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INGESTION OF ICE-NUCLEATING ACTIVE BACTERIA INCREASES THE SUPERCOOLING POINT OF THE LADY BEETLE *HIPPODAMIA CONVERGENS*

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Abstract—Regulation of the temperature at which insects freeze is a critical factor in their overwintering survival. Although the role of ice-nucleating active bacteria in promoting frost-damage in plants is well known, their interaction with other organisms has not been described. In winter, many freeze-susceptible insects avoid freezing by lowering the temperature at which their body fluids spontaneously nucleate, termed the supercooling point. The lady beetle, *Hippodamia convergens* (Coccinellidae, Coleoptera), is a freeze-susceptible insect that overwinters as an adult in the supercooled state, −16°C. In the control group, none of the beetles fed only water had supercooling points above −15°C, similarly no beetles fed an ice-nucleating negative bacterium had supercooling points above −14°C. Following ingestion of the ice-nucleating active bacteria, *Pseudomonas syringae* and *Erwinia herbicola* (10^9 bacteria/ml), the supercooling point was elevated to −3.5 and −4.4°C respectively, the supercooling point remained elevated for 7 days after *P. syringae* treatment and a dose-response relationship was observed.

Key Word Index: *Pseudomonas syringae*; *Erwinia herbicola*; *Hippodamia convergens*; supercooling point; ice-nucleating agents

INTRODUCTION

Several strains of ice-nucleating active bacteria have been identified as a biogenic source of ice nuclei (Vali *et al.*, 1976; Maki and Willoughby, 1978). These ice-nucleating active bacteria are common epiphytes on the surface of a wide variety of plants and function as an ice catalyst by promoting the freezing of plant tissues at high subzero temperatures, −1.5 to −5°C (Lindow, 1983, 1987). In the absence of these ice-nucleating active bacteria, many plants supercool to −8 to −10°C before spontaneous ice nucleation occurs within the tissues (Lindow, 1983). However, the presence of these bacteria on plants limits their supercooling capacity. Thus, if environmental temperatures fall below 0°C, as may happen in early spring or autumn, the plant's capacity to supercool is reduced by the presence of these ice-nucleating active bacteria, resulting in a significant increase in frost injury and economic crop loss. To date, research has focused primarily on the nature of the ice-nucleation process, the genetic components involved and the significance of ice-nucleating active bacteria in promoting frost injury in crops (Lindow *et al.*, 1978, 1982; Lindow, 1983; Kozloff *et al.*, 1984; Kim *et al.*, 1987; Warren, 1987; O'Brien and Lindow, 1988). The possible influence these bacteria may have on other organisms is unknown. The question addressed here is how these ice-nucleating active bacteria affect other organisms exposed to freezing temperatures, in particular insects that feed on or are in contact with plants and their epiphytic bacteria.

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In biological systems the supercooling limit is identified by the presence of non-aqueous nucleating agents that promote heterogeneous ice nucleation within body fluids (Lee, 1989). Ice-nucleating activity is described for a few inorganic compounds such as silver iodide (Vonnegut, 1949), organic compounds including some amino acids and steroids (Head, 1961; Power and Power, 1962; Barthakur and Maybank, 1963) and proteins and lipoproteins from insect haemolymph (Duman and Horwath, 1983; Duman *et al.*, 1984). However, all these substances are relatively inefficient nucleators, only able to induce nucleation at relatively low subzero temperatures, −8°C or below. Thus, these ice-nucleating active bacteria are remarkable for their ability to induce nucleation at temperatures 1–2° below 0°C.

Insects are commonly divided into two categories: freeze-tolerant species which survive ice formation in their body fluids and freeze-susceptible species which do not tolerate freezing. In preparation for winter, freeze-susceptible insects commonly depress the temperature at which ice spontaneously nucleates within the body, termed the supercooling point. Although depression of the supercooling point is required for freeze-intolerant insects to survive low winter temperatures, a number of recent studies have demonstrated that additional mechanisms of cold-hardening may be required to survive exposure to temperatures near the supercooling point (Lee and Denlinger, 1985; Bale, 1987; Lee *et al.*, 1987).

The convergent lady beetle, *Hippodamia convergens* Guerin (Coccinellidae, Coleoptera), is widely distributed in North America and is an important predator of aphids. During the winter, this freeze-susceptible species avoids freezing by depressing the

supercooling point of body fluids to temperatures below −15°C (Bennett and Lee, 1989) and overwinters in massive aggregations on the forest floor (Hagen, 1962).

The purpose of this study was to investigate the potential for ice-nucleating active bacteria to regulate the supercooling point of insects. The lady beetle was selected as an insect model because they are available in large numbers as cold-hardy adults and they maintain a low supercooling point even after removal from the cold (Lee, 1980; Bennett and Lee, 1989). Specifically we examined the effects of the ingestion of ice-nucleating active bacteria, *Pseudomonas syringae* and *Erwinia herbicola*, on the supercooling point of *H. convergens*.

METHODS AND MATERIALS

Rearing

Bacterial strains of *P. syringae* provided by S. E. Lindow (University of California, Berkeley) (strain cit 7), *E. herbicola* provided by L. R. Maki (The University of Wyoming) (strain 265G-2) and *Escherichia coli* K12 derived from strain AB301 provided by J. R. Johnson (Miami University, Oxford, Ohio) were identified and confirmed by their biochemical reactions utilizing the API 20E system (Analytab Products, Plainview, NY 11803). Bacteria were maintained at 20 ± 0.1°C on Bacto nutrient agar (Difco) plates with 2.5% glycerol added to enhance ice-nucleating activity and 0.5% cycloheximide to retard fungal growth. To maximize expression of ice-nucleating activity, plates were incubated at 20°C for 7 days prior to preparation of bacterial suspensions utilized in subsequent feeding trials. Adult *H. convergens* were collected from overwintering aggregations in California, shipped to our laboratory and held unfed at 4°C until used for experimentation.

Supercooling point determination

Supercooling point values were determined by positioning beetles in contact with a 30-gauge copper–constantan thermocouple within a 1.5 ml polypropylene tube. These tubes were placed into glass test tubes suspended in a 0°C refrigerated bath and allowed to equilibrate for 5 min before cooling at approx. 0.3°C/min. The lowest temperature reached prior to the release of the latent heat of crystallization was recorded as the supercooling point.

Bacterial suspension and lady beetle treatment

In determining the number of bacteria/ml of solution, suspensions of 0.5 absorbance, at 660 nm (Baush and Lomb Spectronic 20) were made in sterile distilled water for each strain. Bacterial counts were determined utilizing a custom-made haemocytometer with a reduced cell depth of 0.02 mm and improved double Neubauer ruling (Hausser Scientific Co.). Aqueous serial dilutions of each bacterial species to be used in ice-nucleating active assays and feeding experiments were made from 10^9 to 10^5 bacteria/ml sterile water based upon initial total bacterial counts.

Approximately 50 adult lady beetles were placed in a 150 × 15 mm Petri dish and fed the bacterial suspensions using 200 μl capillary tubes plugged at one end with cotton and filled with sterile water or one of the 3 aqueous bacterial suspensions (*P. syringae*, *E. herbicola* or *E. coli*). Five capillary tubes were placed in each Petri dish containing the beetles and transferred to a 20°C incubator. Each treatment group was fed for 48 h on the bacterial suspension prior to supercooling point determination. Beetles were monitored initially to be certain they were drinking from the tubes. Fresh bacterial suspensions were made daily.

Effect of ice-nucleating active bacterial concentrations on the supercooling point

Twenty beetles were placed in Petri dishes containing varying suspensions, 10^9, 10^8, 10^7, $10^{6.75}$, $10^{6.5}$, $10^{6.25}$ or 10^5 of *P. syringae*/ml sterile water. Supercooling points ($N = 12$) were determined after 48 h of feeding. Fresh bacterial suspensions were made daily.

Recovery of ice-nucleating active bacteria from the insect gut

Lady beetles fed ice-nucleating active bacteria for 48 h were dissected under aseptic conditions. Gut content was plated onto nutrient agar containing 2.5% glycerol and 0.5% cycloheximide. The plates were incubated 7 days at 20°C and a suspension in phosphate-buffer saline was made of each morphologically distinct colony (absorbance 0.5% approx. 10^9 bacteria/ml sterile phosphate-buffer saline). From this suspension ten 10 μl-drops were placed on an aluminum boat, floating on a refrigerated alcohol bath. A surface temperature −6.5 ± 0.3°C was maintained. The droplets were observed for 1–5 min for freezing; initially droplets appear "clear" and turn opaque after freezing occurs. Questionably frozen droplets were touched with a sterile probe; if freezing occurred as a consequence of probing, these data were not included with droplets that froze spontaneously (Vali, 1971).

Retention of ice-nucleating active bacteria following ingestion

Approximately 50 beetles were placed in a Petri dish and fed *P. syringae* (10^9 bacteria/ml sterile water). A fresh bacterial suspension of *P. syringae* was made daily. After 48 h of feeding the suspension was replaced with sterile distilled water and supercooling points checked at 1, 3, 5 and 7 days post *P. syringae* treatment. Fresh water was added daily.

RESULTS

Effect of ice-nucleating active bacteria on the supercooling capacity of water and phosphate buffer

The freezing profile of sterile distilled water, phosphate-buffer solution and *P. syringae* (10^9 bacteria/ml sterile phosphate-buffer solution) is shown in Fig. 1. All three treatments were run concurrently, on individual boats, with twenty 10 μl-droplets (a total of 40 droplets/solution) placed on each boat floating on a refrigerated bath. Both phosphate-buffer solution and sterile distilled water supercooled extensively and began freezing only after temperatures of −17 and −13°C respectively were reached. This result contrasts sharply with the freezing profile of *P. syringae* where droplets began freezing at −1.6°C with 100%

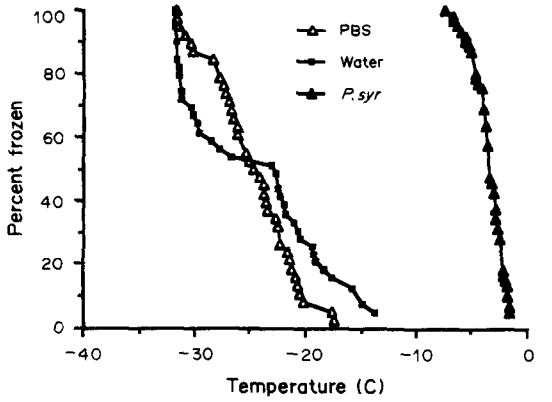

Fig. 1. Cumulative freezing profile of forty, 10 μl-drops each of phosphate buffer (—△—), sterile distilled water (—■—) and a suspension of 10^9 bacteria/ml sterile distilled water of *P. syringae* (—▲—).

frozen by −7.4°C. Thus, the addition of *P. syringae* to phosphate-buffer saline greatly reduced its supercooling capacity. Since this saline proved lethal to the insects, all subsequent bacterial suspensions were made with sterile water.

Effect of ice-nucleating active bacteria on the supercooling point of beetles

Unfed adult beetles normally supercool to -16.0 ± 0.6°C when tested immediately after removal from 4°C (Figs 2 and 3). Beetles fed a suspension of *P. syringae* (10^9 bacteria/ml sterile water) showed a dramatic elevation in the mean supercooling point to -3.5 ± 0.2°C, an increase of 12.5°C. A second type of ice-nucleating active bacteria, *E. herbicola*, was fed to the beetles for 48 h (10^9 bacteria/ml sterile water), and resulted in a significant supercooling point elevation, -4.4 ± 0.6°C (Fig. 3). Controls include: beetles fed only distilled water or unfed and held at 20°C for 48 h, supercooling points for all individuals in both groups were below −15°C, and beetles fed a non-ice-nucleating active bacteria, *E. coli* at 10^9 bacteria/ml sterile water, mean supercooling point, -16.0 ± 0.4°C, indicating, as expected, a lack of ice-nucleating activity in this control species (Fig. 3).

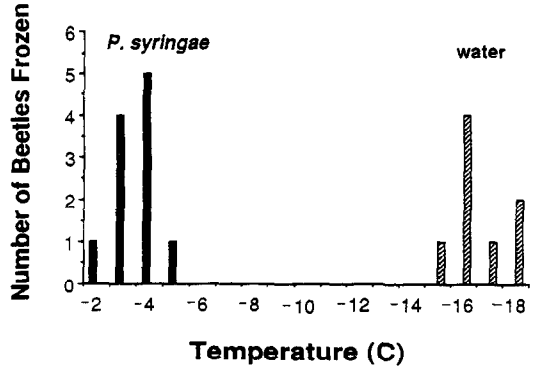

Fig. 2. Effect of ingesting ice-nucleating active bacteria on the supercooling point of the adult lady beetle, *H. convergens*. Supercooling points were determined for beetles ($N = 11$) fed a suspension of *P. syringae* (10^9 bacteria/ml sterile water) vs those ($N = 8$) fed only sterile water.

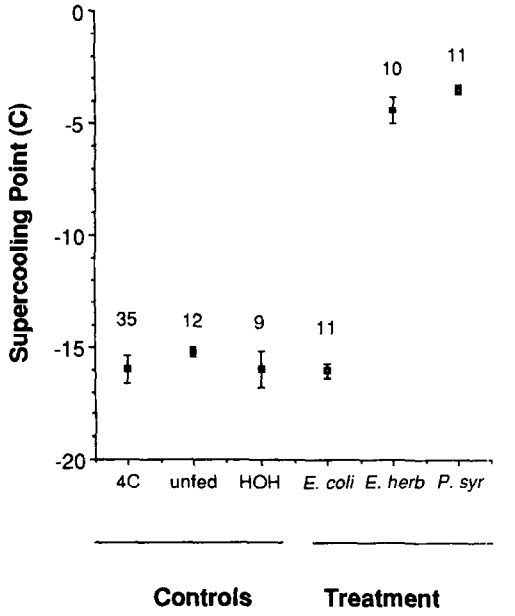

Fig. 3. Effect of ingesting ice-nucleating active bacteria on the supercooling point of adult lady beetles (*H. convergens*). Within the control groups, the 4°C label represents beetles taken directly from 4°C prior to testing, while the remaining groups (unfed for 48 h, or fed sterile distilled water) were held at 20°C. The treatment label corresponds to beetles fed solutions of 10^9 bacteria/ml in sterile distilled water [*E. coli* (*Escherichia coli*), *E. herb* (*Erwinia herbicola*) and *P. syr* (*Pseudomonas syringae*)]. All values reported are mean ± SEM, number of beetles tested is noted above the data points.

Retention of ice-nucleating active bacteria in the intestinal tract of H. convergens

Lady beetles were fed a suspension of *P. syringae* (10^9 bacteria/ml sterile water) for 48 h when the feeding tubes were replaced with ones containing sterile distilled water. Supercooling points of the beetles were tested at 1, 3, 5 and 7 days after the *P. syringae* suspension was removed (Fig. 4). Mean

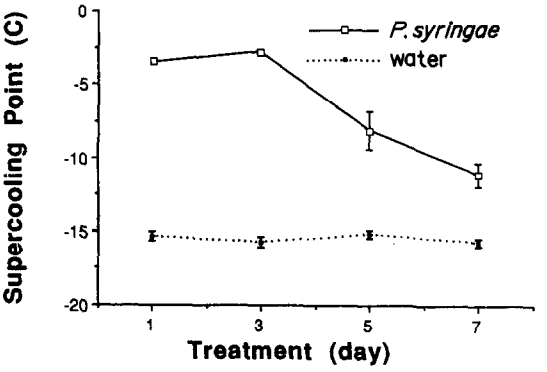

Fig. 4. Retention of ice-nucleating active bacteria and its effect on the supercooling point of adult lady beetles (*H. convergens*). Beetles were fed either sterile distilled water (···■···) or a suspension of *P. syringae* (—□—) (10^9 bacteria/ml sterile water) for 48 h at 20°C. Beginning on Day 0 the beetles were feed only water. All values reported are mean ± SEM.

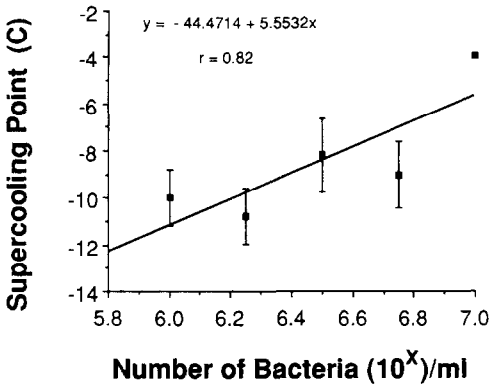

Fig. 5. Effect of feeding lady beetles (*H. convergens*) varying concentrations of the ice-nucleating active bacterium, *P. syringae*, on the supercooling point. All values reported are mean ± SEM.

supercooling points for beetles 1 and 2 days after treatment were −3.5 and −2.8°C respectively, a substantial elevation in supercooling point values with little variation among individual values within the treatment groups. A decrease in the supercooling point was observed on days 5 and 7 which coincided with an increase in the variability of the supercooling point values. At 5 days after treatment 6 of 12 individual supercooling point values were between −3.6 and −4.6°C with the remainder between −10.1 and −14.8°C. Even after 7 days, beetles maintained supercooling points that were slightly higher than control beetles fed only distilled water. Mean supercooling point values at 1, 3 and 5 days after treatment were significantly greater than control means ($F = 28.39$, $P = 0.0001$). Furthermore, it was possible to recover *P. syringae* from the gut of beetles after being fed this bacterium.

Effects of various bacterial concentrations on the supercooling point

Various concentrations of *P. syringae* were fed to beetles for 48 h (Fig. 5). Serial dilutions of *P. syringae* between 10^6 and $10^{6.75}$ bacteria/ml sterile water slightly elevated the mean supercooling points to approx. −9.5°C. A suspension of 10^7 was the lowest concentration of *P. syringae* that showed a marked elevation in the supercooling point −3.9 ± 0.1°C (Fig. 5). Supercooling points were positively correlated ($r = 0.82$) with increasing concentrations of the ice-nucleating active bacteria.

DISCUSSION

In this report we demonstrate for the first time that ingestion of ice-nucleating active bacteria by insects causes an elevation in the freezing temperature of an insect. Ingestion of both *P. syringae* and *E. herbicola* significantly increased the supercooling point of the lady beetle, *H. convergens*. Previous authors have speculated that bacterial gut flora and/or contents may play a role in supercooling point regulation (Somme, 1982; Baust and Rojas, 1985; Cannon and Block, 1988). Until now, no direct evidence has been reported linking ice-nucleating active bacteria to supercooling point elevation in insects.

In the autumn, freeze-susceptible insects enhance their cold-hardiness by lowering their supercooling point, thereby decreasing the chance they will freeze. Salt (1953, 1961) reported that gut material contained abiotic nucleators acquired via ingestion, which reduced the supercooling capacity of the insects tested. As these nucleators passed through the digestive system their nucleation efficiency varied. Using cryomicroscopy Shimada (1989) found that the alimentary canal of *Trichiocampus populi* nucleated at a significantly higher temperature than did the rest of the body. However, in Shimada's study the substances acting as the ice-nucleating catalyst in the alimentary canal were not identified. Cessation of feeding and gut evacuation increases the supercooling capacity of some insects (Salt, 1953, 1961; Somme, 1982). Young and Block (1980) have found that in the Antarctic mite, *Alaskozetes antarcticus*, feeding is associated with a 10 to 20°C reduction in the supercooling capacity (i.e. supercooling point elevation). However, other studies show no definitive relationship between supercooling point and gut content (Somme, 1982; Baust and Rojas, 1985).

In our study, overwintering adult lady beetles fed ice-nucleating active bacteria in the laboratory showed a pronounced increase in their supercooling point from approx. −16 to −2°C; an elevation of 14°C! The supercooling point of the beetles remained elevated for 7 days after they ceased feeding on *P. syringae*. In addition, the bacteria were recovered from the beetles' gut with ice-nucleating activity still maintained.

Although *E. herbicola* and other potential ice-nucleating active bacteria are normal flora in the gut of insects, their ice-nucleating activity has not been investigated. The bacterium *E. herbicola* is found in the gut of field collected fruit flies, *Dacus* spp. (Lloyd *et al.*, 1986), and the bacterium *Pseudomonas fluorescens* is normal flora in both *Diabrotica undecimpunctata howardi*, the southern corn rootworm (Tran and Marrone, 1988), and *Melanoplus sanguinipes*, a migratory grasshopper (Mead *et al.*, 1988). Currently we are investigating the relationship between these ice-nucleating active bacteria and the regulation of ice nucleation *in vivo*.

It may not be necessary for ice-nucleating active bacteria to be normal gut flora before causing an increase in the supercooling point. Dew or rain on a leaf may contain in suspension ice-nucleating active bacteria from the surface of the plant which insects may passively ingest when they drink. In addition, an insect walking or crawling across the surface of a leaf may accumulate a sufficient number of bacteria on its cuticle to elevate the supercooling point. Other studies in our laboratory show that the presence of ice-nucleating active bacteria on the cuticle can initiate inoculative freezing of insects (Strong-Gunderson *et al.*, 1989).

Ice-nucleating active bacteria may have an affect on the cold-hardiness and winter survival of some freeze-tolerant and freeze-susceptible insects. Normally freezing is initiated at relatively high subzero temperatures in freeze-tolerant insects by mechanisms that are not completely understood (Baust and Rojas, 1985). Ice-nucleating proteins have been described in a variety of insects which function as ice catalysts

in insect haemolymph (Duman and Horwath, 1983; Duman *et al.*, 1984). By initiating freezing in the extracellular space at relatively high subzero temperatures the site and rate of ice-crystal growth can be controlled. It is possible that freeze-tolerant insects may rely on ice-nucleating active bacteria instead of, or in addition to, ice-nucleating proteins, to ensure extracellular freezing at high subzero temperatures. We have shown that lower concentrations of bacteria (10^6 bacteria/ml) initiate freezing in the range of -8 to $-10°C$, a temperature range similar to that in which ice-nucleating proteins induce nucleation in freeze-tolerant insects (Fig. 5).

Currently, we are investigating the significance of ice-nucleating active bacteria in the regulation of insect cold-hardiness under natural conditions. In our laboratory studies we have shown that ice-nucleating active bacteria have the capacity to elevate the supercooling point when ingested or applied to the cuticle; therefore, it is conceivable that the application of these bacteria to freeze-susceptible insects in their hibernacula may be used as a control mechanism to increase overwintering mortality. The identification and characterization of novel indigenous ice-nucleating active bacterial strains isolated from field-collected insects will be reported in a subsequent paper.

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