LONG-TERM RETENTION OF ICE-NUCLEATING ACTIVE *PSEUDOMONAS FLUORESCENS* BY OVERWINTERING COLORADO POTATO BEETLES

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SUMMARY

Ice nucleating-active *Pseudomonas fluorescens* F264C was fed to Colorado potato beetles to determine bacterial retention in the beetle gut and its effect on the cold-hardiness of this insect pest. The bacterium was present in beetles recovered after overwintering in the field, seven months after their initial exposure to *P. fluorescens*. Retention was evident not only in the detection of the *P. fluorescens* ice nucleation gene, *inaW*, in bacterial cultures from beetle guts but also in the elevated supercooling points of some of the treated beetles.

Key words: *Pseudomonas fluorescens*, biological control, cold-hardiness, ice-nucleating active bacteria.

INTRODUCTION

Some Gram-negative bacteria of the genera *Erwinia, Pseudomonas*, and *Xanthomonas* can catalyze ice crystal formation at temperatures above -10°C (9, 11, 12, 13). This phenomenon, termed ice-nucleating activity, is conferred by the presence of *ina* genes (also called *ice* genes) that code for proteins located on the outer membrane of the bacterium (15). These proteins may aggregate and serve as templates promoting ice crystal formation (16).

To date, the majority of the described ice-nucleating active bacteria are epiphytes that cause plant frost injury. However, several species were reported recently as normal flora in the guts of frogs (6) and insects (5, 8). The presence of these biological ice nucleators in the insect gut reduces the insect's capacity to supercool (i.e., remain unfrozen at or below the equilibrium freezing point of its body fluid).

Except for a few species capable of surviving extensive freezing of their body water, most insects are freeze-intolerant (3). In winter, many of these freeze-intolerant insects depress their supercooling point, the temperature at which ice nucleation spontaneously occurs in their body fluid, to survive subzero winter temperatures. Since the supercooling point represents the lower temperature limit for survival in these insects, exposure to ice-nucleating active bacteria and, consequently, elevation of their supercooling point could
decrease their cold tolerance (7, 14). These observations are the basis of a novel strategy of using ice-nucleating active bacteria to reduce the winter survival of freeze-intolerant insect pests.

We are exploring the use of ice-nucleating active bacteria as potential biological control agents using the freeze-intolerant Colorado potato beetle (*Leptinotarsa decemlineata*) as a model system. Progress to date has shown that ice nucleating bacteria are capable of elevating the supercooling point of this beetle (7) and that they can also be retained in the diapausing beetle's gut for weeks after initial exposure under laboratory conditions (2). Since Colorado potato beetles overwinter in the soil, control strategies would require beetles to be treated with ice-nucleating active bacteria in late summer or early autumn before they burrow into the ground. Thus, the efficacy of these bacteria as control agents depends on their long-term retention inside the beetle gut through winter, until beetles are subjected to soil temperatures low enough to cause freezing and death. In this study, we exposed adult Colorado potato beetles to ice-nucleating active *P. fluorescens* in early autumn and determined bacterial retention in overwintering beetles based on elevation of supercooling points and detection of the *inaW* gene using Polymerase Chain Reaction (PCR) technique.

**MATERIALS AND METHODS**

**Bacteria and growth conditions**

*Pseudomonas fluorescens* strain F264C, an ice-nucleating active bacterium isolated from the wood frog *Rana sylvatica*, was chosen for this study because of its ability to initiate freezing at high temperatures and its ability to persist in the gut of Colorado potato beetles weeks after ingestion. The bacterium was grown on nutrient agar with 2.5% glycerol for 4 d at 20°C, a regimen known to enhance expression of ice nucleating proteins (2). A bacterial suspension was prepared in sterile deionized water, and cell concentration was determined using a hemocytometer and by plating serial dilutions on nutrient agar plates with glycerol. Ice nucleation activity of bacterial culture was determined by placing 10 μl aqueous suspension of 10⁸ bacterial cells/ml in a 20-μl capillary tube to which a 30-ga copper constantan thermocouple was attached. The capillary tube was placed inside a glass test tube, which was plugged with foam and suspended in a refrigerated bath initially set at 0°C. After thermal equilibration of the sample for 5 min, the temperature was decreased at a rate of 0.3°C/min. The thermocouple was connected to a thermologger (RD3752, Omega Electronics, Stanford, CT, USA) which recorded the temperature of the capillary tube at 30 sec intervals. The highest temperature at which freezing of bacterial suspension occurred was indicated by an exotherm (i.e., release of latent heat of crystallization).

**Experimental animals and field studies**

Adult Colorado potato beetles were collected in early August from cultivated potato fields at Hancock Agricultural Station in central Wisconsin. Beetles were then shipped overnight to Miami University, where groups of 200 beetles were transferred to plastic shoe boxes containing 400 g of autoclaved sand moistened with 40 ml of deionized water. Beetles were held at 15°C, 12:12 (L:D) h, and fed slices of potato tubers.

Prior to exposure to ice-nucleating active *P. fluorescens* F264C, the beetles used in this study were starved for 24 h at 23°C, 12:12 (L:D) h. Then, groups of 20 beetles were placed
in a sterile Petri dish (100 x 15 mm) with a thin slice of potato tuber (50 mm diameter x 5 mm thick) coated evenly with 0.4 ml of bacterial suspension (10⁶ cells/ml) and allowed to feed for 24 h. Afterwards, beetles were removed from feeding dishes and combined with similarly treated beetles into groups of 200, forming one replicate for the field study. There were three replicates each of treated and control beetles. Control beetles were given slices of potato tubers moistened with 0.4 ml of deionized water. Additional beetles were also treated with the bacteria for measurement of supercooling points immediately after feeding. Beetles were then shipped overnight to Wisconsin for release in test arenas at the Hancock Agricultural Research Station.

Groups of 200 adults were released in each of six arenas comprised of bottomless, square steel boxes (58 x 58 x 35 cm deep) which were driven 25 cm into the soil. Beetles were placed on the soil surface in arena centers and provided with potato slices as food. Arenas were covered to prevent beetle escape. A temperature logger (Hobo, Onset Computer Corporation, Pocasset, MA, USA) was installed in one of the arenas at a depth of 10 cm below the soil surface to record temperature fluctuations throughout autumn and winter.

In May of the following year, prior to natural spring emergence of the beetles, all arenas were excavated to a depth of 30 cm. Soil was sieved using a 1/8" wire mesh to recover the beetles. Live and dead beetles from each replicate within a treatment were collected, counted, and placed in separate containers before being shipped overnight to Miami University for supercooling point and PCR studies.

**Measurement of supercooling point**

The supercooling points of Colorado potato beetles were determined as previously described for the ice-nucleating active bacteria, except the thermocouple was in contact with the beetle inside a 1.5 ml polyethylene tube (7). The supercooling points of all live treated and control beetles recovered from the field were determined and compared to values taken from beetles sampled prior to field release.

**Extraction of bacterial genomic DNA**

To detect the presence of ice nucleating bacteria in treated beetles, PCR amplifications were conducted using DNA extracted from bacterial cultures that were isolated from the digestive tract of recovered beetles. Twenty-four treated and 10 control beetles were aseptically dissected and their digestive tracts triturated in 200 µl of sterile saline in 1.5 ml polyethylene tubes using sterile wooden sticks. An aliquot (100 µl) of each suspension was used to inoculate 10 ml nutrient broth with 2.5 % glycerol in 125 ml flasks and cultured overnight in an incubator shaker set to 150 rpm at 24°C. Cells from one ml of broth were used for DNA extraction using the QIAamp tissue kit (Quiagen Inc., CA, USA).

**PCR assays**

Ice nucleating *P. fluorescens* was detected using primers designed from a published sequence of the *inaW* gene from *P. fluorescens* (17): INA-W3 (5'-GCGGTCTGGTAGGCTATTT-3') and INA-W4 (5'-CCGCGCATCGCTATTGTC-3'). The PCR reaction mixture consisted of PCR buffer (100 mM Tris-HCl, pH 8.3; 1 x 10⁻³ M EDTA, pH 8.0), 1.5 mM MgCl₂, 200 µM each of ATP, CTP, GTP, and TTP, 0.5 µM of each primer, 100 ng of template DNA, and 0.5 units of *Taq* polymerase (Quiagen Inc., CA,
USA) in a 50-μl volume and overlaid with mineral oil. Amplifications were performed in a thermal cycler (Gene Amp PCR system 2400, Perkin Elmer Cetus) programmed for an initial denaturation at 94°C for 4 min, followed by 35 cycles of 1 min at 94°C, 1 min at 50°C, 3 min at 72°C, and a final extension of 10 min at 72°C. Three amplifications were carried out for each sample. For each PCR run, a positive control using pure *P. fluorescens* F264C DNA and a negative control using sterile water were used.

**Culture of gut bacteria**

To recover the ice-nucleating active *P. fluorescens* F264C from treated beetles, gut suspensions were plated on *Pseudomonas* isolation agar (Fisher Scientific, PA) and incubated at 4°C. The ability to grow at 4°C is characteristic of *P. fluorescens* and distinguishes it from other *Pseudomonas* species (4). Some of the bacterial colonies recovered from treated, as well as control beetles were identified with API 20E biochemical tests (BioMerieux, France) and their ice nucleating activity assessed after 4 days of growth on nutrient agar with glycerol.

**RESULTS AND DISCUSSION**

One objective of our field experiment was to study the effect of ice-nucleating active bacteria on cold-hardiness of overwintering adult Colorado potato beetles, however the exceptionally warm winter of 1997 - 1998 did not lower soil temperatures to sufficiently impact beetle survival. At a depth of 10 cm below the soil surface, where most of the overwintering beetles can be found, the lowest recorded temperature was -2.8°C. This temperature was not low enough to initiate freezing in Colorado potato beetles since even beetles fed ice-nucleating active bacteria had supercooling points lower than this value. Although the effect of ice-nucleating active bacteria on field survival of overwintering beetles can not be assessed, laboratory studies on recovered beetles permitted detection of retained ice nucleating bacteria and their effect on the supercooling points of treated beetles.

After a twenty-four hour exposure to *P. fluorescens* F264C, beetle supercooling points were significantly (ANOVA: \( F = 57.98, P < 0.0001 \)) elevated, -4.9 ± 0.6°C (range of -3.4 to -6.3°C, \( n = 20 \)) compared to controls, -8.8 ± 2.2°C (range of -5.8 to -13.0°C, \( n = 20 \)). After seven months, when beetles were recovered from the field, some treated beetles froze at temperatures as high as -3.8°C, whereas control beetles did not start freezing until -5.8°C. Although there was no significant difference in the mean supercooling points of treated versus control beetles, the fact that 18.5% of the treated beetles were already frozen before the first control beetle froze indicates that these beetles retained *P. fluorescens* F264C (Fig. 1). The supercooling points of treated beetles ranged from -3.8 to -7.7°C (Table 1), suggesting that the efficacy of this bacterium and/or its retention by beetles varied.

Long-term retention of ice-nucleating active bacteria in the guts of Colorado potato beetles is affected by the bacterial strain ingested, as well as the host source of the bacterium (2). Laboratory studies by Costanzo et al. (2) showed that Colorado potato beetles retained ice-nucleating active *P. fluorescens* for 10 weeks despite the beetle’s purging of its gut prior to overwintering. Our field study not only confirmed long-term retention of the ice-nucleating active *P. fluorescens* but also showed retention under field conditions for the duration of winter. The persistence of *P. fluorescens* F264C in the gut of Colorado potato beetles may be accounted for by the presence of this species in insect digestive tracts as a
normal flora (1). We also found *Pseudomonas* spp. as normal flora in the gut of these beetles (unpublished data). Although the bacterial strain we used was isolated from a frog, the presence of *P. fluorescens* in the guts of other insects and species of *Pseudomonas* in Colorado potato beetles indicate that its gut is hospitable to this species.

Efficacy of the ice-nucleating active bacteria in elevating the supercooling point of Colorado potato beetles may be affected by variability in ice nucleating activity of individual bacterial cells, even from the same colony. In a given population of bacterial cells of the same strain grown under the same condition, the ability of each cell to serve as an ice nucleator may vary (10). For example, while a few cells catalyze crystal formation at -2.0°C, other cells only induce ice formation at lower temperatures. This variability was evident in the range of supercooling points (-3.4 to -6.3°C) exhibited by beetles that had recently ingested *P. fluorescens*. Because the activity of ice nucleating bacteria can vary, retention studies that rely only on elevated supercooling points can be misleading.

Due to variability observed in the phenotypic expression of ice nucleating activity in *P. fluorescens*, we developed another approach for detecting ice-nucleating active bacteria retained by Colorado potato beetles. Since ice nucleation in *P. fluorescens* is conferred by the *inaW* gene, molecular probes for detecting this gene were designed and used in PCR assays of bacterial cultures recovered from beetle guts. Using the PCR technique we detected presence of the ice nucleating bacteria not only in beetles with elevated supercooling points, but also in beetles with low supercooling points (Table 1). A band of approximately 4.5 Kb, corresponding to the *inaW* gene, was detected in treated beetles exhibiting varied supercooling points (Fig. 2). The low supercooling values in some of the treated beetles were likely due to the retention of few bacteria and/or retention of cells that expressed little or no ice nucleating activity. Complimentary cultural studies (data not

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Supercooling point range (°C)</th>
<th>Presence of putative <em>inaW</em> band*</th>
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<tbody>
<tr>
<td>Fed <em>P. fluorescens</em></td>
<td>-3.8 to -4.8 (n = 8)</td>
<td>6/8</td>
</tr>
<tr>
<td></td>
<td>-5.0 to -6.8 (n = 8)</td>
<td>2/8</td>
</tr>
<tr>
<td></td>
<td>-7.0 to -7.7 (n = 8)</td>
<td>0/8</td>
</tr>
<tr>
<td>Control</td>
<td>-5.8 to -6.9 (n = 10)</td>
<td>0/10</td>
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*Number of beetle gut cultures which were positive for the selected *inaW* gene probe per total number of Colorado potato beetles tested.
Fig. 1. Cumulative freezing profile based on individual supercooling points of Colorado potato beetles after overwintering in the field for 7 months after initial exposure to *Pseudomonas fluorescens* F264C (control, n = 10; fed with *P. fluorescens*, n = 65). Hatched area indicates cumulative percent of treated beetles that froze before the first control beetle.

<table>
<thead>
<tr>
<th>Supercooling point (°C) of CPBs</th>
<th>Fed with <em>P. fluorescens</em></th>
<th>Control</th>
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<tbody>
<tr>
<td>M</td>
<td>-3.8</td>
<td>-6.8</td>
</tr>
<tr>
<td></td>
<td>-4.1</td>
<td>-6.9</td>
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<tr>
<td></td>
<td>-5.8</td>
<td><em>P. fluorescens</em></td>
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<td>-6.3</td>
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Fig. 2. PCR amplifications of *Pseudomonas fluorescens* *inaW* gene in gut cultures from recovered Colorado potato beetles (CPB). Lane 1, molecular weight marker (M); lanes 2 to 5 represent beetles fed with *P. fluorescens* F264C; lanes 6 and 7 represent two unfed (control) beetles; lane 8 represents *P. fluorescens* F264C.
shown) to recover the ice-nucleating active bacteria from treated beetles confirmed the presence of *P. fluorescens* F264C and also showed variable ice nucleating activity in recovered bacterial colonies. Other fluorescent *Pseudomonas* spp., with no ice-nucleating activity, were also recovered from treated as well as control beetles. The presence of other *Pseudomonas* spp. as part of the beetle normal gut flora complicated the estimation of introduced *P. fluorescens* F264C and the correlation of number of ice-nucleating active colonies to beetle supercooling point. For ease in detecting the ice nucleating bacteria in Colorado potato beetle guts, we are exploring the use of green fluorescent protein for tagging *P. fluorescens*.

Results of this study showed that the ice nucleating *P. fluorescens* F264C was retained in overwintering Colorado potato beetles during the course of winter, seven months after initial exposure in early autumn. Retention was evident not only by the detection of the *inaW* gene in gut bacterial cultures, but also in the elevated supercooling points of some of the treated beetles. While further studies are needed to determine factors affecting retention and maintenance of ice nucleating activity of the bacterium in the insect gut, these observations lend further credence to the potential use of ice-nucleating active bacteria as biological control agents for overwintering Colorado potato beetles.

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