REVIEW

Insect Cold-hardiness and Ice Nucleating Active Microorganisms Including Their Potential Use for Biological Control

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Since most insects are unable to survive internal ice formation a key factor in their overwintering survival is the regulation of the temperature at which they spontaneously freeze. To enhance their supercooling capacity overwintering insects eliminate endogenous ice nucleators, accumulate low-molecular-weight polyols and sugars, and synthesize hemolymph antifreeze proteins. A number of freeze-tolerant species contain proteins/lipoproteins or insoluble crystals that are ice nucleating active at relatively high subzero temperatures. Only recently have ice nucleating active bacteria and fungi been identified as normal flora in the gut of insects. These microorganisms are the most efficient class of heterogeneous ice nucleators that have been found in insects. Ice nucleating active microorganisms can regulate the supercooling capacity of insects when ingested or applied topically. These unique microorganisms may offer a novel means for the biological control of insect pests during the winter.

INTRODUCTION

Insects rely on a variety of ecological and physiological adaptations to survive low temperatures. Recent articles have reviewed biochemical, physiological and ecological aspects of insect cold-hardiness and overwintering biology (Baust and Rojas, 1985; Zachariassen, 1985; Tauber et al., 1986; Bale, 1987; Danks, 1987; Cannou and Block, 1988; Storey and Storey, 1988; Lee, 1989; Somme, 1989; Block, 1990; Duman et al., 1991). In addition, Lee and Denlinger (1991) recently edited a book on insects at low temperature.

Since most insects are unable to survive internal ice formation, a key factor in their overwintering survival is the regulation of the temperature at which they spontaneously freeze, termed the supercooling point or the temperature of crystallization (Lee, 1991). This temperature is significant to both freeze-tolerant and freeze-intolerant insects. Freeze-intolerant insects do not survive internal ice formation; consequently, for them the supercooling point represents the absolute lower lethal temperature. However, without careful testing it must not be assumed that a given species can survive at temperatures above the supercooling point: some insects die due to cold shock, a form of chilling injury that occurs in the absence of ice formation, at temperatures 10°C or more above the supercooling point (Lee and Denlinger, 1985; Knight et al., 1986; Bale, 1987; Lee et al., 1987). Even for those insects that survive the freezing of their body water, the supercooling point results in a major change of physiological state. During freezing, only water molecules join the growing ice lattice, while the excluded solutes become concentrated in the extracellular fluids (Mazur, 1984). Also, cells are progressively dehydrated, and metabolism is depressed as tissues become anoxic (Storey and Storey, 1988).

This review will summarize factors that influence supercooling capacity and ice nucleation in insects. Specifically, we will discuss the role of ice nucleating active microorganisms, primarily bacteria, in the regulation of the supercooling capacity of insects. Lastly, we will discuss the potential for the use of ice nucleating microorganisms in the biological control of insect pests.

SUPERCOOLING AND ICE NUCLEATION

It is possible to cool small volumes of purified water to approx. –39°C without freezing (Angell, 1982). Even...
a few microliters of tap water in a capillary tube readily supercool (i.e. remain unfrozen at temperatures below the melting point) to −15 to −20°C before spontaneous ice nucleation occurs.

In aqueous systems ice nucleation requires the presence of nuclei upon which water molecules can aggregate until a sufficient size is reached to promote ice crystal growth (Knight, 1967). If the nucleus is composed solely of water molecules, the process is referred to as homogeneous nucleation (Angell, 1982). It is generally believed in insects and other biological systems that ice nucleation is initiated by a heterogeneous mechanism (Franks, 1985). In this case water molecules accumulate on a seed nucleus other than water.

The most efficient ice nucleator within the aqueous system determines the temperature at which ice nucleation begins. A commonly used criterion for identifying a relatively efficient heterogeneous ice nucleator is the capacity to nucleate water at temperatures at or above −10°C. A representative list of heterogeneous nucleators is provided in Table 1. Inorganic compounds and non-proteinaceous organic compounds function as efficient nucleators only when they are in the crystalline form, and lose their ice nucleating activity when they are dissolved in water (Vali, 1991). Certain insects produce soluble ice nucleating proteins and lipoproteins that are active in the range of −6 to −9°C (Duman et al., 1991). Ice nucleating active bacteria and fungi are the most efficient heterogeneous ice nucleators known (Table 1); these microorganisms induce freezing at temperatures as high as −1°C.

Since insects are essentially small volumes of water it is perhaps not surprising that many insects supercool many degrees below the melting point of their body fluids before ice nucleation begins. Using thermocouples or other temperature sensing devices placed in contact with the insect cuticle, the limit of supercooling is readily detected by the release of the latent heat of crystallization as body water freezes. The supercooling point of most freeze-intolerant insects varies seasonally. Although values in summer are often between −4 and −8°C, as the insect cold-hardens in the autumn the supercooling point may decrease to −20°C or lower (Somme, 1982; Lee, 1991). Several gall-forming insects in Alaska depress their supercooling points to −60°C in winter (Miller, 1982).

**TABLE 1. Maximal threshold of ice nucleating activity of representative heterogeneous ice nucleators**

<table>
<thead>
<tr>
<th>Nucleator</th>
<th>Threshold of ice nucleating activity (°C)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inorganic compounds</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fluorophlogopite and other micas and silicas</td>
<td>−1 to −9</td>
<td>Shen et al. (1977)</td>
</tr>
<tr>
<td>Silver iodide crystals</td>
<td>−4</td>
<td>Vonnegut (1947)</td>
</tr>
<tr>
<td>Organic compounds</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amino acid crystals</td>
<td>−4 to −7</td>
<td>Power and Power (1962); Power and Power (1962); Head (1961)</td>
</tr>
<tr>
<td>Cholesterol and other steroid crystals</td>
<td>−5 to −7</td>
<td>Head (1961)</td>
</tr>
<tr>
<td>Insect ice nucleating proteins/lipoproteins</td>
<td>−6 to −9</td>
<td>Zachariassen and Hammel (1976); Neven et al. (1989)</td>
</tr>
<tr>
<td>Microorganisms</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Bacteria</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plant epiphytes</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Pseudomonas syringae</em></td>
<td>−1</td>
<td>Maki et al. (1974); Vali et al. (1976)</td>
</tr>
<tr>
<td><em>Pseudomonas fluorescens</em></td>
<td>−2</td>
<td>Lindow (1982)</td>
</tr>
<tr>
<td><em>Pseudomonas viridiflava</em></td>
<td>−3</td>
<td>Paulin and Luisotti (1987)</td>
</tr>
<tr>
<td><em>Erwinia herbicola</em></td>
<td>−2</td>
<td>Lindow et al. (1978a)</td>
</tr>
<tr>
<td><em>Xanthomonas campestris pv. translucens</em></td>
<td>−2</td>
<td>Kim et al. (1987)</td>
</tr>
<tr>
<td>Insect gut flora</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Enterobacter agglomerans</em></td>
<td>−2</td>
<td>Lee et al. (1991)</td>
</tr>
<tr>
<td><em>Enterobacter taylorae</em></td>
<td>−2</td>
<td>Lee et al. (1991)</td>
</tr>
<tr>
<td><em>Erwinia herbicola</em></td>
<td>&gt;−10</td>
<td>Kaneko et al. (1991a,b)</td>
</tr>
<tr>
<td>Frog gut flora</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Pseudomonas fluorescens</em></td>
<td>−5</td>
<td>Lee M. R. et al. (1992a)</td>
</tr>
<tr>
<td><em>Pseudomonas putida</em></td>
<td>−2</td>
<td>Lee M. R. et al. (1992a)</td>
</tr>
<tr>
<td>Fungi</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Fusarium avenaceum</em></td>
<td>−5</td>
<td>Pouleur et al. (1991)</td>
</tr>
<tr>
<td><em>Fusarium acuminatum</em></td>
<td>−2</td>
<td>Pouleur et al. (1991)</td>
</tr>
<tr>
<td>Lichen mycobiont</td>
<td>−5</td>
<td>Kieft (1988)</td>
</tr>
<tr>
<td><em>Rhizoplaca chrysoleuca</em></td>
<td>−2</td>
<td></td>
</tr>
<tr>
<td>Insect gut flora</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Fusarium sp.</em></td>
<td>−5</td>
<td>Tsumuki et al. (1992)</td>
</tr>
</tbody>
</table>

*Isolated from Hippodamia convergens (Coleoptera) and Cerotoma trifurca (Coleoptera).

*bIsolated from Plutella xylostella (Lepidoptera).*

*cIsolated from Chilo suppressalis (Lepidoptera).*
WHAT FACTORS REGULATE THE SUPERCOOLING CAPACITY OF INSECTS?

The capacity for extensive supercooling is closely tied to the small size of insects. That supercooling potential is inversely related to fluid volume is well established from physical studies (Angell, 1982). Insect eggs, Colembola and mites weighing tens to hundreds of micrograms commonly exhibit supercooling points in the range of -20 to -40°C (see review by Somme, 1982). Generally larger insects have a more limited capacity to supercool and freeze at temperatures between -5 and -20°C. The inverse relationship between insect mass (i.e. fluid volume) and supercooling capacity is also evident intraspecifically among larval stages of the mealworm *Tenebrio molitor* (Johnston and Lee, 1990). Consistent with the effect of body size on supercooling capacity is the observation that the larger, cold-adapted amphibians and reptiles, even freezing intolerant ones, generally freeze at -3°C or higher (Schmid, 1982; Costanzo et al., 1988; Storey and Storey, 1988).

Another factor enhancing insect supercooling is the accumulation of low-molecular-weight polyols and sugars. Although glycerol is the most common cryoprotectant in overwintering insects, other commonly reported compounds are sorbitol, mannitol, glucose, fructose and trehalose. The accumulation of glycerol, sometimes in multimolar concentrations, frequently correlates with increased supercooling capacity (Salt, 1959; Somme, 1964; Baust and Miller, 1972; Young and Block, 1980). In his 1985 review, Zachariassen concluded that polyols depress the supercooling point approximately twice as much as they colligatively depress the melting point.

Not all insects use cryoprotectants to depress the supercooling point. Even in the absence of accumulated cryoprotectants, many species supercool to -20°C (Zachariassen, 1985; Lee, 1991), apparently by removing or inactivating heterogeneous ice nucleating agents. For example, in the flesh fly, *Sarcophaga crassipalpis*, supercooling points decrease from -11 to -23°C during the larval-pupal transition in non-diapause-destined flies (Lee and Denlinger, 1985). This enhancement of supercooling capacity occurs when glycerol titers are decreasing, not increasing (Lee et al., 1987).

Another mechanism by which insects freeze is inoculative freezing (Salt, 1963). In this case contact with external ice catalyzes ice nucleation with body fluids. In some species wetting of the cuticle severely limits supercooling capacity (Danks, 1971; Fields and McNeil, 1988; Bale et al., 1989; Layne et al., 1990). In the freeze-tolerant cranefly (*Tipula sp.*), freezing begins at an unusually high temperature of -3°C due to inoculative freezing (Gehrken and Southon, 1992). In the drosophilid, *Chymomyza costata*, diapausing larvae freezing by contact with external ice survive, while isolated larvae supercool to -20°C and die upon freezing (Enomoto, 1981; Shimada and Riihimaa, 1988).

In a number of freeze-tolerant insects, ice nucleating proteins and lipoproteins are found in the hemolymph of overwintering insects (Zachariassen and Hammel, 1976; Duman et al., 1991). These proteinaceous nucleators function as heterogeneous ice catalysts that are active in the range of -6 to -9°C (Table 1). This ice nucleating activity of these proteins remains high even in the presence of high concentrations of polyhydric alcohols and sugars (Lee et al., 1981; Duman et al., 1991).

Some freeze tolerant insects have relatively high supercooling points, even though they lack efficient nucleating agents in the hemolymph. One such example is the overwintering third-instar larva of the goldenrod gall fly, *Eurosta solidaginis*. During the summer these larvae are freeze-intolerant and supercool to temperatures below -12°C (Morrisey and Baust, 1976). During the autumn the larvae acquire freeze-tolerance and supercooling points rise to approx. -9°C. Soluble ice nucleators must be lacking because the hemolymph readily supercools to -18°C (Bale et al., 1989). Research in our laboratory revealed large crystalloid spheres, identified as calcium phosphate, in the Malpighian tubules that have ice nucleating activity closely matching the supercooling point of the intact insect (Lee R. E. et al., 1992b). We have also shown that uric acid, calcium phosphate and other crystals commonly formed in insects have significant ice nucleating activity. Since a variety of crystals are known in insects, these compounds may represent a new class of ice nucleating agents that regulate the supercooling point.

Aside from ice-nucleating proteins/lipoproteins and these newly reported ice nucleating crystals, the identification of other specific classes of ice nucleators in freeze-susceptible insects remains elusive. A number of investigators, however, have attempted to identify the location at which freezing begins. Baust and Zachariassen (1983) reported ice nucleators associated with the cell matrix of warm-acclimated beetles, *Rhagium inquisitor*; the ice nucleating activity of these nucleators was similar to the supercooling point, -8°C, of the intact beetle. Nucleation was also examined in the excised legs of insects (Salt, 1968). Recently, Tsumuki and Konno (1991) identified muscle and epidermis as the primary site of freezing in overwintering larvae of the rice stem borer, *Chilo suppressalis*.

Although these reports identify ice nucleation in other anatomical locations, the gut is the most frequently reported site of ice nucleation. In large part, this idea stems from the observation that feeding is associated with an elevation of the supercooling point (Salt, 1936, 1966). Furthermore, cessation of feeding and emptying of the gut are often associated with increases in supercooling capacity (Cannon and Block, 1988). Using cryomicroscopy, Shimada (1989) observed that ice nucleation began in the lumen of the anterior midgut, and once freezing began the ice lattice rapidly grew across the gut wall into the haemocoel.

Based on the ice nucleating activity of a number of inorganic crystals (Table 1), it has been suggested that...
food or incidentally ingested dust particles within the gut may function as ice nucleating agents (see review by Cannon and Block, 1988). Others have speculated that ice nucleating bacteria in the gut may regulate the supercooling point (Lindow, 1982; Somme, 1982; Baust and Rojas, 1985; Cannon and Block, 1988; Strong-Gunderson et al., 1989). Only recently has this notion been confirmed (Strong-Gunderson et al., 1990a; Kaneko et al., 1991a, b; Lee et al., 1991).

ICE NUCLEATING MICROORGANISMS

Bacteria

A search for atmospheric ice nuclei led researchers to decaying vegetation as a primary source of efficient nuclei (Schnell and Vali, 1972, 1973). In turn, this study led to the discovery of ice nucleating activity in Pseudomonas syringae isolated from decaying leaves (Maki et al., 1974; Vali et al., 1976). Subsequently, several other species of epiphytic ice nucleating bacteria were discovered, including Erwinia herbicola and Pseudomonas fluorescens (see reviews of Lindow, 1982, 1983). These Gram-negative, rod-shaped, asporogenous bacteria are the most efficient ice nucleators known catalyzing ice formation at temperatures as high as 

\[-1^\circ C\] (Table 1).

The molecular biology of ice nucleating bacteria is the subject of several recent reviews (Warren, 1987, Warren and Wolber, 1987; Wolber and Warren, 1989). Ice nucleating active bacteria synthesize large outer-membrane proteins (120 kDa) that apparently aggregate within the outer wall, associate with membrane lipid and carbohydrate components, and, subsequently, function as a template for the formation of small ice crystal seeds, called “ice nuclei” with sizes estimated to range from 150 kDa for activity at about 

\[-12^\circ C\] to 19,000 kDa for activity at 

\[-2^\circ C\] (Corotto et al., 1986; Warren et al., 1986; Govindarajan and Lindow, 1988; Wolber and Warren, 1989; Mueller et al., 1990). Cell-free ice nuclei, which are borne on outer membrane vesicles, may be shed by ice nucleating active strains of E. herbicola into the surrounding environment and possess ice nucleation activity at 

\[-3^\circ C\] (Phelps et al., 1986). Bacterial ice nucleating activity is heat labile, sensitive to 65°C, inactivated by heavy metal ions, such as Hg²⁺, extreme pHs and sulfhydryl reagents. Activity is stable to lyophilization, and variably affected by certain bacteriocidal antibiotics, such as streptomycin, a protein-inhibiting antibiotic (Kozloff et al., 1983; Maki et al., 1974; Anderson and Ashworth, 1986). Culture conditions, particularly the growth medium, and incubation temperature affect bacterial ice nucleating activity (Lindow et al., 1978a; Rogers et al., 1987; Pooley and Brown, 1991). Addition of 2.5% glycerol to the growth medium, incubation at 22–24°C, and low temperature conditioning at 5°C increase ice nucleating activity (Lindow et al., 1978a, b; Rogers et al., 1987). Genes responsible for ice nucleation have been isolated and partially sequenced.

Repetitive primary structure and possibly tertiary structure of one ice-nucleating protein, encoded by the inaZ gene sequence, is believed to be crucial to the structure of the ice nucleating template (Green and Warren, 1985; Wolber et al., 1986). Analysis of the consensus sequence of ice nucleation proteins encoded by the genes iceE, inaW, and inaZ from E. herbicola, P. fluorescens and P. syringae respectively, has revealed consistent similarities and conserved features (Warren et al., 1986; Warren and Corotto, 1989). Furthermore, evidence has been presented that the lipid, phosphatidylinositol, is a component of the ice nucleation site and is synthesized by an enzymatic translation product, phosphatidylinositol synthetase, of the ice nucleating genes (Kozloff et al., 1984).

The threshold temperature is the highest temperature at which an individual cell functions as an ice nucleus within a specified time interval (Warren, 1987). Within a given bacterial culture with presumably identical genotypes, threshold temperatures may vary greatly from cell-to-cell. Cells with ice nucleation activity at the highest temperatures are uncommon relative to ones active at lower temperatures. For example, in a population of 1 million bacterial cells, only one cell might be active at 

\[-2^\circ C\], whereas 1000 cells are active at 

\[-6^\circ C\] and 10,000 cells have activity at 

\[-10^\circ C\]. Consequently the ice nucleating activity of bacterial populations is usually reported as cumulative ice nucleation spectra of threshold temperatures. These spectra describe the proportion of cells that are active as ice nuclei at a particular temperature. The most common method for reporting these data is the freezing droplet assay of Vali (1971) in which the activity of a bacterial suspension is reported as \[\text{log(ice nuclei/cell)}\].

The majority of reported ice nucleating active bacteria are ubiquitous, pathogenic epiphytes that not only nucleate water in direct contact with them, but also induce freezing of plant tissues. The significance of ice nucleating active bacteria in promoting frost-injury in plants is clearly established. Each year these bacteria are responsible for substantial amounts of frost-related crop losses throughout the world (Lindow et al., 1982a, b; Lindow, 1983). In 1985 frost-related losses were estimated at one billion dollars (Lindow, 1985). The recognition that these bacteria are responsible for frost damage to crops has led to a number of control strategies including the use of bactericides and ice nucleation inhibitors (Lindow, 1983). A particularly novel approach is the application of genetically engineered, antagonistic, non-ice nucleating bacteria to selected crops in an attempt to competitively displace natural populations that have activity (Lindow, 1983; Warren et al., 1986). Not surprisingly, the release of genetically engineered organisms into the environment has proved controversial; however, initial studies suggest that this approach is feasible (Halvorson et al., 1985; Lindow, 1985; Lindow et al., 1989). Plants colonized with ice minus mutant strains of P. syringae before challenge with ice plus strains had fewer ice nucleation active bacteria, and reduced
numbers of ice nuclei active at $-5^\circ\text{C}$ (Lindow, 1987). Furthermore, plants treated with ice minus strains had less frost injury. Field studies also demonstrated significant reductions in colonization by ice plus strains on plants previously colonized by ice minus strains (Lindow, 1987).

Early in most discussions of ice nucleating microorganisms the question arises as to the selective advantage of this trait in natural populations. Although no definitive answer is available, a number of hypotheses have been proposed. Since injured plants are more easily invaded by plant pathogens, including *Fusarium*, Lindow (1983) suggested that the ice nucleating activity of epiphytic bacteria may facilitate bacterial invasion into plant tissues and, thus, increase their access to nutrients. Another contention is that the ice nucleation trait may promote condensation onto airborne bacteria that have been carried into the upper atmosphere and, thereby, rescue them as they return to earth in precipitation (Warren, 1987). Kieft (1988) suggested that lichens may benefit from their mycobiont ice nucleating activity by increasing their available water through condensation. It is also possible that this trait enhances low temperature survival of bacteria, since the ice nucleation phenotype is more common in areas experiencing frost than those that do not (Hirano and Uper, 1990). Freeze-tolerant plants may benefit from epiphytic ice nucleating bacteria by insuring that plant tissues begin to freeze at high subzero temperatures (Lindow, 1982). This process initiates freezing in the extracellular fluids, thus avoiding lethal intracellular freezing.

Fungi

Ice nucleating activity is not restricted to bacteria. Epilithic lichens from three genera, *Rhizoplaca*, *Xanthoparmelia* and *Xanthoria* have ice nucleating activity at temperatures in the range of $-2.3$ to $-8^\circ\text{C}$ (Kieft, 1988). Kieft and Ruscetti (1990) associated ice nucleating activity with protein from axenic cultures of the lichen fungus, *Rhizoplaca chryssoleuca*. Lichen and bacterial ice nucleation sites are similar in both molecular size and in a characteristic logarithmic increase in size with a linear increase in nucleation temperature (Kieft and Ruscetti, 1992). Unlike bacteria, ice nuclei from *Rhiz. chryssoleuca* exhibit activity over a wide pH range from 1.5 to 12, are relatively heat stable, and their activity does not require membrane lipids (Kieft and Ruscetti, 1990). These latter observations suggest significant differences between the nature of fungal and bacterial ice nuclei.

Ice nucleation activity has also been reported in the free-living fungi, *Fusarium avenaceum* and *Fusarium acuminatum* (Pouleur et al., 1991). Ice nucleation activity occurs at temperatures as high as $-2.5^\circ\text{C}$ in *F. avenaceum*. Like *Rhiz. chryssoleuca*, the *Fusarium* species retain ice nucleating activity at a wide range of pHs (1-13), and following heat treatment of up to $70^\circ\text{C}$.

**ICE NUCLEATING ACTIVE MICROORGANISMS AS NORMAL FLORA IN THE INSECT GUT**

**Bacteria**

Two independent research groups have recently isolated ice nucleating active bacteria from the gut of insects (Strong-Gunderson et al., 1990a; Kaneko et al., 1991a, b; Lee et al., 1991). These reports constitute the first demonstrations of close associations between ice nucleating active bacteria and animals (Table 1).

Several species of ice nucleating active bacteria have been isolated from the gut of the beetles, *Cerotoma trifurcata* and *Hippodamia convergens* collected during the summer (Strong-Gunderson et al., 1990a, b; Lee et al., 1991). We identified strains of *Enterobacter agglomerans* and *Enterobacter taylorae* with ice nucleating activity. Ice nucleating activity has not previously been reported in *E. taylorae*. The droplet freezing assay was used to characterize the ice nucleating activity of these two species. Both species exhibited maximal threshold activities of approx. $-2^\circ\text{C}$ (Fig. 1). These spectra differ significantly from that of the non-ice nucleating bacterium *Escherichia coli*. The ice nucleating activities of the two *Enterobacter* species were only slightly less than that observed in the highly efficient epiphytic bacterium, *P. syringae* (Fig. 1).

We confirmed the ice nucleating activity of *E. agglomerans* and *E. taylorae in vivo* by feeding bacterial suspensions to our insect model, the lady beetle *H. convergens* (Strong-Gunderson et al., 1990a, b; Lee et al., 1991). Overwintering adults of *H. convergens* are freeze-tolerant and supercool to approx. $-16^\circ\text{C}$ (Lee, 1980; Bennett and Lee, 1989), but when beetles were fed bacterial suspensions of the ice nucleating active bacteria of insect origin, their supercooling points increased by $12-13^\circ\text{C}$ (Table 2). Indeed, some beetles froze at temperatures as high as $-1.5^\circ\text{C}$.

Both *E. taylorae* and *E. agglomerans* are members of the Enterobacteriaceae, a family of asporogenous, Gram-negative, facultative anaerobic rods. Members of this group are commonly found as digestive tract

**FIGURE 1.** Comparison of the ice nucleating activity of the epiphytic bacterium, *P. syringae*, with *E. agglomerans* and *E. taylorae* isolated from insects. Data from a non-ice nucleating bacterium, *E. coli*, are included for comparison. Data from Lee et al. (1991).
TABLE 2. Effect of treatment with ice nucleating active microorganisms on the supercooling point of insects

<table>
<thead>
<tr>
<th>Insect (stage)</th>
<th>INA Microorganism</th>
<th>Supercycling point (°C)</th>
<th>Untreated</th>
<th>Treated</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Coleoptera</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cryptolestes ferrugineus (adult)</td>
<td>Pseudomonas syringae*</td>
<td>-17.0 ± 1.0</td>
<td>-8.1 ± 0.5</td>
<td>Fields (1991)</td>
<td></td>
</tr>
<tr>
<td>Cryptolestes pusillus (adult)</td>
<td>P. syringae*</td>
<td>-14.0 ± 1.0</td>
<td>-12.0 ± 1.5</td>
<td>Fields (1992)</td>
<td></td>
</tr>
<tr>
<td>Diabrotica undecimpunctata howardi (adult)</td>
<td>P. syringae*</td>
<td>-7.5 ± 0.8</td>
<td>-3.2 ± 0.2</td>
<td>Strong-Gunderson et al. (unpublished data)</td>
<td></td>
</tr>
<tr>
<td><em><strong>Gibbium pyloides (adult)</strong></em></td>
<td><em><strong>P. syringae</strong></em></td>
<td>-10.7 ± 0.9</td>
<td>-6.0 ± 0.5</td>
<td>Lee R. E. et al. (1992b)</td>
<td></td>
</tr>
<tr>
<td>Hippodamia convergens (adult)</td>
<td>P. syringaeb</td>
<td>-16.0 ± 0.5</td>
<td>-2.8 ± 0.2</td>
<td>Strong-Gunderson et al. (1990b)</td>
<td></td>
</tr>
<tr>
<td><em><strong>Erwinia herbicola</strong></em></td>
<td><em><strong>P. syringae</strong></em></td>
<td>-16.0 ± 0.5</td>
<td>-4.4 ± 0.6</td>
<td>Strong-Gunderson et al. (1990b)</td>
<td></td>
</tr>
<tr>
<td>Enterobacter aggleromercaned</td>
<td>Enterobacter tayloriae d</td>
<td>-16.0 ± 0.5</td>
<td>-3.1 ± 0.1</td>
<td>Lee et al. (1991)</td>
<td></td>
</tr>
<tr>
<td>Fusarium acuminatum</td>
<td>-14.9 ± 0.5</td>
<td>-11.0 ± 0.7</td>
<td>Lee et al. (1992b)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Hemiptera**

<table>
<thead>
<tr>
<th>Insect (stage)</th>
<th>INA Microorganism</th>
<th>Supercycling point (°C)</th>
<th>Untreated</th>
<th>Treated</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oryzaphilus surinamensis (adult)</td>
<td>P. syringae*</td>
<td>-13.7 ± 1.9</td>
<td>-11.0 ± 1.3</td>
<td>Fields (1992)</td>
<td></td>
</tr>
<tr>
<td><strong>Plodia interpunctella</strong> (larva)</td>
<td>P. syringae</td>
<td>-10.3 ± 0.4</td>
<td>-5.4 ± 0.5</td>
<td>Lee R. E. et al. (1992b)</td>
<td></td>
</tr>
<tr>
<td><em>Rhynocerosa dominica</em> (adult)</td>
<td>P. syringae*</td>
<td>15.2 ± 0.6</td>
<td>3.3 ± 0.1</td>
<td>Lee R. E. et al. (1992b)</td>
<td></td>
</tr>
<tr>
<td>Sitophilus granarius (adult)</td>
<td>P. syringae</td>
<td>-14.3 ± 0.8</td>
<td>-7.8 ± 0.5</td>
<td>Fields (1992)</td>
<td></td>
</tr>
<tr>
<td>S. granarius (adult)</td>
<td>P. syringae</td>
<td>-15.7 ± 1.0</td>
<td>-8.0 ± 0.6</td>
<td>Lee R. E. et al. (1992b)</td>
<td></td>
</tr>
<tr>
<td>Tenebrio molitor (larva)</td>
<td>P. syringae</td>
<td>-16.0 ± 0.7</td>
<td>-5.4 ± 0.7</td>
<td>Strong-Gunderson et al. (unpublished data)</td>
<td></td>
</tr>
<tr>
<td><em>Tricholoma costatum</em> (adult)</td>
<td><em>P. syringae</em></td>
<td>-15.1± 0.6</td>
<td>-2.7 ± 0.3</td>
<td>Strong-Gunderson et al. (unpublished data)</td>
<td></td>
</tr>
<tr>
<td>Tribolium castaneum (adult)</td>
<td>P. syringae</td>
<td>-13.9 ± 0.8</td>
<td>-4.7 ± 0.4</td>
<td>Lee R. E. et al. (1992b)</td>
<td></td>
</tr>
<tr>
<td><em>Tribolium castaneum</em> (adult)</td>
<td><em>P. syringae</em></td>
<td>-12.3 ± 1.0</td>
<td>-5.8 ± 0.3</td>
<td>Fields (1992)</td>
<td></td>
</tr>
</tbody>
</table>

**Diptera**

<table>
<thead>
<tr>
<th>Insect (stage)</th>
<th>INA Microorganism</th>
<th>Supercycling point (°C)</th>
<th>Untreated</th>
<th>Treated</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Syrphus erassipalps (larva)</td>
<td>P. syringae</td>
<td>-13.8 ± 0.9</td>
<td>-3.6 ± 0.1</td>
<td>Strong-Gunderson et al. (unpublished data)</td>
<td></td>
</tr>
<tr>
<td><em>Hemiptera</em>*</td>
<td><em>P. syringae</em></td>
<td>-20.0 ± 0.5</td>
<td>-8.7 ± 1.0</td>
<td>Strong-Gunderson and Lee (unpublished data)</td>
<td></td>
</tr>
<tr>
<td>Lepidoptera</td>
<td>Chilo suppressalis (larva)</td>
<td>Fusarium sp.</td>
<td>-20.1 ± 0.9</td>
<td>-5.7 ± 0.6</td>
<td>Tsumuki et al. (1992)</td>
</tr>
<tr>
<td><em>Galleria mellonella</em> (larva)</td>
<td><em>P. syringae</em></td>
<td>-10.4 ± 0.1</td>
<td>-4.0 ± 0.3</td>
<td>Strong-Gunderson et al. (unpublished data)</td>
<td></td>
</tr>
</tbody>
</table>

*Treated with 1000 ppm dry, powdered *P. syringae*.
*Misted with 10⁶ bacteria/ml water.
*Treated with 1000 ppm dry, powdered *P. syringae*.
*Ingestion of 2 x 10⁶ bacteria/ml water.
*Misted with 3 mg/ml water.
*Misted with 10⁶ bacteria/ml water.
*Ingestion.

microflora in insects (Cruden and Markovetz, 1987; Mead et al., 1988). Our most frequent bacterial isolate, *E. agglomerans*, was phenotypically classified in the broad, heterogeneous "E. agglomerans-Erwinia herbicola complex". Although historically *Erw. herbicola* was considered a synonym of *E. agglomerans*, it is now known that some bacteria phenotypically classified within this complex are not closely related genotypically (Brenner et al., 1984). Since phenotypic biochemical identification schemes are not yet established to enable one to identify to species a new isolate initially placed within this broad complex, this problem has been addressed by the creation of 13 DNA-DNA hybridization groups, based on genotypic relatedness within this complex (Brenner et al., 1982, 1984). DNA genotypic relatedness studies are currently in progress to determine the taxonomic identity of our ice nucleating active isolates. Studies of this type are critical for a full understanding of the relationship between ice nucleating active bacteria isolated from insects and their relationship to epiphytic ice nucleating active bacteria.

An ice nucleating active bacterium was isolated from pupae of the diamondback moth, *Plutella xylostella* (Kaneko et al., 1991a, b). In a preliminary study of this species, some pupae that fed as larvae on germinating radish seeds had relatively higher supercooling points in the range of -5 to -12°C (Kaneko et al., 1989). When these pupae were held for 5 days at 5°C, the proportion of individuals having high supercooling points increased. This result suggests an enhancement of ice nucleating activity due to low temperature conditioning similar to that previously observed in *Erw. herbicola* (Rogers et al., 1987). In contrast, larvae reared on aseptic radish seeds produced pupae that did not have elevated supercooling points. Based upon this study Kaneko et al. (1991a) recently isolated three bacterial isolates with ice nucleating activity from pupae. These isolates were later identified as *Erw. herbicola* (Kaneko et al., 1991b).

**Fungi**

An ice nucleating active fungus has also been shown to influence the supercooling capacity of an insect. In rice stem borer larvae, *Chilo suppressalis*, the supercooling points of the gut (-8.4°C) and the frass (-6.5°C) correspond closely to the supercooling point of the whole body, -8.3°C (Tsumuki and Kondo, 1991). Other body compartments supercool to -12°C or lower. Further investigation isolated an ice nucleating active fungus...
(Fusarium sp.) from the gut (Tsumuki et al., 1992). Sterile larvae fed a non-ice-nucleating fungus, Penicillium sp., supercool extensively to below −20°C, while larvae fed a mycelial suspension of Fusarium have supercooling points near −5°C. Furthermore, application of the fungus to the larval surface produced a similar elevation in the supercooling point.

Other animals having ice nucleating active bacteria

Ice-nucleating active microorganisms may also play a role in the freezing tolerance of intertidal marine invertebrates. In a preliminary report, Loomis (1990) described the presence of an ice-nucleator in the palial fluid, that was not present in hemolymph, of the bivalve mollusc, Geukensia demissa. The presence of an ice-nucleating bacterium was suggested because filtration significantly reduced the ice-nucleating activity of the palial fluid. Additional circumstantial evidence was the isolation of ice-nucleating active bacteria from seawater surrounding the bivalve. Ice-nucleating activity is known from marine microorganisms; Fall and Schnell (1985) identified a marine pseudomonad that was phenotypically similar to P. fluorescens.

Recent studies in our laboratory have demonstrated the association of ice-nucleating active bacteria and freeze-tolerant vertebrates (Lee M. R. et al., 1992a). P. fluorescens, E. agglomerans and P. putida were isolated from the gut of the freeze-tolerant wood frog, Rana sylvatica, a species that routinely encounters and survives freezing in nature (Table 1). Ice-nucleating activity was previously undescribed in P. putida.

Since ice-nucleating active microorganisms are normal flora in a variety of insects and are associated with other freeze-tolerant animals, the question arises as to the ecological and evolutionary significance of this relationship. In freeze-tolerant insects, they may function to promote ice-nucleation at warm sub-zero temperatures (Lee, 1991). In this case they would function like ice-nucleating proteins/lipoproteins that limit supercooling of the insect’s body fluids, insuring that ice formation begins in the extracellular compartment (Zachariassen and Hammel, 1976; Duman and Horwath, 1983). Insect ice-nucleating proteins/lipoproteins are relatively less efficient nucleators compared to ice-nucleating active bacteria (Table 1). Ice-nucleating active microorganisms would function even better in this role, since even though only a portion of the cells are active at the highest subzero temperatures (about −2°C), only one cell in the population need be phenotypically active at this temperature to nucleate an ice crystal that will then spread throughout the insect’s body. Ice-nucleating microorganisms that regulate the supercooling point of freeze-tolerant insects, as in the case of the rice stem borer (Tsumuki and Konno, 1991; Tsumuki et al., 1992), represent a mutualistic relationship in which the microorganism enhances the freeze tolerance of the insect and by doing so preserves its host and food source.

The significance of the presence of ice-nucleating active microorganisms in freeze-intolerant insects is unclear. The freezing-intolerant lady beetle, H. convergens, overwinters as an adult in reproductive diapause (Lee, 1980; Bennett and Lee, 1989). Although these beetles are unlikely to experience subzero temperatures in summer, actively feeding adults have relatively high supercooling points of −7°C, possibly due to the presence of E. agglomerans and E. taylorae found in the gut during the summer (Lee et al., 1991). To enhance supercooling, either these bacteria must be evacuated from the gut prior to overwintering or the expression of this phenotype in the gut flora must be diminished or eliminated. Since the members of the genus Enterobacter are commonly found in the insect gut, the ice-nucleating trait may be incidental to the primary role of these microorganisms. Many insects evacuate the gut in preparation for overwintering, thus the ice-nucleating bacteria may be removed allowing the insect to supercool in winter.

USE OF ICE NUCLEATING MICROORGANISMS FOR BIOLOGICAL CONTROL

The rationale for the use of ice-nucleating microorganisms for biological control is based on their potential for reducing the cold-hardiness of insect pests. In practice these microorganisms would increase winter mortality by decreasing the effectiveness of natural mechanisms of supercooling. With this application in mind, a number of investigators have examined the use of ice-nucleating microorganisms to manipulate the supercooling point and thereby decrease the cold tolerance of insects.

Bacteria

Ingestion of ice-nucleating active bacteria causes a rapid and significant increase in the supercooling points of insects (Strong-Gunderson et al., 1990a, b). Adults of H. convergens that ingested water or a non-ice-nucleating active bacterium, Escherichia coli, have supercooling point values of −15°C or lower. In contrast, beetles that are fed bacterial suspensions of P. syringae and Erw. herbicola (10⁸ bacteria/ml) have supercooling points that are 12−14°C higher. Even beetles that are allowed to drink for as few as 10 s show an immediate elevation in the supercooling point. The magnitude of the increase is related to the concentration of the bacterial solution ingested: a P. syringae concentration of 10⁶ cells/ml produced a mean supercooling point of −10°C, while a concentration of 1⁰ cells/ml resulted in a value of −3.9°C. Following ingestion of bacteria the supercooling points remain elevated for at least 7 days.

In retrospect it is perhaps not surprising that the ingestion of a potent ice-nucleator should cause a dramatic decrease in the supercooling capacity of an insect. However, it is surprising that the application of these bacteria to the surface of the insect should have an equally dramatic effect on the supercooling point. When suspensions of P. syringae were misted onto beetles whose mouths had been sealed, the supercooling points increased similar to that observed following
ingestion (Strong-Gunderson et al., 1989, 1992). Even beetles that were misted with *P. syringae* and allowed to dry had elevated supercooling points for at least 3 days following the treatment. That surface application of a potent nucleator rapidly and significantly increases supercooling points has obvious significance for the potential use of these bacteria as biological insecticides.

A variety of insects have been tested for the effect of ice nucleating active bacteria on their supercooling points (Table 2). All four species of ice nucleating active bacteria that were applied as living suspensions have the potential to significantly elevate the supercooling point. Dusting insects with a freeze-dried, killed preparation of *P. syringae* also causes a significant increase in the supercooling point (Fields, 1991; Lee R. E. et al., 1992b). This effect is widespread as 15 species from four orders of insects show an increase in the supercooling point following the application of ice nucleating bacteria to their surface. The magnitude of the increase in the supercooling point differs among insects. Treatment with *P. syringae* elevated the supercooling point by only 2°C in the flat grain beetle, *Cryptolestes pusillus* (Fields, 1992), whereas values increased by 11.9°C in the lesser grain borer, *Rhyzopertha dominica* (Lee R. E. et al., 1992b).

**Fungi**

Recent studies in our laboratory demonstrated that surface application of the filamentous ice nucleating active fungus *Fusarium acuminatum* suspensions elevate the supercooling point of *H. convergens* (Lee M. R. et al., 1992b). Using an aqueous suspension of *F. acuminatum* (0.01 g/3 ml sterile distilled water) 50% of the 10 µl drops freeze at −6.1°C or warmer temperatures. When beetles were misted with this suspension, their supercooling points increased slightly from −14.9°C (misted with water only) to −11.0°C. In an attempt to further increase the supercooling point, we added surfactants to the fungal suspension. A 1% solution of Tween 80 caused an elevation of the supercooling point to −13.3°C. In contrast, when the fungal suspension contained a 1% solution of Tween 80 the supercooling point increased to 5.8°C. Application of Tween 20, Triton X-100, and gum arabic with the fungal suspension had minimal effect in elevating the supercooling point. These preliminary results suggest that surfactants used in combination with ice nucleating active microorganisms may be useful in the development of protocols for the control of some insect pests (Lee M. R. et al., 1992b).

**APPLICATION OF ICE NUCLEATING BACTERIA TO STORED PRODUCT INSECT PESTS**

Exposure to temperature extremes is a commonly used means of controlling stored-product insects (Mullen and Arbogast, 1979; Hunter and Taylor, 1980; Fields, 1991, 1992). Since this approach relies on a physical means of control it offers obvious advantages over the use of pesticides, such as malathion and phosphine, with respect to residues in foods and safety concerns for applicators (Fields, 1991). However, the use of low temperatures for insect control is generally slower acting than chemical application and is often costly in terms of electrical power for ventilation and refrigeration (Fields, 1991).

Fields (1991) proposed the use of ice nucleating active bacteria to control stored grain insects. He used a freeze-dried, killed preparation of *P. syringae* to reduce the cold-hardiness of the freeze-intolerant rusty grain beetle, *Cryptolestes ferrugineus* (Fields, 1991). Supercooling point increases were directly related to the concentration of bacteria applied. At the highest concentration tested (1000 ppm), supercooling points were −8.1°C, an increase of 11°C above that of untreated controls. Survival at subzero temperatures closely correlated with the supercooling point data; exposure for 24 h at −10°C caused only 8% mortality in untreated control groups, while 81% of the beetles treated with 1000 ppm of *P. syringae* died. In a simulation of field conditions in granaries, Fields (1992) treated *C. ferrugineus* with various concentrations of *P. syringae*; application of as little as 10 ppm produced significantly reduced insect survival.

Additional studies in our laboratory further examined the effect of *P. syringae* on the cold tolerance of a variety of stored grain pests (Lee R. E. et al., 1992b). The application of 100 ppm of ice nucleating active bacteria to wheat or corn containing insect pests markedly decreased the insect's supercooling capacity. Following treatment with 100 ppm of *P. syringae*, the mean supercooling points of five species increased by as much as 11.9°C above untreated controls (Table 2). Treatment with *P. syringae* also decreased the insect's capacity to survive a 24-h exposure to −5°C in every stored grain pest tested: Indian meal moth larvae [*Plodia interpunctella* (Hubner)], red flour beetle adults [*Tribolium castaneum* (Herbst)], flat grain beetle adults [*C. ferrugineus* (Schonherr)], rusty grain beetle adults [*C. ferrugineus* (Stephens)], *Gibbium psylloides* (Czenpinski), lesser grain borer adults [*R. dominica* (F.)], yellow mealworm larvae [*Tenebrio molitor* (L.)] and granary weevil adults.

![FIGURE 2. Survival of stored grain insect pests exposed to 100 and 1000 ppm of dry, powdered *P. syringae* in wheat for 24 h at 23°C before 24 h exposure to −5°C. Insects (n = 28–30) used were larvae of *T. molitor* and *P. interpunctella* and adults of *S. granarius* and *C. pusillus*. All differences between untreated control and groups treated with *P. syringae* were significant at P < 0.05. Asterisk indicates no survivors. Data from Lee R. E. et al. (1992b).](image-url)
[Sitophilus granarius (L.)] (Fig. 2). Insects, such as C. pusillus, that showed significant mortality in the control group at -5°C, experienced even greater mortality with the application of P. syringae. In some cases, increasing the treatment dose from 100 to 1000 ppm increased mortality by 25-50%. Although this study used non-cold-acclimated insects, no evidence to date suggests that cold acclimation decreases the effectiveness of ice nucleating active bacteria in decreasing the supercooling capacity of insects (Strong-Gunderson et al., 1990a, b; Fields, 1991, 1992; Lee R. E. et al., 1992b).

The use of ice nucleating active microorganisms as biological insecticides requires that insects be exposed to temperatures below their supercooling points. Probably they would be most effective and economical against freeze-intolerant pests that overwinter in aggregated or restricted microhabitats that naturally experience subzero temperatures during the winter. However, it is possible that refrigeration could be combined with the application of the biological nucleator. Since these microorganisms are field isolates, their use circumvents the problems associated with the release of genetically engineered organisms or toxic synthetic compounds into the environment. Furthermore, since this control method would be applied after the growing season when microhabitat temperatures of the insect fall below 0°C, it would be particularly attractive in its compatibility with other control measures of integrated pest management programs.

A current limitation of the use of ice nucleating active bacteria for biological control is their susceptibility to a loss of activity upon warming. Bacterial cultures conditioned at low temperatures have greater ice nucleating activity than ones held at higher temperatures (Rogers et al., 1987). Similarly, the activity of concentrated, freeze-dried and killed preparations of P. syringae, such as the ones used by Fields (1991) and Lee R. E. et al. (1992b), are also high-temperature sensitive. This temperature sensitivity was evident when adults of C. ferrugineus were treated with various concentrations of P. syringae for 1 or 7 days at 30°C prior to subzero exposure (Fields, 1991). Ice nucleation activity at three concentrations of P. syringae decreased by 33-40% between day 1 and 7. It is interesting to note that, despite this decrease in activity, mortality was highly correlated with the index of ice nucleating activity during this interval (Fig. 3). Possibly increased bacterial application could be used to reach a specific level of mortality. It would be desirable for ice nucleating activity to be maintained in granaries over extended periods because this would allow treatment of the grain at the time it was being loaded into the granaries in the autumn. Several months are often required in the northern U.S. and Canada before grain cools to subzero temperatures. Alternatively this problem could be addressed by developing bacterial or fungal strains or formulations that retain ice nucleating activity at high temperatures.

It may also be possible to augment the effectiveness of ice nucleating active microorganisms in elevating the supercooling point. Inoculative freezing involves water on or possibly in the insect cuticle. In the wheat stem sawfly, Cephus cinctus, the addition of a detergent to the water applied to the cuticle increased inoculative freezing as compared to soaking the sawfly with water alone (Salt, 1963). Using a similar approach with plants, Bunster et al. (1989) reported that the surfactant Tween 80, added to a bacterial suspension, increased the wettability of the leaf cuticle and facilitated homogeneous distribution of the bacteria across the surface. Since leaf and insect cuticles are both hydrophobic, the utilization of surfactants in the bacterial suspensions may increase cuticle wettability, thereby increasing the efficacy of inoculative freezing. As discussed previously, the addition of a surfactant to F. acuminatum suspensions significantly increased the supercooling point as compared to the use of the fungal suspension alone (Lee M. R. et al., 1992a). In addition to these synthetic surfactants, natural biosurfactants produced by bacteria

![Graph](image)

**FIGURE 3.** Effect of ice nucleating activity of P. syringae on the mortality of the rusty grain beetle, C. ferrugineus exposed to low temperature. Index of ice nucleating activity is the log of the number of ice nucleating sites per gram of P. syringae. Wheat containing C. ferrugineus was treated with various concentrations of P. syringae and held for 1 or 7 days at 30°C prior to exposure to -10°C for 24 h. Data are from Table 2 of Fields (1991).
might be combined in culture with ice nucleating active microorganisms to promote inoculative freezing. We have observed that, in general, strains of *Pseudomonas fluorescens* (a less efficient nucleator than *P. syringae*) have a much weaker surface tension than *P. syringae* strains. Even though a particular ice nucleating active strain is a weaker nucleator, it may be more effective as a transcuticular inoculant because of its inherent ability to lower water's surface tension. By promoting the spread of ice nucleating microorganism over the insect, there will be a greater chance of it entering a spiracle or other body opening that is in direct contact with body water. By combining the reduction in surface tension provided by *P. fluorescens* with the high nucleation efficacy of *P. syringae* (or one of the ice nucleating active bacteria of insect origin) it may be possible to increase the effectiveness of inoculative freezing above that obtained for the ice nucleating active bacteria used alone. If wettability is altered and inoculative efficacy increased due to the presence of surfactants, this technique could be useful in the development of effective procedures for using ice nucleating bacteria as biological insecticides.

**FUTURE DIRECTIONS**

Only a very few laboratories have systematically searched for ice nucleating active microorganisms in insects or other animals. However, considering the relatively large number of ice nucleating bacteria and fungi that have been identified in the few insects that have been studied, we suspect that these microorganisms may play an important role in regulating the supercooling capacity of overwintering insects and possibly other cold-tolerant animals.

As often occurs when an area of scientific investigation is first opened, results from the initial studies give rise to many new questions. What is the adaptive and ecological significance of ice nucleating active microorganisms? In particular, why do freeze-susceptible species harbor these microorganisms in their gut? Do overwintering freeze-tolerant insects commonly use ice nucleating active microorganisms to limit their supercooling capacity? If so, what is the significance of this apparent symbiotic relationship? In other words, would the insect be unable to survive freezing if the bacteria were removed? What factors regulate the ice nucleating activity in the insect gut? Does cold acclimation enhance the expression of the ice nucleating active phenotype *in vivo* in the insect, as occurs in some epiphytic ice nucleating active bacteria *in vitro*? Are these microorganisms commonly found on the surface of insects and do they promote inoculative freezing? Is it possible to convert a normally freeze-susceptible insect that supercools extensively into a freeze-tolerant species by inducing freezing at high subzero temperatures using ice nucleating active bacteria? Is it possible to develop methods for the use of ice nucleating active microorganisms as biological insecticides? For example, is it possible to colonize the gut or body surface of a freeze-susceptible insect pest with these bacteria or fungi? If so, could or how rapidly would insects develop resistance to this type of control? The answers to these questions will enhance our understanding of natural interactions between insects and ice nucleating microorganisms as well as aid in the evaluation of their potential for use in biological control.

**REFERENCES**


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