characteristics. The onset temperature for freezing is measured in samples containing large numbers of bacteria: it represents only the warmest nucleation threshold of all the ice nuclei present. For samples containing large enough numbers of bacteria, the standard error of such a measurement is surprisingly small, owing to the steepness of most cumulative nucleation spectra towards lower frequencies. Warmest nucleation temperature is most appropriate for the preliminary analysis of large numbers of samples, for example in the detection of mutants. It is a very poor indicator of changes in nucleation frequency.

STUDIES ON WHOLE CELLS

The first type of analysis carried out on the bacterial ice nucleators was the characterization of their cumulative spectra, sometimes under a variety of growth conditions. It was found that for most strains, ice nuclei with thresholds above −4°C were the most scarce; next in abundance were nuclei with thresholds in the range of −4°C to −8°C, while nuclei with thresholds below −8°C formed the most numerous class. Ice nuclei in these classes were designated as types I, II, and III, respectively (Yankofsky *et al.*, 1981): a convenient but artificial division for a continuous spectrum of threshold temperatures. It was found that growth conditions have a large influence on the cumulative spectrum: low growth temperature (below 24°C) is critical for obtaining high nucleation frequencies in *P. syringae*, but reportedly it is unimportant for *E. herbicola* (Yankofsky *et al.*, 1981). Besides looking at nucleation in the aqueous phase, Maki and Willoughby (1978) also tested a *P. syringae* and a *P. fluorescens* strain for ice nucleation of water vapour in an isothermal cloud chamber and, surprisingly, found that only *P. fluorescens* was effective. This experiment would bear repeating with a larger variety of strains and species. The processes of ice nucleation in liquid and vapour phases of water have been distinguished by the terms 'freezing' and 'contact nucleation', respectively (Hobbs, 1974). A structure should be capable of both processes unless it is altered by the desiccation necessary for testing contact nucleation.

Yankofsky, Nadler and Levin (1983) experimented with treatments that interfere with DNA synthesis: UV irradiation, exposure to mitomycin-C or nalidixic acid, and thymine deprivation of a thymine auxotroph. They found that all of these treatments induced an increase in nucleation frequency by a strain of *E. herbicola* after a time lag of about 2 hours. The induction was prevented by rifampicin or streptomycin, which block transcription and translation respectively; this indicates that gene expression is necessary for this induction. A similar phenomenon has not been observed in *Pseudomonas*.

A number of agents have been used to determine the nature of the ice nuclei of bacteria *in situ*: an agent with a more or less specific effect is applied, its effect on nucleation is noted, and inferences are drawn about the nature of the nucleus from its observed sensitivity to the testing agent. These experiments were carried out before the ice-nucleation protein had been identified; the protein's fate during such treatments is now of interest but has not been investigated. Maki *et al.* (1974) found that exposure to a temperature of 65°C inactivated ice nuclei, which suggested that they were unlikely to be made purely of carbohydrate. In fact, ice nuclei can be shown to be sensitive to much less severe temperature treatments: nuclei produced during 24°C growth are severely affected by a 30°C temperature treatment; nuclei with warmer threshold temperatures are lost while those with cooler thresholds are reduced in frequency to lesser extents. Ice nuclei were also found to be sensitive to cetyl-pyridinium chloride, to the dyes crystal violet, methylene blue and safranin, and to mercuric ions (Maki *et al.*, 1974). Zagory, Lindow and Parmeter (1983) found that smoke inactivated ice nuclei, which may be relevant in the use of smudge pots to control frost. However, the significance of such findings to a molecular understanding is not yet clear.

Kozloff, Schofield and Lute (1983) extended these studies with more specific types of reagent. It should be noted that their observations on nucleation response were limited to measurements of warmest nucleation temperature in large populations of bacteria; cumulative spectra were not reported. As noted above, this method tends to underestimate small effects (i.e. less than two or three orders of magnitude) on nucleation frequency. Their observations that metal-chelating compounds and pH changes between 5·0 and 9·2 did not affect nucleation are suspect for this reason. Borate compounds inhibited ice nucleation to varying extents: butyl borate had little effect while phenyl- and *m*-nitrophenyl borate had the greatest effect; borate itself was intermediate. These compounds have a similar order of affinity for *cis*-hydroxyls: the correlation could be explained if their site of action was, for example, a sugar group. A similar implication resulted from studies of lectin inactivation of ice nuclei: only certain lectins were effective, and these shared a specificity for mannose and glucose residues. Three compounds with a specificity for reacting with sulphydryl groups (*N*-ethylmaleimide, iodoacetamide, and *p*-hydroxymercuribenzoate) were found to inhibit ice nucleation very effectively, but they also killed the cells and thus (as discussed in the next section) it is possible that their inactivation of ice nuclei is a secondary effect of their causing a degradation of other cellular structures. Kozloff and co-workers went on to extrapolate the number of ice nuclei present per cell at time zero from the inactivation kinetics. However (in my view), these calculations rest on unjustified assumptions, for example, an implicit assumption that individual ice nuclei have first-order kinetics of inactivation; the inferences from such calculations will not be discussed here.

In a later paper, Kozloff, Lute and Westaway (1984) presented evidence that the lipid phosphatidylinositol (PI) may be a component of the ice nuclei of *P. syringae* and *E. herbicola*. They originally hypothesized the involvement

of such a molecule based on the observation that the carbohydrate *meso*-inositol has a steric arrangement of hydroxyl groups topotactic with that in ice. PI was shown to cause haemagglutination of the lectins which had previously been shown to inhibit ice nucleation: thus it is conceivable that PI might be the target of such lectins. A C_{11} lipase with specificity for phosphatidylinositol was purified from *Bacillus cereus*, and an activity inhibitory to ice nucleation (as judged by population threshold temperatures) was shown to co-purify with it. Lastly, phosphatidylinositol synthase (CDP diacylglycerol–inositol 3-phosphatidyltransferase, EC 2.7.8.11) was assayed in a series of bacterial strains: these included Ina^+ and Ina^- strains of *P. syringae*, *E. herbicola*, and *E. coli*. PI-synthase activity was found in all the Ina^+ and none of the Ina^- strains. This constitutes the strongest evidence for the involvement of PI in ice nucleation, although the PI-synthase assay yielded extremely variable results in duplicate experiments. The possibility that PI has a role is exciting, and it will be interesting to see whether other laboratories can confirm and extend these findings. Kozłoff *et al.* (1984) proposed that PI synthase was the product of the *ina* genes cloned from *E. herbicola* and *P. syringae* by Orser *et al.* (1983). The further characterization of *ina* genes (*see* pp. 116–122) has as yet neither supported nor refuted this proposal. However, such a proposal is not the only way to explain the conditional detection of PI synthase: for example, an endogenous PI synthase might become induced if ice nuclei sequestered PI and depleted its cellular pool.

The size of the ice nuclei with different threshold temperatures was estimated in intact cells by Govindarajan and Lindow (1984b). Freeze-dried cells of *E. herbicola*, *P. syringae*, and *E. coli* were subjected to gamma radiation, followed by rehydration and assaying of ice nucleation frequency. The dose-dependence of the reduction in nucleation frequency was thus observed for ice nuclei in various ranges of threshold temperature. For *P. syringae* and *E. coli*, the nucleation frequency in each range of threshold temperature decreased as a first-order function of radiation dose: this indicated that the ice nuclei were each vulnerable to a single hit. The size of target which each type of ice nucleus presented to the gamma rays could therefore be calculated from the dose-dependence. For both *E. coli* and *P. syringae*, the estimated size of the ice nucleus varied from 620 kD for a −9·0°C threshold to 19 000 kD for a −2·0°C threshold. The overall trend of increasing size with increasing temperature, indicated by this valuable experiment, seems beyond doubt. The exact magnitudes of these estimates should, however, be subject to at least two qualifications. First, some or all hits may degrade the threshold temperature of an ice nucleus, rather than destroy it. This would mean, for example, that while some −9·0°C nuclei were destroyed, others were being created from −8·0°C nuclei, and so forth: this would lead to an over-estimation of the effective target size for ice nuclei in the lower ranges of threshold temperature, where the cumulative nucleation spectra are usually not steep. Second, ice nuclei may be vulnerable to 'near misses' in which a nearby molecule becomes chemically activated and subsequently reacts with the ice nucleus. This would lead to over-estimation of the sizes of all ice nuclei.

STUDIES ON CELL-FREE ICE NUCLEI

In the earliest experiments of this sort, bacterial cells were fragmented and it was observed that Type I ice nuclei were destroyed whereas Type III and some Type II nuclei remained active. The nuclei were found in the sedimenting fraction, not the soluble fraction (Maki *et al.*, 1974; Yankofsky *et al.*, 1981). Thus, they are associated with macromolecular structures, rather than being free in the cytoplasm. The differential sensitivity of the Type I nuclei was interpreted to mean that they required a physiologically normal cell. In view of later findings (*see* p. 116), however, it may instead indicate that they require a cofactor which is diluted out upon fragmentation of the cells in buffer.

Sprang and Lindow (1981) went on to demonstrate that ice nuclei were associated with the membrane fraction of cells (of both *E. herbicola* and *P. syringae*) treated with lysozyme (EC 3.2.1.17) prior to sonication. Upon separation of inner and outer membranes, 80% of the ice nuclei were found in the outer membrane fraction. It is simple and attractive to infer that the ice nuclei are in fact attached to the outer membrane, but it is possible instead that they just happened to have similar separatory properties. The same caveat must be applied to the observations of Wolber *et al.* (1986), who fractionated membranes from *E. coli* and found an approximately equal association of ice nuclei with inner and outer membrane fractions.

Govindarajan and Lindow (1984a) utilized ice nucleation-active preparations of outer membranes (from *P. syringae*) to probe for lipids. (The nuclei present were mostly of Type III.) Three delipidating treatments each reduced nucleation frequency at −9·0°C and showed a linear correlation between loss of phospholipid and loss of ice nuclei; the treatments were incubations with the enzyme phospholipase A_2 (EC 3.1.1.4) or the detergents sodium cholate or SDS. It was also demonstrated that removal of detergent by dialysis could allow the reconstitution of ice nuclei: in the future this technique may permit the analysis of essential components by experimental reconstitution from more highly purified fractions.

Perhaps the most promising source of cell-free ice nuclei for experimentation is that described by Phelps *et al.* (1986). They found that at temperatures below 20°C, growing cultures of Ina^+ *E. herbicola* cells shed ice nuclei into the medium. The shed nuclei could pass through 0·2 μm filters which retain viable cells. Because these ice nuclei appear to be shed by living cells without provocation, they are likely to consist of structures representative of normal, cell-associated ice nuclei. This is supported by the observation that they have cumulative spectra similar to the cells from which they derive.There is strong evidence that shed nuclei are associated with membrane vesicles, which were shown to be shed only under the same conditions as the nuclei. The shedding of membrane vesicles occurred similarly in Ina^- strains of *E. herbicola*: thus it seems likely that when ice nuclei are present in the membrane they are carried along into vesicles passively. This is further reason to expect that their association with the membrane vesicles is representative of their normal situation.

The cell-free ice nuclei possessed a temperature sensitivity similar to that observed previously in whole cells. Phelps and co-workers also tested their preparations with some of the same reagents used earlier on whole cells. The responses were strikingly different. Whereas the sulphydryl-modifying reagents (*N*-ethylmaleimide and *p*-hydroxymercuribenzoate) killed cells and inactivated whole cell-associated ice nuclei in the control experiment, they had no effect on the cell-free nuclei. Conversely, proteases inactivated cell-free but not cell-associated ice nuclei, and did not kill cells. The possibilities of indirect effects on the integrity of ice nuclei via cell damage or death, when whole cells are treated in various ways, are certainly supported by these observations. It seems likely that ice nuclei in whole cells are vulnerable to components of other cellular compartments; the release of these destructive components can always be invoked to explain inactivation of nuclei in whole cells. On the other hand, the living cell, with its complex compartmentalization and physiological homeostasis, may shelter its ice nuclei from the effects of certain agents capable of destroying them. Both qualifications limit the usefulness of most biochemical experiments performed on whole cells.

Phelps and co-workers also reported that the Type I nuclei in their cell-free preparations required 10 mM Mg^{2+} for activity: when Mg^{2+} was removed or increased to 100 mM the ice nuclei with warm thresholds were lost. This observation may be related to the current view that proper adhesion, between peptidoglycan and the inner and outer membranes of a Gram-negative bacterium, is mediated by magnesium ions.

Genetic analysis

GENES

The strategy initially devised to identify ice-nucleation genes was to isolate deficient (Ina^-) mutants, and to screen cloned sequences for complementation of the mutations. In following such a strategy for obtaining ice-nucleation genes from *P. syringae* and *E. herbicola*, Orser *et al.* (1983, 1984a, 1985) found that it was unnecessary to return the cloned genes to their original host species in order to detect their phenotype, because they imparted ice nucleation activity (Ina) to the heterologous host *Escherichia coli*. A 4·5 kb fragment of DNA from *P. syringae*, and a 5·7 kb fragment from *E. herbicola*, conferred the Ina^+ phenotype on *E. coli* and were also sufficient to complement all Ina^- mutations in the original species. Corotto, Wolber and Warren (1986) similarly identified a 5·7 kb DNA fragment from an Ina^+ strain of *P. fluorescens*.

The cumulative spectrum conferred by the *P. syringae* sequence in *E. coli* was similar to that of *P. syringae*, both in the relative frequencies for different temperatures and in the absolute frequency (per cell) for each temperature. The spectra conferred by the *E. herbicola* and *P. fluorescens* sequences were similar in shape (i.e. in relative frequencies), but higher in absolute frequencies, when compared with those of *E. herbicola* and *P. fluorescens*. It is reasonable to conclude, from the shapes of the spectra, that the forma-

tion of ice nuclei from the cloned genes' products is very similar in *E. coli* to that in the original organisms. The frequency differences may be attributable to corresponding differences in the transcription and translation levels of the ice-nucleation genes. Because ice nuclei seem to be formed normally in *E. coli*, not only can we study them in the most convenient host possible, but also we can infer that the action of the ice-nucleation gene products is likely to be quite autonomous—their action cannot depend on anything specific to one species of bacteria.

The nucleotide sequences of two Ina-conferring regions from *Pseudomonas* have been determined: in each case a single long open reading frame, indicating a single gene, was found (Green and Warren, 1985; Warren, Corotto and Wolber, 1986). The *inaZ* gene from *P. syringae* was substantially similar to the *inaW* gene from *P. fluorescens*. The consensus of their predicted translation products is reprinted in *Figure 2*. Orser and co-workers (1985) have shown by Southern blots that another *ina* gene from *P. syringae* has homology to the Ina-conferring region from *E. herbicola*: therefore it is reasonable to expect that a single, substantially similar gene also encodes Ina in *Erwinia*.

The *inaZ* and *inaW* genes differ sufficiently that, while their common ancestry is beyond doubt, it can be concluded that the retention of certain similarities must be due to selection for a common function. The most striking feature of both genes is the complex periodicity of their central portions (*Figure 2*). Each gene has about 120 contiguous repeats of an eight-codon motif. Alternate eight-codon units have greater mutual similarity than adjacent units: thus a more perfect 16-codon periodicity is superimposed throughout the region of eight-codon periodicity. A further order of periodicity, at a 48-codon interval, is superimposed on the first two orders in two regions of each gene. These orders of periodicity place certain constraints on the modelling of protein structure, as discussed later. The highest order of periodicity is broken at approximately the mid-point of both genes (*see Figure 2*), although the break is not colinear in the two genes.

Internally repetitive genes have the capacity to evolve rapidly through the action of recombination systems, which can cause deletions or further internal duplications. Green and Warren (1985) found that the codon usage for serine provided a strong indication that *inaZ* had evolved by amplification of the eight-codon motif. The potential instability dictates that caution should be exercised in interpreting the significance of such genes' structure. Repeats may be more mutually similar than is required for functionality, just because they originate from a recent duplication. The instability may cause some repeats which confer no selective advantage to be present, or it may cause the number of repeats to be suboptimal for function. There may be selection for the divergence of repeats merely to reduce instability. As in other repetitive structural genes (Uhlen *et al.*, 1984; Guss *et al.*, 1986), the functional significance of the repetition would be indicated by the conservation of protein structure during the divergence of DNA sequence between repeats. Warren, Corotto and Wolber (1986) found such a divergence between repeats throughout *inaZ* and in half of *inaW*, indicating that similarity between

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   1    M--DK-LVLRTCANNM-DHCGL-WP--G-VE-R-W--T-R-ENGL-G-LW
  51    G-G-SA-LS--ADARW-VCEV---D-I-LE----VKFPRAEVVHVG-R-S
 101    A---IS---------S-------------------S--LP----------
 151    ------------T-----------

 176                      A-YGSTL-----S-LI AGYGS--T-G--S--I

 208    AGYGSTGTAGSDS-L- AGYGSTQTAGGDS-LT AGYGSTQTA--GSNLT
 256    AGYGSTGTAG-DSSLI AGYGSTQT-GGDSSLT AGYGSTQTAQ-GS-LT
 304    AGYGSTGTAGSDSSLI AGYGSTQT-GGDSSLT AGYGSTQTAQ-GSNLT
 352    AGYGSTGTAG-DSSLI AGYGSTQT-G-DS-LT AGYGSTQTAQ-GS-LT
 400    AGYGSTGTAGSDSSLI AGYGSTQT-G-DSSLT AGYGSTQTAQ-GS-LT
 448    AGYGSTGTAG-DSSLI AGYGSTQT-G-DS-LT AGYGSTQTAQ-GS-LT
 496    AGYGSTGTAG-DSSLI AGYGSTQT-G-ESSLT AGYGSTQTA--GS-LT
 544    AGYGSTGTAGADSSLI AGYGSTQT-G-ESSLT AGYGSTQTAQ-GS-LT

 592                                      -GYGST-TAG--S-L-
 608    -GYGSTGTAG-ES--- AGYGSTQTAGH-SILT AGYGSTQTA-DGS-LT
 656    AGYGST-TAG--SSLI AGYGSTQTA---S-LT AGYGSTQTA-E-S-L-
 704    -GYGSTSTAG-NSSLI AGYGSTQT-G--SILT AGYGSTQTAQE--SL-
 752    -GYGSTSTAGYSSSLI AGYGSTQTAGYESTLT AGYGS-QTAQE-S-LT
 800    TGYGSTSTAGYSSSLI AGYGSTQTAGY-STLT AGYGSTQTAQE-S-L-
 848    -GYGSTSTAGYASSLI AGYGSTQTAGYESTLT AGYGST-TAQE-S-LT
 896    -GYGST-TAGF-SSL- -GYGS-QTAGY-STLT AGYGSTQ-AE--S-LT
 944    AGYGST-TAGQDSSLI

 960    AGYGSSLTSG-RS-LT AGYGSTLI-GL-SVLI AGYGSSLTSG-RS-LT
1008    AGYGSNQIAS--SSLI AGHESIQ-AG-KSMLI AGKGSSQTAG-RSTLI
1056    AGA-SVQ-AGDRSRL- AGA-S-QTAGDRSKLL AG-NSYLTAGDRSKLT
1104    -G-DC-LMAGD-SRLT AGKN-VLTAGA-SKLI GSEGSTLS-GE-SILI

1152    FR-WDGKRY---V-RTG---VEAD-PY---ED-----K-DE--D-----*
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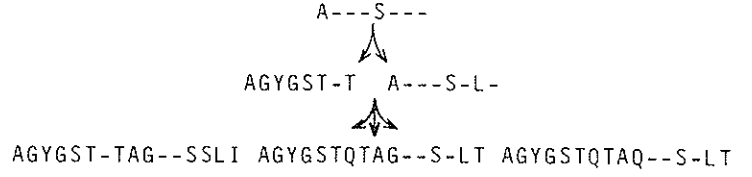

Figure 2. (a) The consensus of InaZ and InaW proteins. The proteins were aligned as described by Warren, Corotto and Wolber (1986). Hyphens denote positions of non-identity, with the following exceptions: basic residues arginine (R) and lysine (K) are treated as identical, and the residue which is in InaZ is arbitrarily presented as the consensus; acidic residues aspartate (D) and glutamate (E) are treated similarly. (b) The relationship of first-, second-, and third-order periodicities is illustrated by the tree, thought to represent their evolution also.

repeats is indeed required for whatever function has been subject to natural selection. (It is possible that ice nucleation is not the function on which selection acts: *see* Discussion.) Can we therefore conclude that the repeated units have evolved to a near-optimal consensus structure? No: the most feasible type of model for protein structure is that in which peptide repeats are in contact with each other in a regular way. This could mean that while a particular set of changes instituted in all repeats (that is, a change in

consensus) would enhance function, the same change in a single repeat might degrade function because of inappropriate interactions between the old and new repeats (*Figure 3*). The observed consensus may thus represent a type of frozen accident. It is ironic that the repetition may cause this aspect of gene structure to be unusually stable during evolution. It would be interesting to alter the consensus experimentally, but at present it is not technically feasible to alter the large number of repeats even to only one new consensus. (It is also probably undesirable to alter ice nucleation function in this way: a gene with an 'improved' consensus, and having therefore the potential to increase frost damage to plants if established in the epiphytic bacterial flora, would have to be permanently subject to strict containment.)

The gene products predicted from the *inaZ* and *inaW* sequences are also similar in their non-repetitive portions both *N*-proximal and *N*-distal to the repetitive region (*Figure 2*). A colinear correspondence between the two is distinguishable through much of these portions, although there are small regions which are unrelated between the genes.

EXPERIMENTAL MANIPULATIONS

Transposon mutagenesis (and insertion mutagenesis *in vitro*) was conducted on *inaZ* and *inaW* (before knowledge of their nucleotide sequences) to define the extents of the genes and to permit complementation analysis to determine the number of cistrons. It was extremely puzzling to observe that most insertions gave a leaky (incompletely deficient) phenotype (Gies *et al.*, 1985; Corotto, Wolber and Warren, 1986). The presence of an insertion leaves no possibility of translating a protein that resembles the wild type, and in other systems it usually results in a tight deficiency. Various hypotheses were advanced to account for this phenomenon, including intracistronic complementation and the possibility that only a small portion of the protein's

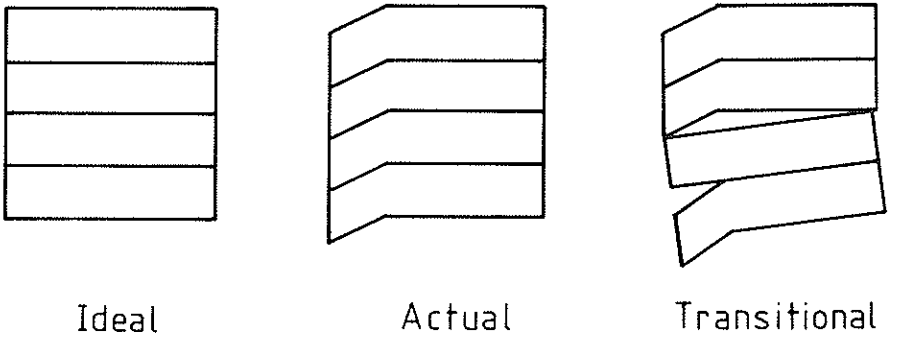

Figure 3. Conceptualized illustration of how peptide repeats may be evolutionarily stable because of mutual contacts. Assemblages of ideal (left) and non-ideal repeats (centre) form smooth, straight columns; a transitional assemblage (right) with a mixture of repeats cannot do so; its dysfunction provides a barrier to the evolution of an ideal assemblage.

carboxy-terminus was essential for ice nucleation. Reversion by removal of the insertion was initially considered unlikely, but knowing the repetitive nature of the coding sequence made such an explanation more plausible. Corotto, Wolber and Warren (1986) were able to demonstrate the formation of pseudorevertants at a frequency sufficient to account for the low level of Ina displayed by a leaky insertion mutant. Such pseudorevertants had lost not only the insertion but a portion of the sequences flanking it, as expected from an excisive recombination event between repeats. Hence, the possibility of reversion must always be borne in mind when considering the phenotype resulting from mutation in an *ina* gene. It is not at present feasible to identify pseudorevertants occurring at a frequency of 10^{-4} or below; therefore we must frequently leave unanswered the question of whether a mutant protein has slight residual activity or none at all, in the analysis of *ina* genes.

The *ina* genes cloned from *Pseudomonas* were shown to be essential for ice nucleation in the strains which were their sources. Ina^- deficiencies were constructed by deletion of sequences internal to each cloned gene, and the deficiencies were introduced into the source organisms by marker exchange (Orser *et al.*, 1984b; Warren *et al.*, 1987). The pseudomonads were observed to become Ina^-, indicating that they did not possess alternative means of forming ice nuclei.

To test the role of the repeats, mutations were introduced into *inaZ* which preserved the reading frame of downstream sequences, but altered the number of repeats. These mutations included additions of extra repeats as well as deletions; some mutations preserved all three orders of periodicity while others broke the periodicity at one or more levels. These experiments were designed to test several predictions based on the theory that the repeats encode a regular structure which is responsible for alignment of water molecules:

1. That since ordering of water molecules would be a collective property of the repeats, no individual repeat would be essential for activity. This was confirmed for all except the repeats towards the carboxy-terminus, where the question is not yet resolved (Green and Warren, 1985).
2. That mutations disrupting periodicity would be more deleterious than those preserving it. This was confirmed at the 48-codon level: it is not certain whether there is an additional penalty for breaking the eight- and 16-codon periodicities (Green and Warren, 1987).
3. That a protein with fewer repeats would be less active in ice nucleation specifically at warmer temperatures. This was confirmed (for two mutations which did not disrupt periodicity) but the observed effect was surprisingly small.)
4. That increasing the number of repeats would extend the range of nucleation thresholds towards warmer temperatures. This was refuted by the results of Green and Warren (1987). Therefore we concluded that minimum threshold approaches an asymptote as the number of repeats increases, and that the content of repeats in the wild-type ice-nucleation gene was already at or near a value in the asymptote. Any

complete model will have to account for the fact that such an asymptote exists; on the most simplistic grounds, it might have been expected that increased water-binding would always lead to warmer thresholds for nucleation, up to the ice–water equilibrium temperature.

To test the roles of the non-repetitive peptide sequences proximal and distal to the repeats, deletions were constructed whose transcription and translation would result in proteins lacking portions of these regions, but otherwise similar to the wild-type protein. The *C*-terminal deletions showed drastic reductions in nucleation at all temperatures, or lacked ice nucleation activity entirely. In contrast, the *N*-terminal deletions were greatly depressed in nucleation frequency for warm threshold temperatures, but much less affected for activity at cooler thresholds. A number of experiments have demonstrated a broad correlation between the expression level of an *ina* gene, and the frequencies displayed at all temperature points of the cumulative spectrum. Orser and colleagues (Orser *et al.*, 1985) found that increasing the copy number of cloned *ina* genes, both in *Pseudomonas* and in *E. coli*, increased the observed activity. When a promoterless *inaZ* gene was placed downstream of a number of promoters whose relative strengths were known, activity was found to vary in exactly the same order as promoter strength (Southworth, Wolber and Warren, 1987). This begs the question—can cell-to-cell variations in the level of gene expression be responsible for the observed distributions of threshold temperature within populations? The expression of *inaZ* from the extremely strong *tac* promoter (Wolber *et al.*, 1986) results in a population of ice nuclei with a median threshold of approximately −4·9°C. However, expression from considerably weaker promoters still generates some nuclei with temperature thresholds as high as −3°C. It therefore appears unlikely that variability within clonal populations could be attributable to differences in gene expression level between cells. Moreover, the variation is strikingly similar for a given protein in its native host and in *E. coli*, whereas there is no reason to expect a parallel control of gene expression in these hosts. It is more reasonable to postulate that post-translational processes involving elements of chance are required to make an InaZ protein participate in ice nucleation. This can explain the similar threshold distributions in different hosts, and explain why increasing the level of gene expression causes a rise in nucleation frequency at all temperatures.

A transcription probe transposon has recently been constructed from *inaZ* (P. Lindgren, B. Staskawicz and N. Panopoulos, unpublished work). This enables a promoterless *inaZ* gene to be transposed into the sequences to be tested; the transcription of the target sequences can then be estimated by the level of ice nucleation activity resulting from expression of *inaZ*. Previously, transcriptional probes have utilized genes such as *lacZ*, the product of which (β-galactosidase) is conveniently assayed where the bacteria to be tested can be harvested in sufficient numbers (for example, *see* Stachel, Flores and Nester, 1985). However, for the study of small bacterial populations, for example epiphytic bacteria on a plant surface, the β-galactosidase assay is

too insensitive. The use of an ice-nucleation gene offers promise for such situations because its activity is easily detected in very small numbers of cells that express it. Lindgren and co-workers have demonstrated adequate sensitivity in trials with some plant-associated bacteria (unpublished data).

Protein analysis

How does the expression of an ice-nucleation gene lead to the formation of ice nuclei? To answer this question it is necessary to identify the gene product and determine its functions, properties, and organization in the cell. On the basis of evidence described above, Kozloff and co-workers (Kozloff, Lute and Westaway, 1984) suggested that phosphatidylinositol synthesis has a key function. In its simplest form, this hypothesis does not require that the gene product should be present at the ice-nucleating site. Conversely, Green and Warren (1985) argued from the inferred primary structure of the InaZ protein that the protein itself performed alignment of water molecules: it would thus be an essential component of the nucleating site, but would not necessarily possess any enzymatic function.

IDENTIFICATION OF GENE PRODUCTS

Ice-nucleation proteins were first seen when comparing the SDS-PAGE patterns of membrane proteins from Ina^+ and Ina^- strains of *E. coli*. Strains expressing the *inaW* gene gave a novel band whose mobility indicated a molecular weight of 180 kD (Corotto, Wolber and Warren, 1986). Likewise, strains expressing *inaZ* gave a novel band with the apparent molecular weight of 153 kD (Wolber *et al.*, 1986). These estimations corresponded rather poorly to the predicted weights of 120 and 118 kD for the *inaW* and *inaZ* products respectively, and other novel bands might have been obscured by co-migrating bands of host protein. The identity of the observed proteins (named p180 and p153) was therefore uncertain until they were purified and their properties (*see* p. 123) lent strength to their identification as the *inaW* and *inaZ* products. However, equivalent experiments comparing Ina^+ and Ina^- strains of *Pseudomonas* failed to identify any proteins until antibodies were used. Antibodies were raised against both a protein isolated from a clone of *inaW*, and a synthetic peptide designed from the consensus repeat of the predicted *inaZ* product. The antipeptide antibody possessed a relatively low titre (this is not unusual for an antibody raised against a synthetic epitope), but nevertheless yielded the more conclusive evidence for identification of the *ina* gene products: because it was directed against a known component of the protein primary structure, rather than a preparation of unknown composition, we could be more confident of its specificity (Muller, Wolber and Warren, 1987). In fact, the other preparation of antibody showed similar specificity (and higher titre) in all subsequent tests (C. Deininger, G. Muller and P. Wolber, unpublished work).

The antibodies identified a single major protein band in each Western blot of an *E. coli* strain carrying an *inaZ* or *inaW* gene; these bands corresponded

in mobility to those identified by SDS-PAGE alone (Muller, Wolber and Warren, 1987). The same bands were identified at lower intensity in membrane preparations of the original *Pseudomonas* hosts of these genes. At higher detection sensitivities, a smear of cross-reacting material was observed below the position of the major band, but this probably represented unprogrammed degradation, rather than specific processing for functional activation. It was therefore assumed that the main bands represent the InaW and InaZ proteins. InaZ differed from InaW in electrophoretic mobility, but each displayed a mobility that was identical in extracts from *Pseudomonas* and from *E. coli*, and anomalous in both. This indicated that there was either the same protein processing in both environments, or no major processing at all. The anomalous molecular weights might be attributable to any of several causes: reduced affinity to SDS caused by the abnormal amino acid content, post-translational modification, or failure to denature fully under electrophoresis conditions (Wolber *et al.*, 1986). It was noted that another protein containing hydrophilic repeats (Dame *et al.*, 1984) displayed a similarly anomalous electrophoretic mobility.

EXTRACTION AND ANALYSIS OF INA PROTEINS

Wolber *et al.* (1986) examined a strain of *E. coli* that was engineered to transcribe the *inaZ* gene at high level. During the preparation of membranes by standard procedures, a novel cell fraction was observed which was greatly enriched in p153. The high density of this fraction permitted a rapid extraction of p153: it was subsequently purified 'to homogeneity'. Its amino acid composition differed from the 'average' protein (Dayhoff, 1969) in a similar way to that predicted for the *inaZ* product: the first nine amino acids at its amino-terminus were determined and found to be identical with those predicted by translating from the first ATG codon in the large open reading frame of *inaZ*. These observations left little doubt that p153 was the InaZ protein. The amino-terminal sequencing also showed that the protein obtained from the over-producing strain had not undergone amino-terminal processing. However, it should be remembered that when a protein is overproduced, the normal pathways of processing may be overloaded, causing most molecules to remain unprocessed. It will therefore be of interest to determine whether amino-terminal processing occurs during localization of InaZ to the membrane.

The first preparation of purified InaZ protein was completely inactive in ice nucleation, but this was hardly surprising since its purification had involved denaturation of the protein. In an attempt to obtain the InaZ protein in an active form, a purification scheme utilizing non-denaturing conditions was developed. Wolber *et al.* were successful in obtaining a preparation of ice nuclei in which only InaZ protein was detectable; however, only one ice nucleus was present per 30 000 InaZ monomers. Thus, the identified, unprocessed InaZ protein was possibly, but not certainly, associated with ice nucleation activity. It is quite conceivable that the activity resided in a

processed form of the protein, or in some other type of contaminant in the preparation.

PROTEIN LOCALIZATION BY MICROSCOPY

The variability of nucleation thresholds between bacterial cells might be explained by differential synthesis or differential organization of Ina proteins between the cells which display differing thresholds. To test this hypothesis, a method is needed which can distinguish the properties of individual cells. (Most methods of biochemical analysis yield results representing only the population average.) Immunofluorescence microscopy was used in an attempt to distinguish the organization of InaZ and InaW proteins in individual cells (Muller, Wolber and Warren, 1987). It was observed that cells were stained in patches, rather than uniformly, and also that the size, number, and fluorescence intensity of these patches varied enormously between cells. Such a result is intriguing because it appears to reflect the cell-to-cell variability in nucleation phenotype. However, it remains possible that much of the observed variability in staining was artefactual, perhaps being due to varying degrees of damage to the cell wall and thus to the cells' varying admittance of antibody. The consideration of possible cell wall damage also made it difficult to be sure whether the stained material was at the cell surface or in the cell interior. Cells grown under different conditions displayed differences in the size and shape of the largest patches; in fact, the mean patch size was greatest after growth under conditions most conducive to ice nucleation at warm thresholds. This observation is again suggestive, but points to the need for a method of greater power than optical microscopy. It may be hoped that immunoelectron microscopy will confirm the validity of our inferences from fluorescence microscopy, and go on to show conclusively how ice nuclei vary in structure between cells and under varying conditions.

DOSE-DEPENDENCE OF NUCLEATION ACTIVITY

Several clues about the organization of ice nuclei may result from an understanding of the dependence of nucleation activity on protein concentration. A linear dose-dependence would indicate that each protein acts independently at the rate-limiting step in forming ice nuclei, whereas a higher power relationship would suggest co-operativity. We might hope to detect different degrees of co-operativity in the formation of ice nuclei with different thresholds, since we suspect that these represent aggregates of different sizes. Moreover, a plateau of activity with increasing dose would indicate the limit of the cell's capacity for converting protein into ice nuclei.

Southworth, Wolber and Warren (1987) varied the rate at which *inaZ* was transcribed in *E. coli* by the substitution of different promoters; the concentrations of InaZ protein in the membrane fraction were then measured. When ice nucleation activity was plotted against the membrane concentration of InaZ protein, the relationship which emerged was interesting:

within a wide range, activity increased as the square of InaZ protein concentration. However, contrary to expectation, ice nuclei of different threshold temperatures all displayed similar power relationships between concentration and activity. This was interpreted to mean that there was a bimolecular combination between molecules of InaZ protein at a rate-limiting step in the assembly of ice nuclei, and surprisingly, that the same step limits the assembly of nuclei with widely different thresholds. In order to accommodate the hypothesis that different extents of aggregation are responsible for differences in temperature threshold, it is necessary to postulate that the bimolecular aggregates undergo further aggregation at a rate which depends linearly on their concentration. This would have interesting implications for the process of ice nucleus assembly—for example, it would suggest that aggregates cannot be in equilibrium with free subunits, but must grow in an irreversible manner, and that the final size of the aggregate is independent of its rate of growth.

Discussion

Ice nucleation research is at an exciting stage: some significant hypotheses may soon undergo rigorous testing, and further problems will undoubtedly be illuminated in the process. Perhaps the most fundamental questions to be answered are these: what is the molecule(s) at the nucleation site whose interaction is stronger with ice than with water, and what is the molecule(s) that provides structural regularity at the nucleation site? The two functions may or may not be performed by the same molecule. We believe that the *ina* gene product performs the second function: its primary structure has a regularity which has been maintained by selection; since such regularity appears to be required for function, it may be presumed to determine a corresponding regularity in the tertiary structure of the mature, functional protein. Moreover, the protein has always been found associated with ice nuclei after various types of cell fractionation, suggesting that it is an essential component of the ice nucleus. The latter point would be strengthened further if antibodies against this protein could be shown to inhibit ice nucleation, either in whole cells or in extracts. (Colleagues have attempted to demonstrate such an effect, so far without success.) However, the weight of evidence is strongly in favour of the regularity being provided by the *ina* gene product.

There is no convincing indication of whether the same protein interacts with water directly, or whether it instead aligns a smaller water-binding molecule, thus organizing water molecules by proxy. The inferences that phosphatidylinositol (PI) is involved in ice nucleation (Kozloff, Lute and Westaway, 1984) are consistent with the latter scenario, since PI contains a hydrophilic group that is topotactic with ice. However, the evidence for involvement of PI is indirect: inferences are drawn from small effects on ice nucleation activity by agents which are believed to affect PI synthase (but which may also affect other factors pertinent to ice nucleation), and from the induction of PI synthase activity. A direct demonstration that a particular cofactor is an essential component of the ice-nucleating site, or is associated with it in stoichiometric quantity, would be more satisfying. Work on the *in*

vitro characterization of ice nuclei appears to be most promising in this regard.

The variability of temperature thresholds between individual cells is a phenomenon attracting considerable attention at present. Variability may be closely connected with the fundamentals of ice nucleus assembly, in which case its understanding will be of basic significance. Why do thresholds vary, and what determines the spectrum of thresholds that a population displays? Thermodynamic considerations demand that increasing the size of a perfect ice-binding array will result in an increasing temperature threshold for nucleation. Therefore a 'first explanation' for variability is that varying numbers of water-binding units can be assembled, resulting in a range of effective sizes for the ice-binding array. For discussion let us postulate that the water-binding unit is a dimer of an Ina protein. The spectrum of thresholds would thus be a consequence of the availability of protein dimers and of their kinetics of aggregation and disaggregation. However, thermodynamic considerations also suggest that the presence of imperfections in a water-binding array will reduce the threshold temperature. Thus, a 'second explanation' (which is not necessarily exclusive of the first) is that different thresholds can be caused by different amounts of doping (inclusion of impurities) in an array of given size. The doping molecules might poison some water-binding sites, thus reducing threshold temperature, or they might improve long-range spacing, as silver bromide does in co-crystals with silver iodide.

So far, there is no evidence for the second explanation. It may be testable by studies *in vitro*: if ice nuclei could be fractionated on the basis of their content of Ina protein, then it would predict that each fraction will retain all or part of the threshold variability of the whole population. The converse would be expected if the first explanation of variability were correct. At first sight the gamma-ray inactivation studies of Govindarajan and Lindow (1984b) lend support to the former explanation, since nuclei with warmer threshold temperatures were demonstrated to present larger targets for the radiation. However, if all water-binding arrays are presumed to be the same size, it follows that those where all parts act co-operatively will nucleate at the warmest threshold, and their activity at that temperature will be sensitive to a single 'hit'. On the other hand, those wherein various portions act independently (whether due to an insufficiency of doping, or an excess) will have cooler thresholds, but each may require several hits for inactivation. Therefore, the results of the gamma-ray inactivation studies cannot discriminate between the explanations of variability. The phenotypes of the *N*-terminal deletions in *inaZ* could be explained by the involvement of that domain in aggregation interactions. It is possible that characterization of the Ina proteins from such mutants *in vitro* will clarify the role of aggregation.

A different type of explanation for varying threshold temperatures has been proposed by Caple *et al.* (1986). They suggest that 'endergonic metabolism' may be responsible: plainly stated, the bacteria may act as miniature refrigerators. A major flaw of this rather Aristotelian hypothesis is that significant thermal gradients are implausible at the scale of bacteria: miniature refrigerators would be too poorly insulated. It was further suggested

therefore, that the endergonic metabolism might trigger other types of physical change in the vicinity of the ice nucleus. There is no evidence for this hypothesis; it may be disproved if completely non-metabolizing cell extracts can be obtained which maintain a full range of threshold nucleation temperatures, but otherwise it will be difficult to test until formulated in more precise terms. The suggestion does have some merit in that it illuminates assumptions which are sometimes implicit, for example, the simplifying but unjustified assumption that the ice nucleus is surrounded by an aqueous phase identical to that surrounding the cell.

If we consider an ice nucleus as an array of identical water-binding units, it is predictable that threshold temperature will increase as a function of the size of the array. In fact, it will approach an asymptotic threshold temperature determined by three factors: (1) how well the individual units bind ice; (2) the spacing, or co-operativity, of the individual units; and (3) the fact that ice cannot be nucleated above the equilibrium temperature for phase transition (0°C for pure water at normal pressure). The cumulative nucleation spectra of bacterial ice nucleators show a precipitous drop in frequency at the warmest temperatures: it is meaningful to ask whether this reflects the graphs' approach to an asymptote of temperature for arrays of unlimited size. If we assume that spacing is perfect, the asymptotic temperature can be used to define, in thermodynamic terms, a minimum estimate for how well the individual unit binds water. Conversely, if the individual unit is assumed to bind water perfectly, the maximum threshold temperature defines a minimum estimate for the efficiency with which the individual units of the array can co-operate.

A more specific set of questions concerns the 'natural history' of the Ina protein: what does it look like, where does it live, and what makes it go there? These questions seem more tangible but less fundamental than those preceding. Their great attraction is that the approach to their resolution by experiment is more easily defined. The localization of Ina protein may be analysed through techniques of cell fractionation, although cautious interpretation will be necessary, since ice nuclei might exist as multimolecular aggregates large enough to mimic the fractionation behaviour of other cellular components, for example, membranes. The results of Sprang and Lindow (1981) and Wolber *et al.* (1986) are subject to this caveat, whereas the demonstration of ice nuclei in membrane vesicles shed from whole living cells (Phelps *et al.*, 1986) provides a less ambiguous indication that (at least some) ice nuclei are associated with the cell's outer membrane.

What determines localization of the Ina protein within the bacterial cell? As only the *ina* structural gene is needed for a normal Ina$^+$ phenotype, specialized processing mechanisms are ruled out, and the determinant must be some feature(s) of the protein's own structure. It is frequently found that the extreme *N*-terminal portion of proteins that are transported across membranes is a 'signal' that is recognized by the system initiating transport, and that is cleaved from the rest of the protein during the transport process. If we could observe that membrane-associated Ina protein was differentiated from that in the cytoplasm by removal of the first 20 or so amino acids, this

would provide a strong indication that signal cleavage was involved in its localization. There is no consensus by comparison with which signal sequences can be recognized, but certain features of a signal sequence are believed to be almost universal: these include hydrophobicity, net positive charge, and an α-helical secondary structure. Both of the Ina proteins of which the sequences are known can be construed to possess these features (the secondary structure being inferred by the predictive algorithm of Garnier, Osguthorpe and Robson, 1978). However, both proteins have at their *N*-termini a feature that is rare among the signal sequences of Gram-negative bacteria (Watson, 1984)—an acidic residue. The role of a signal certainly requires experimental confirmation.

Many proteins are anchored to a membrane by their content of one or more 'transmembrane spans'. These are hydrophobic stretches of approximately 20 residues; they serve as anchors because their preferred state is to be embedded in the hydrophobic interior of the membrane, with the flanking parts of the primary structure entering the membrane from opposite sides. Plots of hydrophobicity (Kyte and Doolittle, 1982) shown in *Figure 4* reveal three or four possible transmembrane spans in the *N*-terminal unique region of InaZ (P. K. Wolber, unpublished work). It is also noteworthy that the unique *C*-terminal region of InaZ is strongly hydrophilic, in common with the cell-wall-binding *C*-termini of some proteins from Gram-positive bacteria (Guss *et al.*, 1986). As was true of the question of protein aggregation, the behaviour of proteins deficient in particular domains may be revealing in the assignment of localization function.

What is the structure of the Ina protein? This question is of considerable interest because its solution may provide clues relevant to other problems, such as the nature of the molecule that performs the alignment function, and the differentiation of localization and aggregation functions between domains. At present there is no direct evidence for any feature of tertiary structure. The similarity of certain features of the repetitive portions of InaW and InaZ indicates that they have been conserved by selection, and therefore they may be presumed to be necessary for the function on which natural selection has acted. Without presuming that the selected function is that of ice nucleation, we may still expect that the conserved repetitive features of the primary structure reflect a functionally significant repetition in the tertiary structure. Because three orders of periodicity are significant, this greatly constrains the building of models: they are restricted to those in which each order of repeat corresponds to a structural segment that is homologous to and aligned with its neighbours. In essence, a regular, repeating structure must be built from only a few types of building block, and in such a way that the smallest blocks combine pairwise to make larger units, which in turn combine in threes to make still larger repeating units (*Figure 5*). Warren, Wolber and Corotto (1986) additionally constrained model building by requiring a correspondence of symmetry in the model to that in an ice crystal: relatively few structural plans could then be accommodated. These consisted of columns of triangular or hexagonal cross-section. If such structures organized their *internal* water, then lateral aggregation would not interfere with

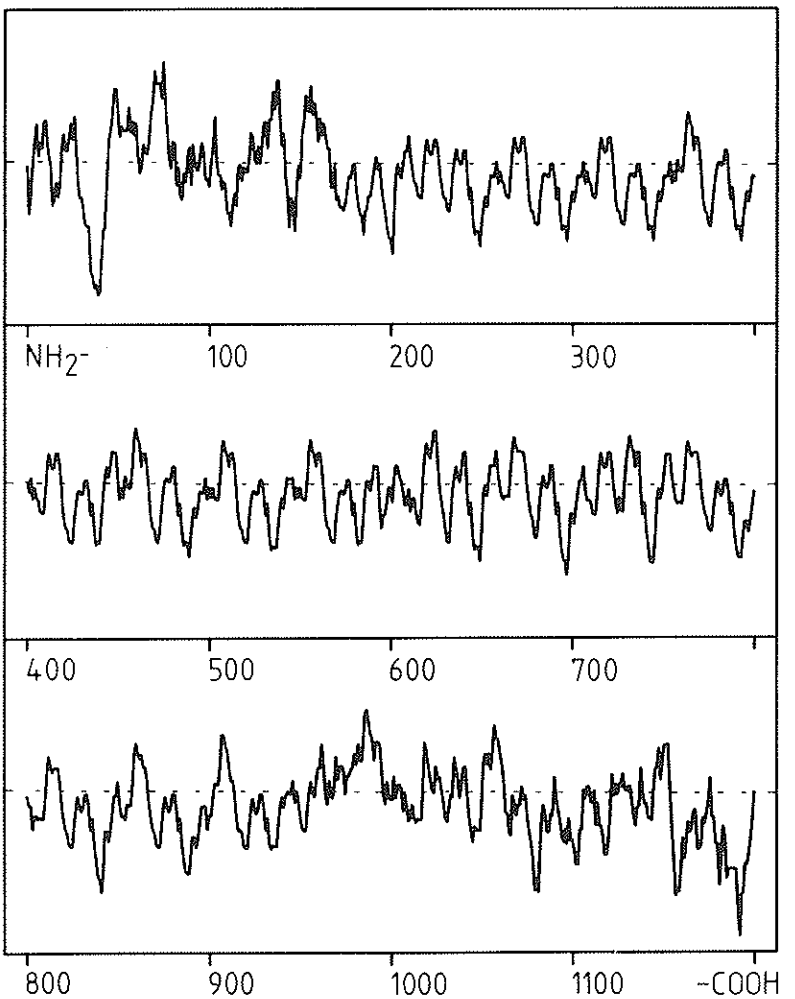

Figure 4. Hydropathy plot for InaZ protein. Mean hydropathy is plotted for a moving window of nine residues, after Kyte and Doolittle (1982). Residue positions are marked below the points of the graph to which they refer. The dashed line indicates the position where a hydropathic index of zero would be plotted; positions above the dashed line represent positive values (indicating more hydrophobic character), and positions below it represent negative values (indicating more hydrophilic character). The upper and lower outlines of the graph are at positions equivalent to hydropathic values of +3·4 and −3·4 respectively.

the function of each unit; such an aggregate would resemble a honeycomb in which each protein represented a columnar cell. This model makes possible a neat explanation of the observed effect of increasing the number of repeats per protein—which was the approach to an asymptotic level of ice nucleation activity. What would determine the activity of such a structure would be the organization of the water at the open end of each column. The level of organization here will increase to an asymptote as the organizing column

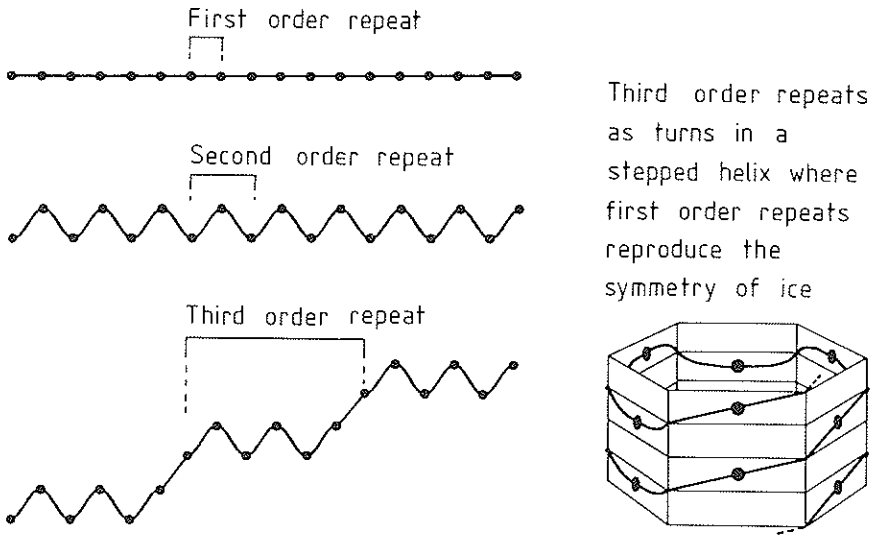

Figure 5. A model to illustrate how different features of structural repetition are expected to correspond to the three orders of repetition in the protein sequence. At left, each order of repetition is differentiated in two dimensions. At right, the structure from lower left is represented in three dimensions as though wrapped on to a hexagonal columnar framework. Such a structure is attractive as a model because it reproduces the symmetry of ice (Warren, Corotto and Wolber, 1986).

grows in height, because there will be a distance beyond which the sides' influence on the end is negligible.

It should be noted that the models embodying these features do not make detailed predictions about atomic positions; they can serve only as rough guides for making hypotheses about structure. Ultimately, it is desirable to determine structure by X-ray crystallography. However, the preparation of protein for crystallization presents a problem: how can we know that most of the protein in such preparations is in an active form (i.e. has the correct structure)? If other molecules are necessary components of the ice nucleus, it would be necessary either to check protein activity by reconstitution with these components, or to crystallize an active complex containing all necessary components. However, as it is not clear how many protein molecules would be needed for a minimal ice nucleus, it might still be very difficult to determine whether most of the protein monomers in a preparation were in the correct conformation. On the other hand, the heterogeneity of all preparations of ice nuclei might defeat attempts to isolate a uniform preparation of the active complex.

In discussions on ice nucleation a basic question is often raised which is simple but frustratingly difficult to answer: why did the ice nucleation gene evolve? Such a question is rarely asked about genes such as *lacZ*: evidently it is harder to see how the trait of ice nucleation can confer a selective advantage on the bacterium carrying it. An advantage might be offered by

the ability to cause damage to plant tissues opportunistically, because cell death and increased nutrient availability is the consequence. Epiphytic bacteria sometimes become airborne (Lindemann *et al.*, 1982) and ice nuclei might rescue bacteria carried into the upper atmosphere by causing condensation around them: the selective significance of such a property is at present a matter for guesswork. Ice nucleation might also increase cold survival as it does in some insects, but there is no evidence for this. Alternatively, ice nucleation may be merely the fortuitous property of a protein that has been selected to perform an unknown function. It is natural that this view should be unpopular among specialists in bacterial ice nucleation, but it may be difficult to explain in other ways the possession of ice nuclei by marine bacteria isolated in temperate waters.

What does our present state of knowledge imply for the applications of ice nucleation? The goal from which all applications would benefit—that is, the more uniform production of ice nuclei with warm thresholds—appears distant but approachable. We do not know why bacteria tend to make populations of ice nuclei with non-uniform thresholds, but the question may be answerable by investigating some of the observations described above, namely, the phenomenon of ice nuclei with different thresholds being affected differentially both by genetic and biochemical manipulations. When the reasons for non-uniformity are known, it should become clear whether greater uniformity is attainable, and if so, how.

For applications in food preparation and signal transduction, the isolation of cell-free ice nuclei is a vital objective. The identification of the Ina protein, and the virtual certainty that it is a structural component of the ice nucleus, make it worthwhile to pursue a more approachable subsidiary objective: the stabilization of Ina proteins and all molecules associated with them, during extraction from the cell. The extraction and preservation of ice nuclei is a biochemical problem, but it is also possible that genetic manipulation will aid in its solution. Membrane vesicles are likely to prove a useful model system for cell-free ice nuclei, and the knowledge already gained from them is useful in understanding the biochemical requirements for stabilization.

In the design of ice nucleation-deficient competitor bacteria for frost control, maintenance of 'fitness' and stability of the deficiency are prime objectives which are impacted by current knowledge. It is probable that complete absence of an Ina protein would be less deleterious to the bacterium than production of a truncated version: therefore removal of promoter or translational initiation sequences will be desirable in future constructions. Our experience with pseudorevertants, and knowledge that the *N*-terminal unique portion is qualitatively inessential for nucleation activity, shows that a deficiency will be most stable if its *C*-terminus, or the majority of its repeats, are removed.

Evidently, ice nucleation presents challenges for understanding and practical application at a number of levels. The disciplines of physical chemistry, biochemistry, molecular biology, and ecology are all involved in taking up the task. As I have remarked to an esteemed colleague in a different discipline, 'This thing is bigger than both of us'.

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