Pergamon

0022-1910(95)00004-6

J. Insect Physiol. Vol. 41, No. 7, pp. 603–608, 1995

0022-1910/95 $9.50 + 0.00

Effect of Biological Ice Nucleators on Insect Supercooling Capacity Varies with Anatomic Site of Application

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Received 6 October 1994; revised 16 November 1994

Topical application of ice nucleating active (INA) bacteria or fungi decreases the cold tolerance of freeze-intolerant insects by raising their supercooling points (SCPs). However, the route by which INA agents come in contact with insect body water is unknown. To determine their effect on the SCP, we topically applied a suspension of INA *Pseudomonas syringae* to four anatomic sites of the freeze-intolerant lady beetle, *Hippodamia convergens*. Aqueous suspensions of either cultured or lyophilized, ultraviolet irradiated (UVI) *P. syringae* produced significantly higher mean SCPs than control treatments when applied to the thoracic spiracle of the insect, −7.7 and −5.6°C, respectively, compared with the control treatment's mean SCP of −14.9°C. Application of an aqueous suspension of UVI *P. syringae* to three other anatomic sites on the beetle produced less dramatic and more varied increases in mean SCP. Application of the INA fungus *Fusarium avenaceum* to the thoracic spiracle significantly elevated the mean SCP to approx. −10°C. Application of powdered UVI *P. syringae* to the thoracic spiracle resulted in a SCP increase from −14.9 to −4.6°C, the most dramatic increase in this study. These results indicate that the efficacy of INA microorganisms in elevating the SCP varies with the microorganism and its site of application.

Supercooling capacity *Pseudomonas syringae* *Fusarium avenaceum* Ice nucleating active *Hippodamia convergens*

INTRODUCTION

Spontaneous ice formation in biological systems is promoted in part by the presence of ice nucleating agents, which act by ordering water molecules in an ice-like array. Once the resulting crystal reaches a critical size, it functions as a seed nucleus. Under ideal conditions, homogeneous ice nucleation does not occur above temperatures as low as −40°C (Bigg, 1953). Inorganic, organic or biological heterogeneous ice nucleators can raise this temperature of crystallization to as high as −1°C (Maki *et al.*, 1974; Lindow *et al.*, 1976; Schnell, 1976).

Ice nucleating active (INA) microorganisms, a category of biological nucleators first discovered among the bacteria in the early 1970s, and recently among the fungi, are defined as those microbes that are able to catalyze ice nucleation at temperatures warmer than −10°C (Maki *et al.*, 1974; Kieft, 1988; Pouleur *et al.*, 1992). The source of ice nucleation activity in INA bacteria is a protein with a highly repetitive amino acid sequence (Green and Warren, 1985; Warren *et al.*, 1986). The structures of INA proteins, based on molecular modeling, are largely planar molecules. One side of the molecule serves as a template for orienting water molecules into an ice lattice whereas the other side of the protein interacts with the outer bacterial membrane (Kajava and Lindow, 1993). These proteins are capable of forming aggregates within the outer membrane, an event associated with an increase in their ice nucleating activity (Govindarajan and Lindow, 1988; Mueller *et al.*, 1990). The common epiphytic bacterium *Pseudomonas syringae* containing INA proteins on its outer membrane nucleates water that is in direct contact with it, and can also induce the freezing of plant tissue (Lindow, 1983). Furthermore, both INA bacteria (Strong-Gunderson *et al.*, 1989; Fields, 1991) and INA fungi (Lee *et al.*, 1992; Fields, 1991, 1993; Fields *et al.*, 1993) initiate the freezing of insect tissues at high subzero temperatures.

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Many insects, including the convergent lady beetle *Hippodamia convergens*, are termed "freeze-intolerant" because they cannot survive extensive ice formation in their extracellular body fluid. *H. convergens* survives winter temperatures as low as −16°C by supercooling, meaning that their body fluids remain liquid at temperatures below their melting point. To enhance their supercooling capacity, freeze-intolerant insects must not only avoid inoculation by external ice, but also eliminate efficient internal ice nucleating agents. The supercooling capacity of insects is also influenced by antifreeze proteins (Duman and Horwath, 1983, Knight and Duman, 1986), glycerol and other low molecular weight polyols and sugars (Bale *et al.*, 1989; Salt, 1961), inoculative freezing by external ice (Salt, 1961) and ice nucleating proteins and lipoproteins (Duman *et al.*, 1985; Neven *et al.*, 1989; Zachariassen and Hammel, 1976).

Either ingestion (Strong-Gunderson *et al.*, 1990) or topical application (Strong-Gunderson *et al.*, 1992) of the INA bacterium *P. syringae* can raise the supercooling point (SCP) of the insect model *H. convergens* from −16 to as high as −2°C. INA bacteria have been isolated from the gut of field collected insects (Lee *et al.*, 1991; Strong-Gunderson *et al.*, 1990), suggesting that biological ice nucleators may influence insect cold-hardiness in nature. Recent studies have shown that the fungus *Fusarium avenaceum* also promotes ice formation in aqueous suspensions at temperatures as high as −2.5°C (Pouleur *et al.*, 1992), and, when topically applied to insects, reduces their supercooling capacity (Fields *et al.*, 1993; Lee *et al.*, 1992, 1994). Potentially these microorganisms may be used for the biological control of insect pests via increasing winter mortality by decreasing supercooling capacity of freeze-intolerant insects (see Lee *et al.*, 1993 for review).

In a previous study in which the mouth of the insect was sealed to prevent ingestion of the INA bacteria, topical application of these bacteria caused a significant elevation of the SCP, although the route of contact between the ice nucleating agent and insect body water was not determined. The beetle *H. convergens* was chosen as an insect model in this study because, as cold-hardy adults, they consistently maintain low SCPs of approx. −16°C. We hypothesized that spiracles may be an efficient route of contact between ice nucleating agents and insect body water because in nature, spiracles serve as a direct conduit between the external environment and internal body water. The two-fold objective of this study was to examine the effect of anatomic site of an ice nucleating agent's application on insect supercooling capacity and to examine the effects of several biological ice nucleating microorganisms on insect supercooling capacity.

MATERIALS AND METHODS

Sources and maintenance of bacteria and insects

The cultured INA bacterial strain *P. syringae* cit 7 was provided by S. E. Lindow (University of California, Berkeley), *Escherichia coli* (ATCC 35421) was obtained from American Type Culture Collection, Rockville, MD. Bacterial strains were maintained aerobically at 20°C, or 37°C on Bacto Nutrient Agar (Difco) with 2.5% glycerol (NAG). A commercial preparation of lyophilized, ultraviolet irradiated (UVI) *P. syringae*, was provided by Genencor International, Rochester, NY, and stored at −20°C. The fungus *F. avenaceum* was obtained from American Type Culture Collection, and maintained at 22°C on potato flakes agar (Rinaldi, 1982) with 2.5% glycerol. Our studies used the vegetative sporulating mycelium of *F. avenaceum*. Lady beetles, *H. convergens*, were obtained commercially (Fountain's Sierra Bug Co., Rough and Ready, CA) and held unfed at 4°C, in the dark until used. Cultured *P. syringae* was suspended at a density of 10^{10} cells/ml in sterile water from a 3 day old culture grown at 20°C, whereas UVI *P. syringae* was suspended at a density of either 1000 or 20,000 ppm in sterile water. Seven day old *F. avenaceum* was suspended at a density of 0.3 mg dry weight *F. avenaceum* per ml of a 1% solution of Tween-80 (Sigma Chemical Co., Detroit, MI) in sterile water, or used as scrapings from fungal mats. The bacterium *E. coli* was suspended in sterile water at a density of 10^{10} cells/ml from a 3 day old culture, and used as a non-INA control.

Activity measurements

The INA of control *E. coli*, cultured *P. syringae*, UVI *P. syringae*, and *F. avenaceum* were assayed by a standard drop-freezing test (Vali, 1971; Lindow *et al.*, 1978). For a suspension containing the preparation to be tested, a set of 40 droplets, each containing 10 μl of the control or test suspension was equilibrated to 0°C, then cooled at a rate of 0.6°C/min until all of the drops had frozen. T_{max} defined the temperature at which the first drop froze. The temperature by which 50% of the set of 40 drops froze (T_{50}) was determined from a cumulative freezing histogram, and taken as a measure of the INA of the suspension tested.

Insect treatment

All control and test suspensions were prepared in sterile water unless stated otherwise. *H. convergens* received a 0.5 μl drop of either sterile water, 10^{10} *E. coli*/ml, 10^{10} cultured *P. syringae*/ml, a suspension containing 20,000 ppm UVI *P. syringae*, or 0.3 mg *F. avenaceum*/ml on one of four anatomic sites: the left thoracic spiracle, the left second abdominal spiracle, the midline on the ventral abdominal surface, and the ventral midline of the union of the head and thorax (ventral midline of the cervix). The beetles were restrained using modeling clay and the drop was applied onto the insect using a micropipeter. Because the application of an agent to the thoracic spiracle of *H. convergens* required removal of a wing, sham-operated controls were run using water, *E. coli*, and no additive.

After allowing 15 min for the inoculum to dry, each beetle was transferred to a 0.5 ml polypropylene

microcentrifuge tube and the SCP was measured by placing a 36-gauge copper–constantan thermocouple in direct contact with the dorsal side of the insect. These tubes were inserted into glass tubes suspended in a refrigerated bath, equilibrated to 0°C, and cooled at approx. 0.6°C/min. The cooling profile was recorded, and the SCP taken as the lowest temperature reached prior to the release of the latent heat of fusion.

Recovery of P. syringae from the thoracic spiracle

After application of cultured *P. syringae* to the left thoracic spiracle, and determination of the SCP of the insect, both the left and right thoracic spiracles were removed. The external portion of spiracles from three beetles were used as inoculum, smears were cultured aerobically at 20°C on NAG. INA was determined for bacteria which grew under these conditions, and the bacteria were identified using an API 20E system for identification of Enterobacteriaceae and other gram-negative bacteria (Analytab Products, Plainview, NY).

Statistical analysis

Mean SCP values for different treatment groups were compared, using log transformed data, analysis of variance (ANOVA) and Tukey–Kramer multiple comparisons test.

RESULTS

Activity of INA agents and control agent

An aqueous suspension of UVI *P. syringae* had the greatest INA compared with all other nucleating agents tested (Table 1), with a T_{50} of -1.8°C. Cultured *P. syringae* had a T_{50} of -4.6°C, and *F. avenaceum* had a T_{50} of -3.2°C. All three microorganisms were considered strong ice nucleators because they nucleated ice formation in sterile water at temperatures > -5°C. *E. coli* was non-INA, as its T_{max} was < -10°C; its T_{50} was -15.1°C.

Controls

The mean SCPs ± SEM of four groups of beetles used in control experiments (untreated, water placed on the abdomen, *E. coli* applied to the ventral mid-cervix, and *E. coli* applied to the left thoracic spiracle) were -16.3 ± 0.2, -15.7 ± 0.5, -15.3 ± 0.3, and -14.9 ± 0.5°C, respectively, and did not differ significantly ($F = 4.9$, $df = 81$, $P > 0.05$). The group in which *E. coli* was applied to the left thoracic spiracle served as the control in subsequent comparisons. It was the most stringent control of the four tested as it illustrated that mean SCP was not influenced by either wing removal or the presence of a non-INA bacterium as a potential source for heterogeneous ice nucleation.

Comparisons among anatomic sites

We chose UVI *P. syringae* (20,000 ppm) as the test treatment for comparison of four anatomic sites as routes of contact between an INA agent and insect body water, as this ice nucleating agent had the highest T_{50} of all agents tested (Table 1). Application of 20,000 ppm UVI *P. syringae* to the thoracic spiracle raised the mean SCP ($F = 33.1$, $df = 119$, $P < 0.001$) from -14.9 ± 0.5 to -5.6 ± 0.4°C, whereas application of UVI *P. syringae* to the second abdominal spiracle raised the mean SCP ($F = 33.1$, $df = 119$, $P < 0.01$) to -11.1 ± 0.8°C (Table 2). Application of UVI *P. syringae* to the ventral cervix resulted in the higher mean SCP value of -8.1 ± 0.8°C, compared to control ($F = 33.1$, $df = 119$, $P < 0.001$) (Table 2).

The distribution pattern of individual SCPs obtained after application of UVI *P. syringae* to the thoracic spiracle was different than those obtained after treatments to either the abdominal spiracle, or the ventral cervix (Fig. 1). Both the SEM and range resulting from application of UVI *P. syringae* to the thoracic spiracle were smaller than after application of the same agent to these other two anatomic sites (Table 2, Fig. 1).

Application of UVI *P. syringae* to the ventral abdominal surface of *H. convergens* resulted in the mean SCP of -13.6 ± 0.8°C, similar to control mean SCP ($F = 33.1$, $df = 119$, $P > 0.05$) (Fig. 1). Again, SEM and range of SCPs were larger than those obtained from application of UVI *P. syringae* to the thoracic spiracle (Table 2).

TABLE 1. Ice nucleating agents and controls used, and their INA activity defined as T_{50} at concentrations corresponding to equivalent concentrations used for insect treatments

Treatment*	Source	Concentration†	T_{50} (°C)
Non-INA control agent			
E. coli	ATCC #35421 (Rockville, MD)	5×10^8 cells/ml	−15.1
INA agents			
P. syringae, cultured	S.E. Lindow (Univ. Calif., Berkeley, CA)	5×10^8 cells/ml	−4.6
P. syringae, UVI	Genencor International (Rochester, NY)	1000 ppm	−1.8
F. avenaceum	S. Pouleur (Univ. Laval, Quebec, Canada)	0.17 mg/ml	−3.2

*All suspensions were aqueous, with *F. avenaceum* in aqueous suspension containing 1% surfactant Tween-80.

†Concentration used for activity measurement corresponded to equivalent concentrations used during treatments, i.e. same number of ice nuclei per 0.5 μl drop applied to insect as contained in 10.0 μl drop used for measurement of T_{50}.

TABLE 2. Supercooling point (SCP) analysis after application of an aqueous suspension of either *E. coli*, or UVI *P. syringae* to four anatomic sites on *H. convergens* ($n = 24$ per site). Mean data calculated using one-way analysis of variance (ANOVA) ($F = 33.1$, $df = 119$, $P < 0.001$)

Agent	Anatomic site	SCP ± SEM (°C)	Range (°C)
Non-INA control, *E. coli*	Thoracic spiracle	−14.9 ± 0.5	−10.0 to −18.0[a]
UVI *P. syringae*	Ventral abdomen	−13.6 ± 0.8	−5.1 to −19.9[a,b]
	Abdominal spiracle	−11.1 ± 0.8	−3.8 to −16.9[b]
	Ventral cervix	−8.1 ± 0.8	−2.9 to −17.0[c]
	Thoracic spiracle	−5.6 ± 0.4	−3.9 to −9.3[d]

Mean values identified by different letters are statistically distinguishable ($P < 0.05$). ANOVA and Tukey–Kramer multiple comparisons test performed on log transformed data.

Effect of INA agents applied to the thoracic spiracle

Following application of either an INA bacterium, *P. syringae*, or an INA fungus, *F. avenaceum*, to the thoracic spiracle of *H. convergens*, the mean SCPs of beetles in all treatment groups were statistically higher than values for the non-INA bacterium, *E. coli*, control group (Table 3). Application of powdered UVI *P. syringae* to the thoracic spiracle of *H. convergens* raised the mean SCP by more than 10°C, relative to the control treatment. Application of the other agents elevated the mean SCP from 4.5 to 9.3°C above control levels. Generally, UVI *P. syringae* treatments resulted in comparable SCP effects, although application of cultured *P. syringae* was less effective in raising the mean SCP, compared to UVI *P. syringae* in powdered form (Table 3). This result is consistent with the greater INA of UVI *P. syringae* reported in Table 1. The mean SCPs after treatment with scrapings of the mycelial mat or an aqueous solution of *F. avenaceum* were similar to each other, but statistically lower than those of any *P. syringae* treatment (Table 3).

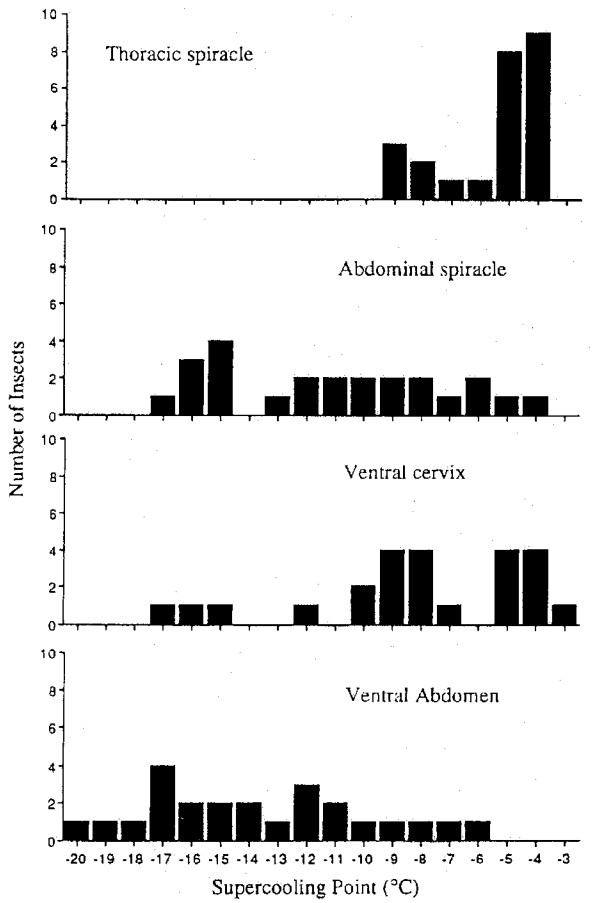

FIGURE 1. Distribution of supercooling points (SCPs) of freeze intolerant beetles, *H. convergens*. Inoculum volume was 0.5 μl of 20,000 ppm UVI *P. syringae*. Site of inoculation listed with corresponding graph.

Recovery of P. syringae from the thoracic spiracle

After application of viable *P. syringae* to the left thoracic spiracle, and determination of the higher SCP of the insect, both the left and right thoracic spiracles were removed from three beetles. The external portion of the spiracles from these lady beetles were cultured aerobically at 25°C on Bacto Nutrient Agar containing 2.5% glycerol (NAG). No detectable bacterial growth was noted on plates inoculated with the untreated, control right thoracic spiracles. In contrast, from each of the left spiracles, morphologically similar colonies of INA bacteria with T_{50}s between −4.3 and −4.5°C were isolated. This INA bacterium was identified using an API 20E system as *P. syringae*.

DISCUSSION

This study explored several possible routes of contact between insect body water and biological ice nucleating microorganisms. It also compared the effects of several ice nucleating agents on insect SCPs when topically applied to an efficient anatomic site for inoculative freezing. Inoculative freezing initiates ice formation in insect body water, hence contact must exist between the growing ice crystal and insect body water. This study identified anatomic sites that readily promoted internal ice nucleation, and other sites which were less efficient. An efficient route of contact between an ice nucleating agent and insect tissue can also be thought of as a vulnerable site on the insect for the potential use of INA microorganisms for biological control.

In this study, the thoracic spiracle provided the least protection against contact of the aqueous ice nucleating agent with insect body water. Application of UVI *P. syringae* in aqueous suspension to the left thoracic spiracle

TABLE 3. Mean supercooling point (SCP ± SEM) of beetles after application of control or INA bacteria or fungi to the thoracic spiracle of *H. convergens*

Treatment	*n*	SCP ± SEM (°C)
Control non-INA agent		
E. coli (10^{10} cells/ml, aqueous)	24	-14.9 ± 0.5^{a}
INA agents		
P. syringae		
UVI (powder)	22	-4.6 ± 0.3^{b}
UVI (1000 ppm aqueous suspension)	18	$-5.9 \pm 0.7^{b,c}$
UVI (20,000 ppm aqueous suspension)	24	$-5.6 \pm 0.4^{b,c}$
Cultured (10^{10} cells/ml, aqueous)	23	-7.7 ± 0.9^{c}
F. avenaceum		
Mycelial mat	12	-10.4 ± 0.7^{d}
Aqueous solution with 1% Tween-80 (0.3 mg/ml)	24	-10.0 ± 0.7^{d}

Mean values identified by different letters are statistically distinguishable ($P < 0.05$). ANOVA and Tukey–Kramer multiple comparisons test performed on log transformed data.

produced the highest mean SCP and smallest SEM of the four sites tested. Spiracles are openings of the insect tracheal system to the outside of the body, for the exchange of respiratory gases. Since the inner surface of the tracheal system is moist and the distal ends of the tracheoles are fluid filled, the spiracles may provide an efficient route for ice nucleating agents to contact body water. Finally since in many insects the thoracic spiracle is the incurrent spiracle for unidirectional flow through the tracheal system while the abdominal spiracles are primarily excurrent, this could account, at least in part, for the observed results.

The abdominal spiracles and the cervical site were less efficient routes of contact between the ice nucleating agent and insect body water, compared to the thoracic spiracle. Even though mean SCPs of these treatment groups were significantly higher than the control mean SCP, these test groups had a greater SEM than the thoracic spiracle treatment group. Also, SCPs for beetles in each of these two treatment groups had a range of more than 13°, compared to a range of <6° for beetles treated with an aqueous suspension of UVI *P. syringae* applied to the thoracic spiracle. Abdominal spiracles, since their diameters are less than half that of thoracic spiracles, may therefore provide less effective conduits between insect tissue and the environment, compared to thoracic spiracles. The ventral cervix is not covered by a hard, sclerotized cuticle. This thin, flexible portion of the integument provided a relatively weak barrier to the action of the ice nucleating agent. The highly sclerotized ventral abdominal surface provided the greatest barrier between INA agent and insect body water compared with the other sites. The mean SCP for this test group was not significantly different from control values. Although the waxy layer of the cuticle has ribbonlike pore canals (Neville *et al.*, 1969), our results indicate that this cuticle was an effective barrier against external ice inoculation nucleated by the aqueous UVI *P. syringae*.

The inherent variability in the INA of individual bacterial cells may account for some of the observed variability in insect SCPs. Within a colony of *P. syringae* cells vary considerably in the temperature at which they can catalyze ice nucleation (Maki *et al.*, 1974). For example, on average only one cell in 10,000 is capable of nucleating ice formation at temperatures as high as −2°C (Maki *et al.*, 1974). Therefore, a decrease in either the concentration of the inoculum, or its volume should result in a decreased chance of contact between a single *P. syringae* capable of ice nucleation at high temperatures and insect body water. Additionally, a single strain of an INA bacterial species contains cell-associated freezing sites active at −2 to −4°C (Group I), at −5 to −7°C (Group II), and at −8 to −10°C (Group III) (Yankofsky *et al.*, 1981). Group I ice nuclei are rare. The same volume of 20,000 ppm UVI *P. syringae* was applied to all anatomic sites, but the smaller size of the abdominal spiracle may have allowed a smaller inoculum volume contacting insect body water via the tracheal system compared to the larger thoracic spiracle. The surface tension of an aqueous suspension may prevent entry into the smaller tracheal tube bordered by the abdominal spiracle.

Following application of several ice nucleating agents individually to the thoracic spiracle, beetles which received the powdered form of UVI *P. syringae* had a mean SCP of −4.6°C that was higher than that of beetles which received an aqueous suspension of the same agent (Table 3). One of the main functions of a spiracle is to regulate gas exchange into and out of the tracheal system; another function of a spiracle is to protect parts of the tracheal system from being filled with fluid. Despite the role of the spiracle to prevent entry of water into the tracheal system, inoculative freezing still occurs after application of an aqueous suspension of UVI *P. syringae* to the thoracic spiracle, but consistent with the spiracle's functions, inoculative freezing initiated by powdered UVI *P. syringae* is more pronounced. Application of either the mycelial mat of, or an aqueous suspension containing the INA fungus *F. avenaceum* produced mean SCPs higher than control values, but not as high as treatment with either cultured or UVI *P. syringae*. These data, consistent with previous findings (Lee *et al.*, 1992), do not correlate with the T_{50} of *F. avenaceum*. *F. avenaceum* is a filamentous fungus which may not adhere to the surface

of the insect or may not be able to enter the insect's tracheal system through the spiracles as efficiently as the relatively small INA bacteria.

We have demonstrated that *P. syringae* was present at the application site throughout treatment by recovering it after SCP determinations. The tracheal system is a moist environment so inoculation of this insect body water by an ice front nucleated by an INA agent is possible. The membrane surrounding the spiracle is extremely flexible and has many folds and depressions. This increase of surface area near the spiracle could provide harbor for the applied bacteria, as INA bacteria are known to adhere to the surface of an insect after topical application (Strong-Gunderson *et al.*, 1992).

This study provides information on several possible routes by which INA microorganisms may come in contact with insect body water. Obviously the efficacy of INA microorganisms on elevating the SCP may be influenced by species that have efficient atrial valves for closing the spiracular opening or whose spiracles are covered by the elytra. Nonetheless these data suggest that the supercooling capacity of insects may be diminished by routes other than through the mouth and support the continued investigation of these unusual microorganisms as a potential means of biological control.

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Acknowledgements—We thank Jon Costanzo and the anonymous reviewers for critically reviewing an earlier draft of this manuscript. We thank S.E. Lindow for providing us with *P. syringae* cit 7. This study was supported in part by Cooperative State Research Service, USDA grant #93-37302-9003, NSF IBN-9305809 and Genencor International Inc.