Bacterial ice nucleation: significance and molecular basis

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ABSTRACT Several bacterial species are able to catalyze ice formation at temperatures as warm as −2°C. These microorganisms efficiently catalyze ice formation at temperatures much higher than most organic or inorganic substances. Because of their ubiquity on the surfaces of frost-sensitive plants, they are responsible for initiating ice formation, which results in frost injury. The high temperature of ice catalysis conferred by bacterial ice nuclei makes them useful in ice nucleation-limited processes such as artificial snow production, the freezing of some food products, and possibly in future weather modification schemes. The rarity of other ice nuclei active at high subfreezing temperature, and the ease and sensitivity with which ice nuclei can be quantified, have made the use of a promoterless bacterial ice nucleation gene valuable as a reporter of transcription. Target genes to which this promoter is fused can be used in cells in natural habitats. Warm-temperature ice nucleation sites have also been extensively studied at a molecular level. Nucleation sites active at high temperatures (above −5°C) are probably composed of bacterial ice nucleation protein molecules that form functionally aligned aggregates. Models of ice nucleation proteins predict that they form a planar array of hydrogen binding groups that closely complement that of an ice crystal face. Moreover, interdigitation of these molecules may produce a large contiguous template for ice formation.—Gurian-Sherman, D., Lindow, S. E. Bacterial ice nucleation: significance and molecular basis. *FASEB J.* 7: 1338–1343; 1993.

Key Words: ice nucleation protein • bacterial ice nuclei • amino acid residues • warm-temperature nucleation activity

The ability of certain bacterial species to catalyze ice formation has proved to be a fascinating phenotype, with both practical and scientific applications. Bacterial ice nucleation represents an unusual case of a phase transition that can be catalyzed by a biological entity. The phenomenon of bacterial ice nucleation was first observed in strains of *Pseudomonas syringae* in 1974 (1). Shortly thereafter, strains of *Erwinia herbicola* (2), *Pseudomonas fluorescens* (3), *Pseudomonas viridiflava* (4), and *Xanthomonas campestris* pathovar *translucens* (5) were demonstrated to have the ability to catalyze ice formation in supercooled water. Although bacterial ice nucleation activity is apparently limited to these gram-negative species, the ubiquitous presence of many of these species on plants and in other natural habitats makes bacterial ice nucleation a common phenomenon in nature. The role of these unique ice catalysts and their practical applications will be addressed to demonstrate their significance to both biological and physical processes. Several recent reviews have detailed development of our understanding of the molecular basis of ice nucleation (6–9), and we refer the reader to them. In this review we will also examine recent efforts to expand our understanding of the structure of ice nucleation proteins in the membrane. Much of this work involves trying to understand how bacterial ice nuclei function at warm temperatures, which distinguishes them from most other ice nucleators and thereby gives them their biological and commercial importance.

BIOLOGICAL FEATURES OF BACTERIAL ICE NUCLEATION

An interesting feature of bacterial ice nucleation is its quantitative and qualitative variability. This is especially true given the previously mentioned fact that a single protein is responsible for the ice nucleation phenotype. A population of cells of a given ice nucleation active (Ice$^+$) bacterial strain, however, contain ice nuclei that are active as ice catalysts in water supercooled to from −2°C to −12°C (10–12). Not every bacterial cell contains an ice nucleus, however. Warm-temperature ice nuclei tend to be rare in a population of cells. For example, usually less than 10^{-6} of all cells in a culture contain ice nuclei active at temperatures warmer than −2°C (12). Ice nuclei active at colder assay temperatures are usually much more numerous than those active at warmer temperatures (11, 12). Even in very active cultures, however, not every cell contains an ice nucleus at a given time. It is unclear whether this represents a "snapshot" of a dynamic situation whereby cells only transiently express ice nucleation activity, or whether only a small subset of the cell population ever produces a functional ice nucleus. All available information suggests that the former hypothesis is correct; subtle changes in the environment of cells can cause preformed ice nucleation proteins in cells to rapidly produce ice nucleating sites (13, 14). Different strains of a bacterial species also differ greatly in their ability to produce ice nuclei (15). For example, there is a log-normal distribution of the frequency of production of ice nuclei among a large collection of strains of *P. syringae* (15). In some strains, nearly every bacterial cell is active in ice nucleation at −10°C; in other strains fewer than one in 10^7 of the cells are active at this assay temperature. Ice nuclei that are active at high subfreezing temperatures have attracted the most interest in bacterial ice nucleation. Because of their ability to produce warm-temperature ice nuclei (ice nuclei active at −5°C or warmer), Ice$^+$ bacterial species in nature are important in ice nucleation-limited situations.

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PRACTICAL APPLICATIONS AND IMPLICATIONS OF BACTERIAL ICE NUCLEI

Bacterial ice nuclei can incite plant frost injury to many plant species. Most plant tissues can supercool extensively (16–18) whereas frost damage occurs at temperatures as high as −2°C. Ice nuclei active at temperatures above −5°C are rare in axenically grown plants, especially in nonwoody plant species (16, 17; **Fig. 1**). Although many natural substances, such as plant tissue and soil particles, contain large numbers of ice nuclei active at temperatures of −10°C or lower, ice nucleating substances active at assay temperatures above −5°C are rare in nature (19–26). Small droplets of pure water will supercool to about −40°C (21). Even large quantities of tap water ($>$ 10 ml) will commonly supercool to about −8°C. Most plant species can supercool to about −5°C, and frost-sensitive plant species can avoid damaging ice formation above −5°C if Ice$^+$ bacterial species are absent. When plants are colonized by even as few as 10^3 Ice$^+$ bacterial cells per gram of tissue, the threshold ice nucleation temperature of the plant tissues is greatly increased (Fig. 1). Ice$^+$ bacterial species are common epiphytic inhabitants of healthy plants in nature (27–35). Populations sizes of Ice$^+$ bacteria are often as high as 10^6 cells per gram of plant tissue (27–35). Thus, plant tissues in the field commonly harbor large numbers of bacterial ice nuclei. Because frost-sensitive plant species are killed upon ice formation (36, 37), the reduced supercooling ability caused by epiphytic populations of Ice$^+$ bacteria increases the likelihood of frost damage. The application of bactericides or antagonistic bacteria to reduce the population size of Ice$^+$ bacteria on plant species in field conditions can reduce the incidence of freezing damage during natural frosts with minimum air temperatures of −5°C or higher (27, 30, 38–40). New methods for frost control based on control of Ice$^+$ bacteria thus promise to minimize frost damage, which amounts to billions of dollars of lost production per year on average, to many frost-sensitive agricultural plant species.

Although bacterial ice nuclei are always damaging to frost-sensitive plant species, they may play a role in allowing frost-hardy plant species to tolerate ice formation. Supercooling is apparently detrimental to frost-hardy plant species that can tolerate ice formation (37, 41). Hardy species can best tolerate ice if it forms slowly in their tissues. After extensive supercooling, nucleated ice can rapidly propagate throughout the tissues, and the movement of liquid water to the intercellular spaces no longer occurs in an equilibrium fashion, leading to intracellular ice formation and cell death (41). Bacterial ice nucleation genes were introduced via *Agrobacterium tumefaciens*-mediated transformation into the frost-tolerant species *Solanum commersonii*, and ice nuclei as active as −3°C were detected in transgenic plants (42). The production of heterologous warm temperature ice nuclei in frost-hardy plant species may lead to an enhanced tolerance of frost damage by limiting the supercooling of water in tissues. Such studies are currently under way in our laboratory.

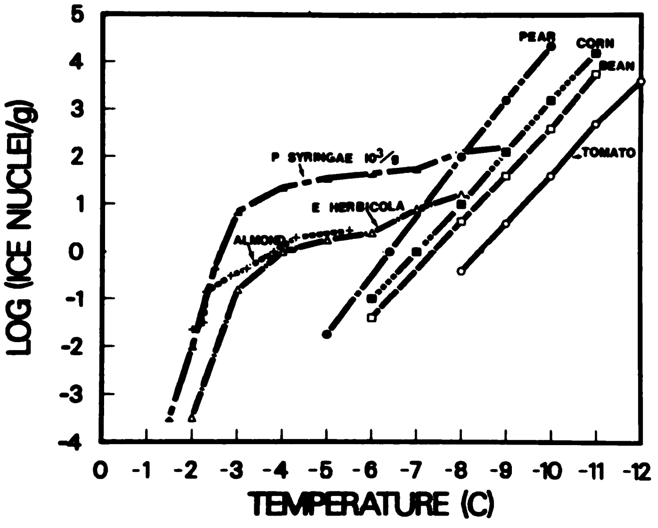

Figure 1. Ice nucleus content of different plant species when grown axenically or when colonized with 10^3 cells/g of typical ice nucleation active strains of either *Pseudomonas syringae* or *Erwinia herbicola*.

Bacterial ice nuclei active at warm subfreezing temperatures may potentially play important practical roles in artificial snow production, weather modification, and in freezing of certain food products. Freeze-dried cells of a *P. syringae* strain are used commercially on a large scale as an additive to water used in artificial snow production (43). By increasing the fraction of water droplets that freeze while they are suspended in the frigid air above ski slopes, more complete freezing of these particles can occur before their impact on the ground. The density of the artificial snow that is produced therefore is lower because freezing is more complete and the amount of ice particles that can be produced by a snow-making machine in a given period of time is increased. It is conceivable that bacterial cells could also be introduced into the atmosphere to initiate ice formation at relatively warm temperatures at the base of clouds, thus enhancing precipitation (44). Although large numbers of bacterial cells would be required for even modest weather modification activities, and methods must be devised to disperse these bacteria within the large volumes of air underneath clouds, they may yet play an important role in enhancing precipitation or mitigating dangerous meteorological events such as hail. Addition of bacterial ice nuclei to foodstuffs can change the pattern of ice crystallization, leading to a more desirable frozen product (45). In all such applications, nucleation of water at the highest possible subfreezing temperatures increases the utility of the bacterial ice nuclei.

SCIENTIFIC APPLICATIONS OF BACTERIAL ICE NUCLEI

Ice nucleation activity has proved to be useful as the basis for a new reporter gene system whereby a promoterless ice nucleation gene is fused with a gene of interest to monitor its transcriptional activity. Ice nucleation activity can be quantified conveniently by a droplet freezing assay, in which environmental samples are suspended without prior preparation in an aqueous solution; small droplets from dilutions of the suspensions are cooled to an assay temperature such as −5°C and the fraction of the droplets that freeze are recorded (11). At −5°C, there is little background ice nucleation activity in soil or other environmental samples we have tested (11). Further, soil particles, plant material, and other environmental constituents do not interfere with measurements of ice nucleation activity. The expression of ice nucleation activity in bacteria is not metabolically demanding; only approximately 300 IceC protein molecules are produced even in actively expressing cells of *P. syringae* (46). Thus the expression of ice nucleation activity does not require cells to be highly active metabolically nor does it significantly alter cell growth or survival in environments with low nutrient contents. Fusions of the *lux* operon, frequently used as a reporter gene, to target genes are estimated to be about

1,000-fold more sensitive in detecting gene activity than corresponding *lacZ* gene fusions (47). Ice nucleation gene fusions, on the other hand, are about 100,000 to 1,000,000-fold more sensitive for measuring transcriptional activity of target genes than corresponding *lacZ* fusions (48). Thus the ice nucleation reporter gene system has a substantially higher sensitivity and suffers less interference from components of environmental samples than other reporter genes. A convenient vector, Tn3-Spice, has been produced to introduce a promoterless ice nucleation gene from *P. syringae* (*inaZ*) into cloned genes (48). This reporter gene system provides the opportunity to measure the activity of interesting genes in natural habitats (48). This system has been used to describe plant-inducible expression of genes involved in pathogenesis of plants (48, 49) and of sensing the availability of ferric ions on plants and in soil using fusions to an iron-regulated promoter (50). As *inaZ* is transcribed and produces functional ice nuclei in a broad range of gram-negative bacteria (48) as well as plants (42), it is a useful tool that can be used in a broad range of both prokaryotic and eukaryotic systems.

Ice nucleation genes have also been used to develop rapid and sensitive assays for pathogenic bacteria. In such assays, the ability of a target organism-specific bacteriophage to transiently transform that strain to an Ice$^+$ phenotype is exploited (8). Because most water and food samples that would be assayed for the presence of human pathogens are devoid of ice nucleation activity at temperatures warmer than −5°C, the presence of even small numbers of pathogenic bacteria can be sensed by the production of ice nuclei upon their infection by broad host-range recombinant phage containing an ice nucleation gene (8). Many possibilities exist for further uses of ice nucleation-based detection methods.

STRUCTURAL ASPECTS OF BACTERIAL ICE NUCLEATION PROTEINS

Bacterial ice nucleation is conferred by single genes in all ice nucleating bacteria that have been examined and have been cloned from nearly all Ice$^+$ species (6, 7). Although ice nucleation genes vary slightly in length, their overall structure is similar; all the genes encode a protein with small unique amino and carboxyl termini but with a similar tandemly repeated 16 amino acid motif in the internal region that encompasses a large fraction of the protein (6, 7). Ice nucleation proteins from *P. syringae* have been shown to be located in the outer membrane (51, 52) and to require a lipid environment to function (53). In *E. herbicola* the production of ice nucleation active outer membrane vesicles demonstrates the outer membrane location of ice nucleation protein in this species (54). Most of the protein is rather hydrophilic, especially the large repetitive region, and therefore it is probably found at the surface of the membrane.

Because bacterial ice nucleation proteins are found in the membrane and have not been sufficiently purified in an active form, almost no work to determine their physical structure or other properties has been reported. Like many other proteins, most studies of bacterial ice nucleation proteins is speculative and based on structures derived from the nucleotide sequence of the encoding genes. We therefore must rely mostly on deduction and inference when considering the structure of these proteins. Deducing the structure of ice nucleation protein is facilitated by theoretical constrains imposed by the function of the protein. For example, ice nucleation theory suggests that an efficient ice nucleator must align water molecules into an ice-like lattice. Therefore amino acid residues spaced at appropriate intervals must be available to hydrogen bond with water molecules. The repetitive domain of the protein would be well suited for this function. It contains highly conserved potential hydrogen bond-forming residues at regular intervals (55). Furthermore, the very high nucleation temperature of the bacterial ice nucleator requires a large size, approaching 20 × 10^6 D at the warmest temperatures (56). Because a monomer of ice nucleation protein is about 120,000 D, theory predicts that an aggregate of the protein nucleates ice at warm temperatures. This has been confirmed by gamma radiation inactivation studies of the protein (12). In this work, high-energy gamma radiation was used to inactivate the protein, with the rate of inactivation positively correlated with molecular size of the functional unit. Gamma inactivation suggests a size range of from 150 kDa for nuclei active at −12°C to 19,000 kDa for nuclei active at −2°C. Aggregation of the protein has also been demonstrated by immunofluorescence and transmission electron microscopy (57, 58). In the former experiments, aggregates of the protein were observed on the surface of bacteria containing ice nuclei active at warm temperatures, but absent from bacteria possessing nuclei active only at colder temperatures. Secondary structure algorithms have predicted that monomers of ice nucleation protein have a beta turn structure throughout the repetitive region. Although the first models of the three-dimensional structure of ice nucleation proteins have used some constraints imposed by the unusual function of this protein, they were developed before it was established that the protein forms aggregates (59, 60). These models did not allow for the close packing of ice nucleation protein in higher-order structures that are necessary for a stable ice-like template. In addition, the cavities proposed in earlier models of ice nucleation proteins contained unsatisfied hydrogen bonds. More recent models attempt to take these properties into account. One such model has been proposed by Kajava and Lindow (61). It is suggested that the repeating structure of the protein consists of a series of beta hairpins, each defined by two octapeptide repeats. The rectangular units formed by groups of three or more hairpin structures would have the ability to interdigitate, forming large planar aggregates. The model is consistent with the widely accepted role of the repeat region as the template for initiating formation of an ice embryo (62). The "interdigitation" of the 48 amino acid units not only would likely stabilize the structure of the protein, but would also allow for quantitative increases in the contiguous area of hydrogen bonding template. The amino- and carboxyl-terminal domains of the protein may form alpha helix and beta structures, with the somewhat hydrophobic amino-terminal domain possibly anchoring the protein in the membrane.

Although the repetitive portion of ice proteins has a logical direct role in ice catalysis, less is known of the role of the remainder of the molecule. Mutational analysis assigns several possible functions to the amino- and carboxyl-terminal domains (63). The amino-terminal domain, in addition to its probable function in export of the protein to the outer membrane, seems to have a role in the formation of small aggregates. However, no structural model currently addresses how the aggregation of two or more monomers might occur. Kajava and Lindow (61) do not address the apparently essential roles of either the amino- or carboxyl-terminal domains (63). It has been proposed that the carboxyl-terminal domain may facilitate the folding of the protein (6). The Kajava and Lindow model suggests that the repeat region itself is at least partially responsible for stabilizing aggregates of the protein. This is contrary to recent suggestions (6, 63) that the amino-terminal unique sequence domain, possibly with some contribution from the unique carboxyl-terminal domain (64), is responsible for aggregation. However, recent work (65) examining single base change mutations that generate Ice protein molecules that appear to be aggregation-deficient (i.e., exhibit reduction of only warm temperature ice nucleation activity) found that all of these mutations were located in the repetitive domain of the protein. Reexamination of earlier work reveals that although deletions in the amino-terminal domain can selectively reduce or eliminate warm temperature nucleation activity, certain deletions that disrupt the regular repeat pattern in the repetitive domain also selectively reduce warm-temperature ice nucleation activity, giving a phenotype similar to those observed in the single base change mutants (63). The phenotypes of mutants in the amino-terminal and repeat domains differ slightly, however, with higher temperature residual ice nucleation activity remaining in mutants in the repetitive region. This suggests that two domains of the protein may be required for the formation of aggregates of different size.

To optimize the practical application of bacterial ice nuclei it is important to understand how warm temperature ice nuclei are formed and stabilized. This is especially true because warm-temperature nuclei are generally much less stable than cold-temperature nuclei (14, 66, 67). Part of the reason for the greater instability of warm-temperature ice nuclei can be inferred from the preceding discussion to result from disruption of aggregates of ice nucleation protein. This contention is supported by the observation that warm-temperature bacterial ice nuclei are unstable above approximately 25°C (13, 14). Lowering temperatures under conditions that are unlikely to allow significant rates of metabolism dramatically increases the number of warm-temperature nuclei present in a cell suspension, suggesting reaggregation of the protein at the lower temperatures (13, 14). It is currently not known whether this temperature effect is due to intrinsic instability of the aggregates, reflecting either the force of interactions between monomers or changes in the structure of the membrane at different temperatures, or both. Some membrane perturbants such as phospholipase CII and 2-phenylethanol also selectively reduce warm-temperature nucleation activity (14, 67). This effect, along with other evidence such as the presence of small amounts of phosphatidylinositol in ice nucleating strains, may indicate that this phospholipid is a component of warm-temperature ice nuclei (68), but may also indicate indirect effects generated by this membrane component. In experiments where delipidated ice nuclei were reconstituted with membrane lipids, only low-temperature nucleation activity (below −7°C) could be recovered (53). It is interesting that −9°C ice nucleation activity could be recovered, as this indicates that oligomers of two or three residues were restored in these experiments. This may indicate either that an additional membrane component or the overall membrane structure, lost during delipidation, is necessary for larger aggregates to form or that larger aggregates may form by an active process. This would support contentions that nuclei active above and below about −8°C have different structures (67) or depend on different domains of protein (see above) for their activity. Suggestions that large aggregates of ice nucleation protein have additional necessary components, such as phosphatidylinositol or mannose (67), associated with them require further experimental support, although it may be difficult to isolate moieties covalently linked to a minor component of a minor membrane protein.

Further study of single amino acid mutations that selectively reduce warm-temperature ice nucleation may reveal changes that might be engineered into the protein to increase the stability of large aggregates or create larger numbers of aggregates per cell. It might also be possible to create Ice proteins that could form even larger aggregates active at temperatures above −2°C. The most recent model of ice nucleation protein aggregates does not suggest a theoretical limit for the size of nuclei (65). However, as the resulting aggregates would be extremely large, they might not be accommodated by bacterial cells. It could be argued that there may be an intrinsic limit to the stability of large aggregates, as ice-nucleating bacteria probably have selected ice nucleation proteins with optimal stability. However, Large nuclei are stable at temperatures below approximately 20°C (13, 14), as would be encountered under typical conditions in the field, so there may not have been any pressure to evolve ice nucleation proteins more stable than the existing ones.

Selection pressure may occur against the formation of large Ice protein aggregates and may suggest a biologically imposed limit to the size of warm-temperature ice nuclei. Mueller and co-workers (57) examined aggregation of ice nucleation protein by in situ immunofluorescence labeling. They noted that only cells expressing warm-temperature ice nuclei showed patches of fluorescence, which is indicative of aggregation. Bacterial cells containing fluorescent patches were curved abnormally toward the patch, suggesting that membrane structure was altered, which may have affected the fitness of the cells. Reduced fitness of bacterial cells possessing warm-temperature ice nuclei would not imply reduced fitness for the entire population of bacteria, as only a small fraction of cells express warm-temperature ice nuclei at a given time. Furthermore, patches examined in this work probably do not represent nuclei active at the warmest nucleation temperatures (about −3°C in this study) as only 1 in 10^2 or 10^3 cells that express nuclei at −5°C express nuclei active at −3°C. Ice nuclei active at −2°C are predicted to be three- to fourfold larger than nuclei active at −5°C, or about 45 nm in diameter (56), and might produce even more severe morphological changes. It should be cautioned, however, that there is no direct evidence that cells possessing warm-temperature nuclei are less fit than others. It is possible that the observed curvature is an artifact of antibody labeling or that this curvature and the underlying structural changes would not reduce fitness. In addition, *Escherichia coli* cells were used in this work rather than *P. syringae*. It would be interesting to examine nonlabeled cells to see whether some are curved under conditions that allow warm-temperature nucleation, and whether such cells divide at a normal rate and are otherwise typical (for instance, whether they show normal motility, etc.). If bacterial production of ice nuclei has any fitness costs, it must be balanced by the benefits that it provides to the cells such as rapid removal from the atmosphere during precipitation events or in facilitating the invasion of plants during pathogenesis (69–71). FJ

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