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Topical Application of Ice-Nucleating-Active Bacteria Decreases Insect Cold Tolerance†

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The majority of overwintering insects avoid lethal freezing by lowering the temperature at which ice spontaneously nucleates within their body fluids. We examined the effect of ice-nucleating-active bacteria on the cold-hardiness of the lady beetle, *Hippodamia convergens*, a freeze-intolerant species that overwinters by supercooling to ca. $-16°C$. Topical application of the ice-nucleating-active bacteria *Pseudomonas syringae* increased the supercooling point to temperatures as high as $-3°C$. This decrease in cold tolerance was maintained for at least 3 days after treatment. Various treatment doses (10^8, 10^6, and 10^4 bacteria per ml) and modes of action (bacterial ingestion and topical application) were also compared. At the highest concentration of topically applied *P. syringae*, 50% of the beetles froze between -2 and $-4°C$. After topical application at the lowest concentration, 50% of the individuals froze by $-11°C$. In contrast, beetles fed bacteria at this concentration did not begin to freeze until $-10°C$, and 50% were frozen only at temperatures of $-13°C$ or less. In addition to reducing the supercooling capacity in *H. convergens*, ice-nucleating-active bacteria also significantly reduced the cold-hardiness of four additional insects. These data demonstrate that ice-nucleating-active bacteria can be used to elevate the supercooling point and thereby decrease insect cold tolerance. The results of this study support the proposition that ice-nucleating-active bacteria may be used as a biological insecticide for the control of insect pests during the winter.

In the early 1970s, an ice-nucleating-active bacterium was isolated from decaying plant material and identified as *Pseudomonas syringae* (29, 37). The effect of this ubiquitous plant pathogen on decreasing plant cold-hardiness has been studied extensively (11, 18). Many plants supercool to temperatures of $-5°C$ or below before freezing occurs (1, 11, 17, 18); however, the presence of these epiphytic, ice-nucleating-active bacteria decreases the capacity of the plant to supercool, causing it to freeze under mild frost conditions ($-2°C$) (16).

Until recently (15, 34), little attention has been paid to the interaction between ice-nucleating-active bacteria and animals. We began by investigating the possible role these bacteria play in regulating the cold-hardiness of freeze-intolerant insects. In winter, these insects survive by depressing the temperature at which their body water freezes to temperatures below those encountered in the field (13). The temperature at which freezing occurs is the supercooling point or the temperature of crystallization (38).

The capacity to supercool is determined by the presence or absence of heterogeneous ice-nucleating agents. To enhance their capacity to supercool, freeze-intolerant insects must eliminate efficient internal ice-nucleating agents and avoid inoculation by external ice. Factors that affect the supercooling capacity include ice-nucleating proteins and lipoproteins (22, 39), antifreeze proteins (9, 12), low-molecular-weight polyols and sugars (3, 26, 32), and inoculative freezing by external ice (27). Some investigators have suggested the gut as the source of heterogeneous ice nucleators (4, 7, 30, 31). Recently, we isolated ice-nucleating-active bacteria from the guts of field-collected insects (15, 33).

Previously, we reported that ingestion of ice-nucleating-active bacteria increases the mean supercooling point of the lady beetle *Hippodamia convergens* by as much as 14°C (34). Here we specifically examine the effect of topically applied ice-nucleating-active bacteria on the supercooling point, the duration of the effect, and comparison of topical treatment with bacterial ingestion. Overall, we report that topical application of ice-nucleating-active bacteria increases the mean supercooling point, thereby decreasing insect cold-hardiness and increasing insect mortality when exposed to subzero temperatures.

MATERIALS AND METHODS

Insects and bacteria used. Adult lady beetles (*H. convergens*) were collected from overwintering aggregations in California, shipped to our laboratory, and held unfed at 4°C. Field-collected adult southern corn rootworm (*Diabrotica undecimpunctata*) were collected near Oxford, Ohio, in September and October and held unfed at 4°C until used for experimentation. Mealworm beetle (*Tenebrio molitor*) adults and larvae were reared at room temperature on bran (light: dark cycle, 12 h:12 h), nondiapausing flesh fly (*Sarcophaga crassipalpis*) larvae were reared on liver as described by Denlinger (8), and laboratory colonies of waxworm (*Galleria mellonella*) larvae were held at room temperature.

The bacterium *Escherichia coli* (ATCC 35421) and the ice-nucleating-active bacterium *P. syringae* cit 7, provided by S. E. Lindow (University of California, Berkeley), were used throughout the study. Bacteria were maintained at 20 ± 0.1°C on Bacto Nutrient Agar (Difco) with 2.5% glycerol added to enhance ice-nucleating activity.

Insect treatment and recovery of ice-nucleating-active bacteria. Direct bacterial counts were performed at an A_{550} of 0.5 and determined to be 3×10^8 bacteria per ml of water for both *E. coli* and *P. syringae*. From these aqueous suspen-

sions, serial dilutions were made of from 10^8 to 10^4 bacteria per ml of water.

The lady beetles were fed various bacterial suspensions and water via capillary tubes as previously described (34). Dispensing the bacteria in this manner prevented insect surface contamination. Topical application of the bacteria was accomplished by misting the solution onto the surface of the lady beetle. Approximately 50 lady beetles (with sealed mouths) were placed in a petri dish and misted with sterile water or P. syringae (10^8 bacteria per ml of water). The beetles were held at 20°C, and the supercooling points were determined immediately (day zero) and 1, 2, and 3 days after misting. The beetles survived only 3 days with their mouths sealed, thus limiting the duration of the experiments.

Supercooling point values were determined by positioning a 30-gauge copper-constantan thermocouple in direct contact with an insect confined in a 1.5-ml polypropylene microcentrifuge tube. These tubes were inserted into glass tubes suspended in a refrigerated bath, equilibrated for 2 to 3 min at 0°C, and cooled at approximately 0.6°C/min. The cooling profile was recorded, and the supercooling point was identified as the lowest temperature reached before the release of the latent heat of fusion.

To determine whether the application of ice-nucleating-active bacteria elevated the supercooling point of a variety of insects, we tested selected insects from three additional orders. These insect species, representing different life stages, were treated with P. syringae as described above; however, their mouths were not sealed and their supercooling points were determined immediately after treatment.

Recovery of ice-nucleating-active bacteria from the insect cuticle. After misting with P. syringae (10^8 bacteria per ml of water), a lady beetle was vortexed and sonicated in 1 ml of sterile distilled water from which a 0.1-ml aliquot was plated onto Bacto Nutrient Agar containing 2.5% glycerol and 0.5% cycloheximide, which inhibited mold growth. The plates were incubated for 3 to 7 days at 20°C, and the total number of bacteria recovered from the insect surface was estimated. The persistence of bacterial ice-nucleating-activity was determined by making an aqueous bacterial suspension (at an A_{550} of 0.5) from which forty 10-μl drops were placed on an aluminum boat with a surface temperature of -6.5 ± 0.3°C (34). The drops were observed for 5 min for freezing; initially, drops appeared clear and then became opaque upon freezing (36).

Scanning electron microscopy. After misting, beetles were prepared for scanning electron microscopy by being quickly frozen at -20°C. After being frozen, the insects were dried at room temperature for 2 to 4 days, mounted onto aluminum stubs, and lightly sputter coated with gold-paladium. Specimens were viewed in a JEOL T-200 electron microscope at a working distance of 8 mm. An accelerating voltage of 10 kV was used to minimize charging.

Statistical analysis. Data were analyzed with an analysis of variance, Duncans multiple-range test, and the Students t test programs of the SAS Institute (28).

RESULTS

Supercooling point elevation. When lady beetles whose mouths had been sealed were misted with water or E. coli (10^8 bacteria per ml of water), they supercooled extensively to -15.9 ± 0.2 and -16.5 ± 0.2°C (mean ± standard error of the mean SEM), respectively, before freezing (Fig. 1). However, beetles misted with P. syringae (10^8 bacteria per

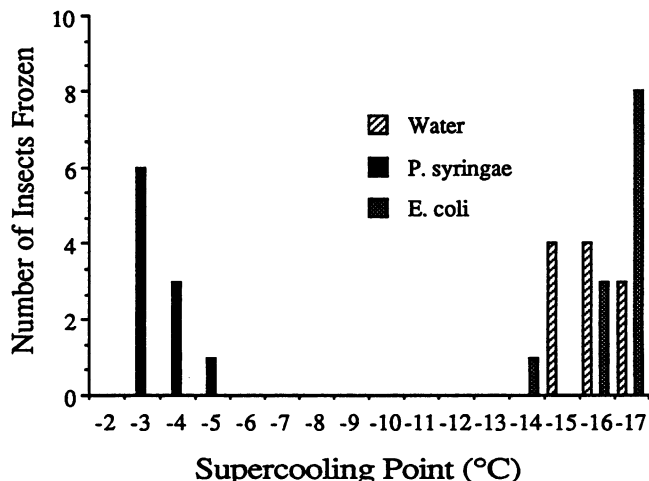FIG. 1. Supercooling points of adult lady beetles (H. convergens) determined immediately after misting with water, E. coli (10^8 bacteria per ml of water), and the ice-nucleating-active bacterium P. syringae (10^8 bacteria per ml of water).

ml of water) exhibited elevated supercooling points of -3.5 ± 0.2°C.

To determine the duration of supercooling point elevation, insects were treated and the supercooling points were measured after 1, 2, and 3 days (Fig. 2). In this experiment, the mouths of the insects were sealed prior to misting. Topically applied ice-nucleating-active bacteria significantly elevated the mean supercooling point for a minimum of 3 days after treatment (-4 to -9°C versus ca. -15°C for water controls; $P < 0.001$).

Two approaches were used to document the presence of bacteria on the cuticle. First, after treatment with ice-nucleating-active bacteria, the insect was washed and the
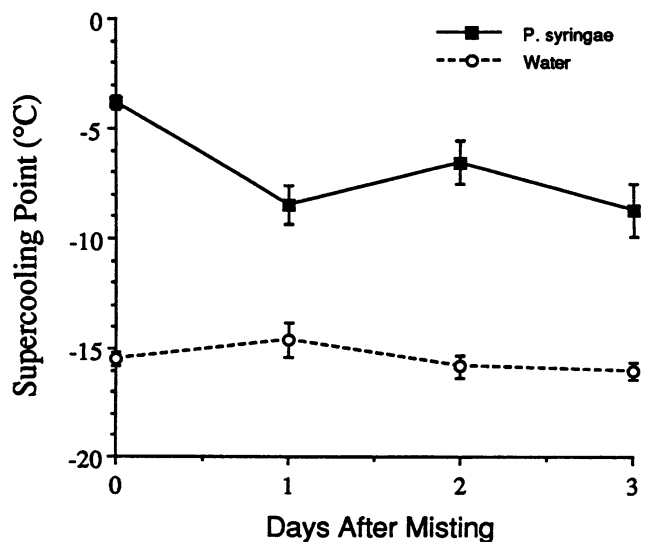

FIG. 2. Supercooling points of lady beetles (H. convergens) at the time of misting (day zero) and 1, 2, and 3 days after misting with water or P. syringae (10^8 bacteria per ml of water). The mouths of the insects were sealed to prevent ingestion. Supercooling points of P. syringae-treated individuals were significantly elevated above control values (n = 10 to 12).
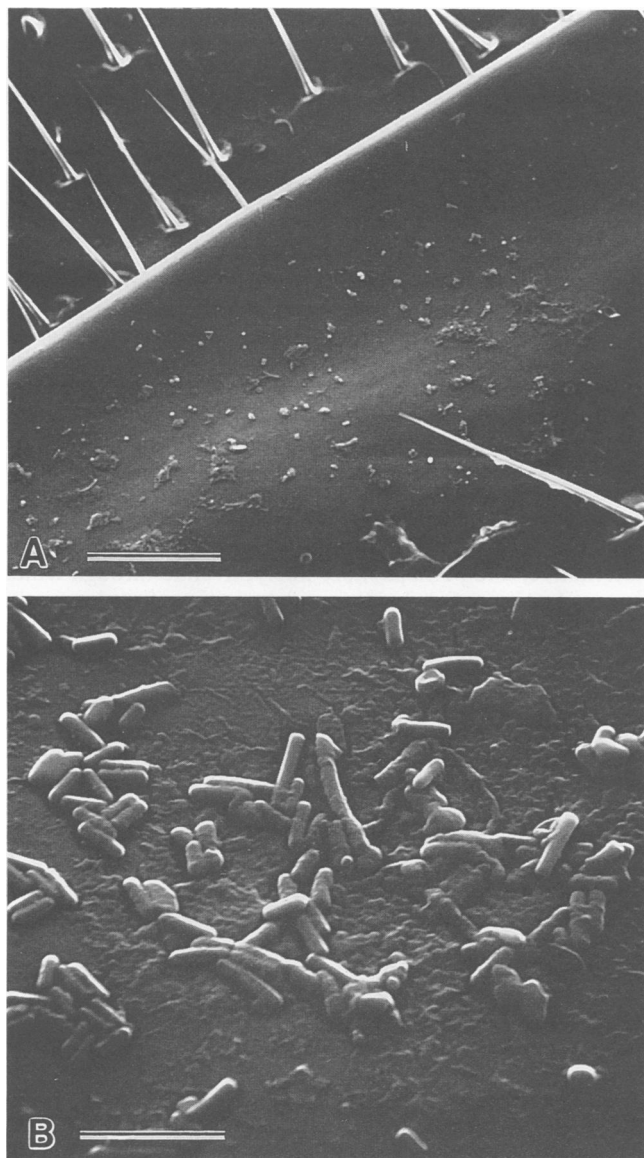
FIG. 3. (A) Posterior abdominal sternite of an adult lady beetle (*H. convergens*) previously misted with 10^8 *P. syringae* bacteria per ml of water. Clusters of bacteria can be seen among the debris. Bar, 50 μm. (B) Magnified cluster of bacteria on the metasternite of an adult lady beetle after misting with 10^8 *P. syringae* bacteria per ml of water. Bar, 5 μm.
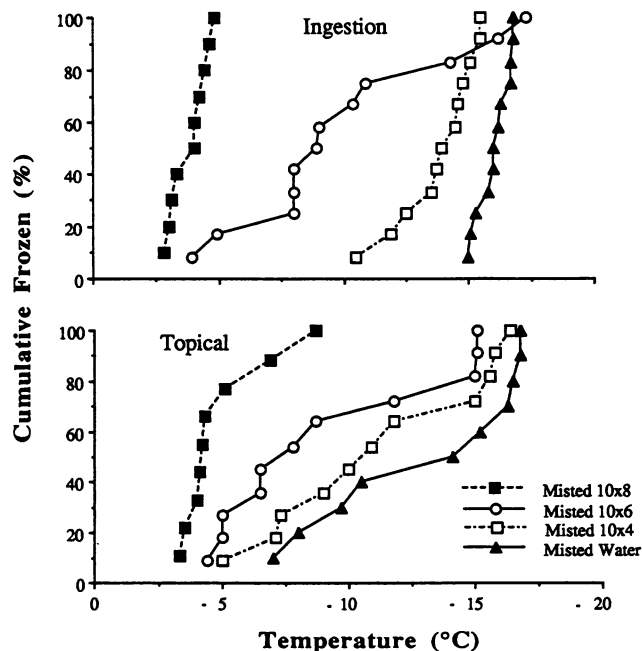

FIG. 4. Cumulative percentage of individual supercooling points of the adult lady beetle (*H. convergens*) after ingestion (top) or topical application (bottom) of sterile water or various concentrations of the ice-nucleating-active bacterium *P. syringae*.

solution was plated onto nutrient agar. Total concentrations of culturable bacteria ranged from 50 to 150 colonies per ml of water (50 to 150 colonies per insect), with approximately one-half of these colonies having ice-nucleation activity at −10°C or above. Beetles misted with only sterile water did not show recoverable ice-nucleating-active bacteria. Second, bacteria applied to the insect cuticle were identified visually by using scanning electron microscopy. Several control insects, unmisted or misted with water, did not have large clusters of bacteria on their surfaces. However, six of seven insects examined after misting with *P. syringae* (10^8 bacteria per ml of water) had large aggregations of rod-shaped bacteria on their cuticles. Bacteria were most fre-

quently observed on the metasternites and posterior abdominal sternites (Fig. 3) of these six insects.

Supercooling point elevation after bacterial ingestion versus that after topical application. A cumulative freezing profile was used to evaluate the efficacy of supercooling point elevation between beetles fed or misted with *P. syringae* (Fig. 4). The supercooling point of individuals fed ice-nucleating-active bacteria increased, indicating a reduction in cold-hardiness. Within a treatment group, coincident with a reduction in bacterial concentration was an increase in the supercooling point variability among individual beetles. Beetles fed water had supercooling points of ca. −15°C, while those fed 10^8 *P. syringae* bacteria per ml of water had supercooling points within a narrow range of −3 to −5°C. This narrow range of variability contrasts with the range of those fed the lowest bacterial concentration (10^4 bacteria per ml of water), in which freezing did not begin until −10°C and only 50% of the insects froze by −14°C (Fig. 4). The minimum concentration necessary to increase the mean supercooling point above −10°C was ca. 10^6 bacteria per ml of water. Similar trends were observed in replicate experiments.

Beetles misted with the bacteria showed trends similar to the effects observed in beetles after ingestion; however, the variability within treatment groups was substantially greater in the beetles misted with bacteria (Fig. 4). Insects misted with 10^8 bacteria per ml began to freeze at −3°C, with 70% freezing by −5°C and the remainder freezing by −9°C. Similarly, insects misted with 10^4 bacteria per ml began freezing at −5°C, with 50% freezing by −11°C. Insects misted with sterile water had supercooling points between −7 and −16°C.

A comparison of the supercooling capacities of beetles fed or misted with ice-nucleating-active bacteria shows that at high concentrations there is no significant difference in the
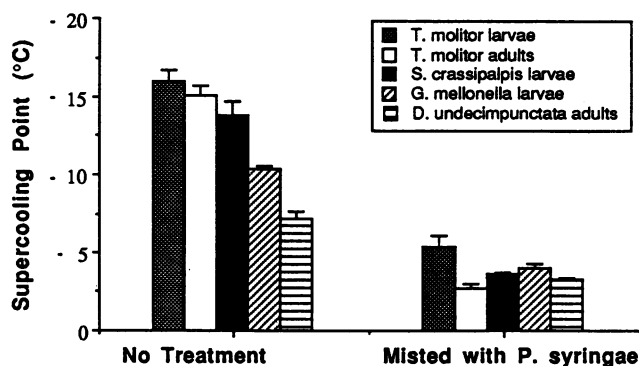
FIG. 5. Supercooling points (means ± standard errors of the mean) of various insects (*T. molitor*, *S. crassipalpis*, *G. mellonella*, and *D. undecimpunctata*) after misting with 10^8 *P. syringae*. All treatments were elevated significantly ($P < 0.005$) above control values ($n = 10$ to 12).

supercooling points. As the bacterial concentration is lowered to 10^4 bacteria per ml, the supercooling points range from as high as $-5°C$ (misting treatment) to $-17°C$ (both misting and ingestion treatments). Of greater significance, for insects misted with 10^4 bacteria per ml, 50% of the individuals froze by $-11°C$, whereas those fed bacteria at this concentration did not begin to freeze until $-10°C$ or lower. These observations of cumulative freezing versus temperature, with both ingested bacteria and topically applied bacteria, were consistent over four replicated experimental runs.

Topical application of ice-nucleating-active bacteria to other insects. An elevation of the supercooling point after treatment with the ice-nucleating-active bacteria was not restricted to the lady beetle. Supercooling points from a variety of insect species (mouths unsealed) were compared before and immediately after misting with *P. syringae* (10^8 bacteria per ml of water) (Fig. 5). In untreated control insects, the greatest supercooling capacity was observed in adults and larvae of *T. molitor* and larvae of *S. crassipalpis*, which supercooled to $-13°C$ or lower before freezing. *G. mellonella* larvae and *D. undecimpunctata* adults supercooled the least (supercooling points of -7 and $-10°C$, respectively). However, after the application of ice-nucleating-active bacteria, all four species, regardless of their initial freezing temperatures, had elevated supercooling point values in the range of -3 to $-5°C$. In all cases, the supercooling points were significantly higher ($P < 0.001$) than those of controls.

DISCUSSION

The mechanisms involved in inoculative freezing are largely unknown. We can, however, speculate on some of the routes that may be involved. By definition, inoculative freezing involves freezing from outside the insect (water on or in contact with the cuticle) inwards. The initial, related questions are the following. Can ice propagate through the cuticle? Is there enough water associated with the cuticle to participate in the propagation of ice "through" this surface?

Bale et al. (2) suggested that inoculative freezing occurred through cuticular apertures; however, they did not specifically identify these openings or suggest a route for ice crystal growth. Salt (27) showed that inoculative freezing of wheat stem sawfly (*Cephus cinctus*) larvae did not increase after

the larvae had been soaked in water. Our studies also showed no significant elevation in the mean supercooling point of lady beetles misted only with sterile water (Fig. 1). However, when a strong nucleating agent (*P. syringae*) was added to the suspension, inoculative freezing occurred at significantly elevated temperatures.

In general, insect cuticle is composed of three acellular layers perforated by pore canals (20). These canals pass through the epicuticle and terminate just beneath the thin outer cuticulin layer (wax and cement layers). The bacteria may adhere to the cuticle and nucleate the surface water. However, for internal body water to freeze, these growing ice crystals would have to propagate through pore canals to the inside. There are several inherent problems with this explanation: initially, the ice would have to penetrate the hydrophobic outer cement or wax layer and continue through the pore canals, which are not cylindrical but ribbonlike (23). Additional circumstantial evidence against this mode of freezing is that the pore canals are primarily thought to transport wax (6), making them hydrophobic and possibly devoid of sufficient water for ice crystal growth. An alternative, and perhaps more likely, avenue through which ice-nucleating-active bacteria might initiate freezing is the respiratory system. The bacteria could enter the trachea through open spiracles and nucleate water located within this moist environment.

The elevation in the supercooling point after misting with ice-nucleating bacteria was not restricted to our model; an additional four pest species from three different insect orders also had elevated supercooling points. Regardless of the developmental stage or species examined, all supercooling points were significantly elevated above control values. The cuticular compositions of these insects differ substantially. It therefore appears that a variety of insects can have their supercooling points elevated by the application of ice-nucleating-active bacteria to their surfaces alone.

To better understand the effect of ice-nucleating-active bacteria on supercooling point elevation and the supercooling point variability observed after treatment, it is important to recognize the differences in the inherent capacity of the bacteria to nucleate supercooled water. Bacterial ice-nucleation structures (membrane proteins) are classified into one of three classes on the basis of their nucleation activity (35). Those in class A are the most potent ice catalysts and initiate freezing at $-4.4°C$ or above; those in class B initiate freezing between -4.8 and $-5.7°C$; members of class C are the weakest, initiating freezing at $-7.6°C$ or below. Within a bacterial suspension of ice-nucleating-active bacteria, class C structures are found on most cells, whereas class A structures are the least abundant. Therefore, as an initial suspension is diluted, the concentration of potent nucleators is greatly diminished. Previous studies with ice-nucleating proteins in insects have shown that as the concentration of nucleators is reduced, the supercooling point variability increases (10, 39).

We examined the efficacy with which two treatment methods (bacterial ingestion and topical application) decreased the supercooling capacity. At high concentrations (10^8 bacteria per ml of water), there was no significant difference between the mean supercooling point values of the insects in the two treatment groups (Fig. 4). However, as the concentration decreased to 10^4 bacteria per ml, topical application induced freezing at higher temperatures than did ingestion. A comparison between insects fed water and those misted with water indicated greater variability in the misted insects. This increased variability may be a result of

a nucleating agent already present on the insect surface coming into contact with water or the accumulation of water on the cuticle that may function to promote inoculative freezing and thereby diminish the supercooling capacity.

Our results indicate that supercooling points remain elevated for at least 3 days after treatment (Fig. 3). Compared with the supercooling points of controls and day zero treatment, the supercooling point values within a treatment group (1, 2, and 3 days) were quite variable, with individual values ranging from -2 to $-15°C$. This variability may be explained by a number of factors. First, if the bacteria are on the insect surface but not in contact with water, they cannot induce inoculative freezing. Second, the number of bacteria that remain attached to the cuticle may be reduced because of the insect surface being a nonhost surface. Third, there may be a loss of ice-nucleating activity because of prolonged exposure to room temperature. In culture, P. syringae expresses optimum ice-nucleating activity (class A nucleation efficiency, $>-4.4°C$) if maintained at temperatures less than or equal to 20°C. We have preliminary evidence indicating that beetles misted with bacteria and held at 4°C (compared with those held at 20°C) maintain elevated supercooling points for at least 7 days after treatment. Insects held at 4°C are not as active as those held at 20°C; thus, the bacteria may remain on the surface longer, and evaporation of surface water is reduced.

We have demonstrated by two methods (surface recovery and scanning electron microscopy) the presence of bacteria on the insect cuticle. Pseudomonads do have adhesive properties, and numerous investigators have discussed the differential adsorption of these bacteria to the plant surface (5). Preece and Wong (25) determined that 52 to 92% of the cells will attach to their host, with only 11 to 30% attaching to a nonhost. Furthermore, they reported that many species of bacteria can adhere to surfaces so strongly that they cannot be removed even by 30 min of washing. In our attempts to estimate the number of bacteria adhering to the insect cuticle, we followed the washing protocol of Lindow et al. (18). Preliminary results recovered a total bacterial concentration of 50 to 150 bacteria per insect, with approximately 50% of these isolates expressing ice-nucleating activity. If these bacteria adhere irreversibly, then our preliminary estimates may be low, and a more appropriate method of recovery would be necessary before an accurate count could be made. Future studies should attempt to quantify the percentage of ice-nucleating-active bacteria that actually adhere to the insect surface.

Once the bacteria are applied to the cuticle, their numbers and position may not be fixed. Mansvelt and Hattingh (21) have shown that P. syringae multiply and move across the leaf surface, invading the plant through stomata. It may be possible for these bacteria to move across the insect surface and invade through the spiracles or other routes, ultimately leading to contact with internal body water. Since P. syringae is a plant pathogen, it is unlikely that it would establish a community on or in the insect. It may be possible for other species of ice-nucleating-active bacteria such as Enterobacter agglomerans and Enterobacter taylorae (members of the family Enterobacteriaceae are common insect gut flora [19]), isolates from field-collected insects (15, 33), to invade and establish colonies on or within the insect host. E. agglomerans, our most common isolate, is biochemically very similar to the erwinias, which have previously been described as having strong adhesive properties (24). These ice-nucleating-active enterobacters may also have adhesive properties and may be able to adhere to the insect cuticle or

enter the respiratory system or the gut and establish a colony.

We previously suggested that ice-nucleating-active bacteria may be used as a biological insecticide (14, 15, 34). These bacteria would function as ice catalysts to diminish the supercooling capacity of insects, causing them to freeze at temperatures only a few degrees below 0°C. Although the actual route (respiratory system, cuticular pores, etc.) of inoculative freezing is currently unknown, the fact that the supercooling point is elevated very rapidly after misting suggests the absence of effective barriers to block ice nucleation. Therefore, it may be difficult for insects to develop resistance to this type of biological control since it would entail blocking all avenues of contact between the bacteria and the body water of the insect. Our study demonstrated that the topical application of ice-nucleating-active bacteria decreases insect cold-hardiness. A decrease in cold-hardiness after topical application is of particular importance for biological control since overwintering insects usually do not eat and only rarely drink. Therefore, if the supercooling point elevation (i.e., a reduction in cold-hardiness) relied solely on ingestion and retention of the bacteria in the gut, the effectiveness of winter pest control would be reduced, since many overwintering insects purge their guts in preparation for subzero temperatures.

ACKNOWLEDGMENTS

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