RAPID COMMUNICATION

Isolation of Ice Nucleating Active Bacteria From Insects

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ABSTRACT In preparation for winter many insects enhance the supercooling capacity of their
body fluids by 25°C or more, thereby avoiding the lethal effects of tissue freezing. A primary factor
limiting supercooling capacity is the presence of nucleating agents that catalyze ice formation at
high subzero temperatures. Two species of ice nucleating active (INA) bacteria, Enterobacter ag-
glomerans and Enterobacter taylorae, the latter with previously unknown ice nucleating activity,
were isolated from the gut of two species of field-collected beetles, Ceratoma trifurcata and Hip-
podamia convergens. Ingestion of these INA bacteria greatly diminished the capacity of our insect
model, H. convergens, to supercool and caused freezing at temperatures as high as −1.5°C. Removal
or masking of endogenous INA bacteria may be a major factor in the cold-hardening of freeze
intolerant insects for winter survival. Furthermore, these bacteria may provide a novel biological
insecticide to control overwintering pest insects by decreasing their natural capacity to supercool.

Although some overwintering insects are able to survive extensive internal ice formation, most
cannot. Many freeze-intolerant species increase their cold tolerance by the synthesis of anti-
freeze proteins, and/or the accumulation of large amounts of glycerol and other low-molecular-
weight polyols and sugars (Baust and Rojas, '85; Duman and Horwath, '83; Lee, '89; Storey and
Storey, '88; Zachariassen, '85). Of particular importance for these insects is the regulation of the
temperature at which the insect spontaneously freezes, termed the supercooling point. As a
freeze-tolerant insect is cooled below 0°C it does not freeze immediately, but typically supercools
many degrees before ice nucleation occurs. The capacity to supercool is critical for winter survival
of freeze-intolerant insects. Increases in the supercooling capacity are correlated with the evacu-
ation of the gut in a number of species (Somme, '82; Cannon and Block, '88); however, the precise
mechanism(s) regulating supercooling in insects is unknown.

In the 1970s a new category of biological nucle-
ators, ice nucleating active (INA) bacteria, was
identified (Maki et al., '74; Vali et al., '76). These
bacteria are unique in their capacity to catalyze
ice nucleation at temperatures as high as 1 to 2
degrees below 0°C. These common epiphytic bac-
teria not only nucleate water that is in direct con-
tact with them, but they also induce freezing of
the plant tissues on which they reside, resulting
in substantial amounts of frost-related crop losses
throughout the world (Lindow, '83). Although the
significance of INA bacteria is clearly established
with respect to frost injury in plants, their impact
on other organisms under natural conditions is
unknown. In an earlier study, we demonstrated
that ingestion of known INA bacteria, Pseudomo-
nas syringae and Erwinia herbicola, caused an in-
crease of up to 14°C in the temperature at which
the lady beetle, Hippodamia convergens, sponta-
naneously froze (Strong-Gunderson et al., '90).
The purpose of this study was to determine if INA
bacteria are found naturally in the gut of insects
and whether they can decrease the supercooling
capacity of insects.

METHODS

To determine whether INA bacteria are normal
flora in the gut, we collected insects from crop

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fields near Oxford, Ohio, aseptically removed the gut, and cultured its contents aerobically on nutrient agar plates containing 2.5% glycerol at 20°C for 7 days. Morphologically distinct colonies were isolated in pure culture, and screened for ice nucleating activity using the droplet freezing assay of Vali ('71). Bacteria exhibiting the phenotype for ice nucleation were identified biochemically with the API 20E system and independently confirmed by Analytab Products, Inc., Plainview, NY. The nucleation temperature of 10 μl droplets (n = 40) cooled at about 0.3°C/min was determined for each bacterial suspension with a concentration of ca. 2 × 10⁶ bacteria/ml sterile water. Data from a non-ice nucleating active bacterium, Escherichia coli, was included for comparison. The proportion of bacterial cells active as nucleators at varying temperatures was reported as [log(ice nuclei/cell)] using the standard methods of Vali ('71) and Lindow et al. ('82).

To determine whether INA bacteria have the potential to decrease supercooling capacity in vivo, we fed suspensions of the novel INA bacteria to our insect model, the lady beetle, H. convergens. We used overwintering adults that are freeze-intolerant and supercool to −16°C (Lee, '80; Bennett and Lee, '89). Supercooling points of H. convergens were determined by placing a 36 gauge copper-constantan thermocouple on the surface of the beetle. Beetles were cooled at 0.3°C/min in air in a refrigerated bath. The supercooling point, sometimes referred to as the temperature of crystallization, was recorded as the lowest temperature reached prior to freezing. Freezing was verified by detection of the release of the latent heat of crystallization.

RESULTS

During the summers of 1988 and 1989, several bacterial isolates with ice nucleating activity were cultured from the gut of bean leaf beetles, Ceratoma trifurcata, and the lady beetle, H. convergens, immediately after being collected from the field. Two bacteria, Enterobacter taylorae and E. agglomerans, were identified phenotypically to be ice nucleating active. The droplet freezing assay was used to characterize the ice nucleating activity of these two species and to compare their activity with the previously studied INA bacteria, P. syringae, and to the non-ice nucleating active bacterium, E. coli (Fig. 1). Droplets containing suspensions of E. coli supercooled extensively, whereas ice nucleating activity of both Enterobacter species was observed at temperatures as high as −2°C. These data demonstrate that phenotypic expression of ice nucleating activity by the new INA bacteria of insect origin is only slightly less than for P. syringae.

To test whether the INA bacteria of insect origin have the potential to reduce the supercooling capacity we fed suspensions of the bacteria to our insect lady beetle model, H. convergens. Unfed beetles, ones fed sterile distilled water, or the non-ice nucleating active bacterium, E. coli, have supercooling points of approximately −16°C (Table 1). When E. taylorae, E. agglomerans, P. syringae, or E. herbicola was fed to the lady beetles their supercooling points increased from −16°C to as high as −1.5°C with mean values in the range of −2.8 to −4.4°C, thus confirming the ice nucleating activity of the Enterobacter spp. in vivo (Table 1).

DISCUSSION

This is the first report of the isolation of INA bacteria from any animal. Furthermore, ice nucleating activity has not been reported previously in E. taylorae. Enterobacter agglomerans is a member of a large, heterogeneous group of bacteria in the Erwinia herbicola–Enterobacter agglomerans complex. Historically, E. agglomerans is a synonym of E. herbicola in which ice nucleating activity has been well documented. There exists a dichotomy between clinical microbiologists and plant pathologists regarding species designation; E. agglomerans is commonly used to describe clinical isolates, whereas E. herbicola refers to strains isolated from plants (Beji et al., '88; Bren-
TABLE 1. Effect of the ingestion of various bacterial suspensions (ca. $2 \times 10^8$ bacteria/ml distilled water) on the supercooling point of overwintering adult lady beetles (Hippodamia convergens)\(^1\)

<table>
<thead>
<tr>
<th>Feeding treatment</th>
<th>Supercooling point (°C) Mean (x) ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td></td>
</tr>
<tr>
<td>Unfed</td>
<td>-16.0</td>
</tr>
<tr>
<td>Water</td>
<td>-16.5</td>
</tr>
<tr>
<td><em>Escherichia coli</em> (non-ice nucleating active bacterium)</td>
<td>-17.1</td>
</tr>
<tr>
<td>Ice nucleating bacteria</td>
<td></td>
</tr>
<tr>
<td><em>Enterobacter taylorae</em></td>
<td>4.3 ± 0.4</td>
</tr>
<tr>
<td><em>Enterobacter agglomerans</em></td>
<td>-3.1 ± 0.1</td>
</tr>
<tr>
<td><em>Pseudomonas syringae</em>(^2)</td>
<td>-2.8 ± 0.2</td>
</tr>
<tr>
<td><em>Erisinus herbicola</em>(^2)</td>
<td>-4.4 ± 0.6</td>
</tr>
</tbody>
</table>

\(^1\)Each point is the mean ± SEM, n = 7–10 individuals.
\(^2\)Groups were compared with ANOVA followed by Scheffe's test. Treatment groups with different letters are significantly different (P < 0.001).
\(^3\)From Strong-Gunderson et al. ('90).

ner et al., '84). Since our isolates are of animal origin we have chosen to use *E. agglomerans*. However, genotypic studies are necessary ultimately to resolve the taxonomic position of our isolates.

Both *E. taylorae* and *E. agglomerans* are members of the family Enterobacteriaceae, which includes many gram-negative, facultative anaerobic rods commonly found in insects (Cruden and Markovetz, '87; Mead et al., '88). The species *E. taylorae* is differentiated phenotypically from *E. agglomerans* based upon biochemical tests to detect the production of lysine decarboxylase, arginine dihydrolase, and ornithine decarboxylase, commonly referred to as the “LAO” reactions. *E. taylorae* isolates are LAO - + +, whereas *E. agglomerans* are LAO - - -. Additionally, *E. taylorae* isolates on nutrient agar do not produce a yellow pigment at either 25 or 37°C after 48 hr, and are negative for fermentation of inositol, d-sorbitol, raffinose, and melibiose; in contrast, *E. agglomerans* are variable for each of these tests (Lennette et al., '85; Farmer et al., '85). Although the *E. herbicola-E. agglomerans* complex is frequently found in the insect microflora, ice nucleating activity in bacteria of insect origin has not been reported previously. Since we have tested only a few species of insects for the presence of INA bacteria, it seems likely that additional insects will be found with these bacteria and, possibly, additional species of INA bacteria will be identified.

In a previous study using *P. syringae* and *E. herbicola* we demonstrated a significant positive correlation between the concentration of INA bacteria ingested and the degree of supercooling point elevation (Strong-Gunderson et al., '90). In that study the ingestion of relatively dilute concentrations (10^5 bacteria/ml) significantly elevated the supercooling point of some beetles. Furthermore, some beetles fed *P. syringae* had an elevated supercooling point for at least 7 days after removal of the bacterial suspension from which they were drinking.

This study provides the first evidence that INA bacteria are normal flora in the insect gut and that the INA bacteria isolated from the insect gut have the capacity, at least under laboratory conditions, to decrease the supercooling capacity of an overwintering insect. These data suggest that INA bacteria may play a role in the natural regulation of supercooling capacity in insects. Future investigations must examine the significance of INA bacteria from an ecological perspective in order to evaluate potential interactions between INA bacteria, plants, and insects. It is possible that naturally occurring strains of INA bacteria may be used ultimately as biological insecticides for the control of insect pests during the winter. These bacteria would be expected to increase overwintering mortality by decreasing the effectiveness of natural mechanisms of supercooling.

A control measure that acts during the winter is a particularly attractive option since it would avoid the problem of crop contamination. Furthermore, this approach would be compatible with other control measures in an integrated pest management program.
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LITERATURE CITED