

## Enhancement of Ice-Nucleating Activity in *Pseudomonas fluorescens* and Its Effect on Efficacy against Overwintering Colorado Potato Beetles

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The application of ice-nucleating bacteria as biological control agents for Colorado potato beetles requires the development of media for mass production that optimize bacterial efficacy against overwintering adults. The production method in our laboratory has been limited to a solid medium, nutrient agar supplemented with 2.5% glycerol (NAG), that is known to enhance ice-nucleating activity. In this study, we report culture conditions that enhance expression of type 1 ice nuclei (i.e., initiate freezing at or above  $-5^{\circ}\text{C}$ ) in the bacterium *Pseudomonas fluorescens* F26-4C grown in liquid media. Expression of type 1 ice nuclei was induced during the exponential to the stationary phase of its growth cycle by a low-temperature shift to either 15 or  $4^{\circ}\text{C}$  for at least 1 h. Growth in media with a limiting level of nitrogen and with 5% dextrose or galactose, coupled with a temperature shift to  $4^{\circ}\text{C}$  for 2 h, resulted in the greatest enhancement of type 1 nuclei. Adult Colorado potato beetles fed *P. fluorescens* cells induced to produce type 1 ice nuclei showed a sustained elevation of supercooling points (i.e., the temperature at which ice formation spontaneously occurs in body fluids) comparable to beetles fed cells grown on NAG. These results suggest that *P. fluorescens* F26-4C produced in liquid media is as persistent in and as effective against overwintering adults as that grown on a solid medium. Furthermore, the ability to culture in liquid media may facilitate the mass production of bacterial cells needed for field applications. © 2001 Academic Press

**Key Words:** *Leptinotarsa decemlineata*; *Pseudomonas fluorescens*; Colorado potato beetle; ice-nucleating bacteria; freeze-intolerant insects; biological control.

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### INTRODUCTION

Some members of the gram-negative bacterial genera *Erwinia*, *Pseudomonas*, and *Xanthomonas* can catalyze ice crystal formation in aqueous solutions at temperatures above  $-10^{\circ}\text{C}$  (Maki *et al.*, 1974; Lindow *et al.*, 1978; Kim *et al.*, 1987). Certain strains can nucleate ice at temperatures as high as  $-2^{\circ}\text{C}$ , making these bacteria among the most potent biological ice nucleators in nature (Lindow, 1990). Ice-nucleating activity is conferred by the presence of *ina* genes (also called *ice* genes), which code for ice-nucleating proteins localized on the bacterium's outer membrane (Lindow *et al.*, 1989; Warren, 1995). These proteins have a 16-amino-acid repeat sequence and function as templates for the formation of small ice crystal seeds termed "ice nuclei" (Wolber and Warren, 1989; Turner *et al.*, 1991).

Although most of the reported ice-nucleating bacteria are epiphytic in nature (Gurian-Sherman and Lindow, 1993), some strains have been isolated from the gut of frogs (Lee *et al.*, 1995) and insects (Lee *et al.*, 1991; Takahashi *et al.*, 1995; Olsen and Duman, 1997). In insects they elevate the temperature at which ice formation spontaneously occurs in body fluids, termed the supercooling point (SCP). Since most freeze-intolerant insects (i.e., incapable of surviving internal ice formation) depress their SCP in winter, the presence of these ice-nucleating bacteria in their gut generally reduces their cold hardiness and increases the likelihood of mortality at subzero temperatures.

The potential use of these microorganisms as biological control agents against freeze-intolerant insects has been investigated (Lee *et al.*, 1992, 1994; Fields, 1993; Fields *et al.*, 1995; Watanabe *et al.*, 2000). One likely target is the Colorado potato beetle, *Leptinotarsa decemlineata* (Say) (Coleoptera: Chrysomelidae), a major arthropod pest of solanaceous crops (for review, see Lee *et al.*, 1998). Diapausing adults minimize their exposure to lethally low temperatures, and, thus, promote their survival during the coldest part of winter, by

burrowing into the ground (Lashomb *et al.*, 1984; Boiteau and Coleman, 1996). Overwintering survival is also enhanced by the adult's capacity to depress its SCP (Lee *et al.*, 1994). When adults are exposed to ice-nucleating bacteria, either by ingestion (Costanzo *et al.*, 1998) or by topical application (Lee *et al.*, 1994), their supercooling capacity (i.e., ability to remain unfrozen below the equilibrium freezing point of their body fluids) is markedly reduced. Accordingly, we propose to decrease the cold tolerance of overwintering adults by feeding them ice-nucleating bacteria which would reduce their survival after exposure to subzero soil temperatures (Lee *et al.*, 1998).

To date we have identified strains of *Pseudomonas fluorescens* Migula capable of significantly elevating beetle SCPs and persisting in the gut of overwintering adults (Costanzo *et al.*, 1998; Castrillo *et al.*, 2000a). One strain, *P. fluorescens* F26-4C, was efficacious in reducing beetle cold tolerance in the field throughout winter (Castrillo *et al.*, 2000b). As we pursue the use of these bacteria as biological control agents for field application, it is necessary to develop production media that optimize expression of bacterial ice-nucleating activity.

Our previous studies indicated that the degree of SCP elevation in treated beetles is affected not only by the number of bacterial cells retained in the beetle gut but also by the variability in ice-nucleating activity of individual bacterial cells (Castrillo *et al.*, 2000a,b). In a culture of ice-nucleating bacteria, only a small fraction of the population expresses nucleating activity at any one time; moreover, the highest temperature at which each active cell nucleates ice differs (Lindow *et al.*, 1982). The ice-nucleation frequency, the ratio between the number of ice nuclei and the number of bacterial cells in a culture, varies with bacterial growth conditions (i.e., incubation temperature, composition of growth medium, and culture age) and can be elevated by various postculture treatments (Lindow *et al.*, 1982; Pooley and Brown, 1991; Nemecek-Marshall *et al.*, 1993; Fall and Fall, 1998). Thus, by optimizing culture conditions that induce ice nuclei expression, one can maximize the proportion of cells that are active at higher temperatures.

Ice nuclei activity has been classified by the range of temperature in which they initiate freezing: type 1 ice nuclei are active between  $-2^{\circ}\text{C}$  and  $-5^{\circ}\text{C}$ , type 2 are active between  $-5^{\circ}\text{C}$  and  $-7^{\circ}\text{C}$ , and type 3 are active between  $-7^{\circ}\text{C}$  and  $-10^{\circ}\text{C}$  (Yankofsky *et al.*, 1981). Bacterial cells with type 1 nuclei could be more effective against Colorado potato beetles because they would elevate the beetles' SCPs to higher values and, thus, increase their susceptibility to freezing in winter.

The objective of this study was to improve efficacy of *P. fluorescens* F26-4C by the maximization of ice-nucleating activity by use of a combination of culture conditions that enhances expression of type 1 ice nu-

clei. Experiments were conducted to determine the level of activity during the bacterial growth cycle, the effects of induction time and temperature, and the effects of different nutrients on type 1 expression. Bacterial cultures with type 1 ice nuclei were also tested against adult Colorado potato beetle for persistence and efficacy in elevating beetle SCP.

## MATERIALS AND METHODS

### *Bacterial Strain and Culture Conditions*

Experiments were conducted using ice-nucleation active *P. fluorescens* strain F26-4C, isolated from the wood frog, *Rana sylvatica* LeConte (Lee *et al.*, 1995). This strain has shown efficacy and persistence in the field and is a potential biological control agent for overwintering adults of Colorado potato beetles (Castrillo *et al.*, 2000a,b). This ice-nucleating bacterium was routinely cultured on nutrient agar with 2.5% glycerol (NAG) for 4 days at  $22^{\circ}\text{C}$  (Lindow *et al.*, 1982).

To determine expression of ice-nucleating activity during the different growth phases, the bacterium was grown in nutrient broth with 2.5% glycerol and in a defined medium, L broth, containing 50 mM 3-[*N*-morpholino]propanesulfonic acid buffer (pH 7.2), 25 mM KCl, 20 mM  $\text{NH}_4\text{Cl}$ , 10 mM  $\text{Na}_2\text{SO}_4$ , 10 mM NaCl, 1 mM  $\text{MgCl}_2$ , 1 mM  $\text{KH}_2\text{PO}_4$ , 0.1 mM  $\text{CaCl}_2$ , 10  $\mu\text{M}$   $\text{FeCl}_3$ , and 0.4% (wt/vol) sorbitol as a carbon source (Nemecek-Marshall *et al.*, 1993). Liquid cultures were shaken at 150 rpm and incubated at room temperature ( $\sim 23^{\circ}\text{C}$ ). Two 1-ml aliquots were taken from 50-ml cultures at regular intervals to determine bacterial cell counts and ice-nucleating activity before and after induction of ice nuclei expression.

### *Measurement of Ice-Nucleation Activity*

Ice-nucleation activity was measured by use of the freeze-drop method (Vali, 1971; Lindow, 1990). Serial dilutions of bacterial cells in sterile I buffer (20 mM  $\text{KPO}_4$ , 10 mM  $\text{mgSO}_4$ , pH 7.4) were prepared and 30  $10\text{-}\mu\text{l}$  drops of the bacterial suspension placed on an aluminum boat in a circulating ethanol cold bath set to  $-10$  or  $-5^{\circ}\text{C}$ . Type 1 ice nuclei were determined by the presence of frozen droplets at  $-5^{\circ}\text{C}$  after 5 min. Cumulative type 2 and 3 ice nuclei were measured at  $-10^{\circ}\text{C}$ . Bacterial cell concentrations were determined by measurement of optical density at 600 nm and confirmed by the plating of serial dilutions of bacterial cells on NAG incubated at  $22^{\circ}\text{C}$  for 2 days. Ice-nucleation activity was expressed as ice nuclei per milliliter per  $\text{OD}_{600}$  in experiments in which activity during different growth phases was determined and as ice nuclei in  $10^9$  bacterial cells in experiments in which cultures were grown to the stationary phase.

### *Effect of Induction Time and Temperature on Expression of Type 1 Ice Nuclei*

Expression of type 1 ice nuclei was induced by a low-temperature shift from ~23 to 15 or 4°C for 1 to 24 h. Bacterial cultures were grown first in 50 ml of growth medium at ~23°C to early stationary phase. Then 15-ml aliquots were transferred to separate flasks to be incubated at 15 or at 4°C. After 1, 2, 4, 6, or 24 h of temperature shift, 1-ml aliquots were collected, bacterial cells were resuspended in I buffer, and freeze-drop assays were conducted. There were three replicates per temperature setting.

### *Effect of Different Carbon Sources and Nutrient Starvation on Expression of Type 1 Ice Nuclei*

Components of L broth were modified to determine the effect of different carbon sources (dextrose, galactose, glycerol, inositol, mannitol, sorbitol, or sucrose) and carbon levels (0.4, 1, 2.5, or 5% wt/vol, except glycerol which was calculated vol/vol) on ice nuclei expression. Nutrient starvation was also tested by a 10-fold decrease in the level of a given nutrient (carbon, nitrogen, phosphorus, or sulfur) or combination of nutrients. Starvation media were designated with lower-case letter(s) to indicate limiting nutrient(s) (i.e., Ln or L broth with limiting nitrogen, for medium with reduced NH<sub>4</sub>Cl). For each experiment, inoculum was first grown in L broth with 0.4% sorbitol to the stationary phase. Bacterial cells were then harvested, washed, resuspended in sterile deionized water, and used to inoculate 50-ml of different liquid media. For each medium tested, cells were grown to the stationary phase before measurement of ice nuclei expression. Ice nuclei were assayed in cultures kept at ~23°C and in 25-ml aliquots shifted to 4°C for induction. All experiments were replicated three times.

### *Effect of Culture Conditions on the Persistence and Efficacy of the Bacterium against Beetles*

*P. fluorescens* F26-4C grown under different culture conditions and induced to produce type 1 ice nuclei was tested for its persistence in and efficacy against adult Colorado potato beetles. These beetles were collected from recently harvested potato fields by use of potato tuber baits at the Hancock Agricultural Station in central Wisconsin in early August 1999. Following Costanzo *et al.* (1998), efficacy was tested by comparison of the SCPs of beetles fed ice-nucleating bacteria versus untreated controls, and persistence was determined by sampling at two times after feeding: 1.5 h and 2 weeks postingestion. A 10- $\mu$ l drop of bacterial suspension (10<sup>8</sup> bacteria/ml) was applied to a small potato cube (~5 mm<sup>3</sup>) inside an individual well in a 24-well tissue culture plate, where a single beetle,

starved for 24 h, was introduced. Control beetles were fed potato slices moistened with 10  $\mu$ l of sterile deionized water. There were 15 beetles per replicate, with three replicates per treatment. Beetles to be assayed 1.5 h after feeding were kept in the tissue culture plate until measurement of their SCP. Only 10 beetles per replicate were assayed for their SCP. Beetles to be sampled after 2 weeks were transferred to deli cups with moist sand and held at 15°C with 10:14 (light: dark) h to induce diapause (Costanzo *et al.*, 1998). Each deli cup held 15 beetles, representing one replicate. After 2 weeks, 10 beetles were recovered from each replicate and assayed for their SCP. A 2-week sampling time after feeding was chosen because ice-nucleating strains persisting for 2 weeks are likely to persist in adult Colorado potato beetles throughout winter (L. A. Castrillo *et al.*, unpublished data). Supercooling points of adult beetles were measured as reported in Castrillo *et al.* (2000a).

### *Statistical Analyses*

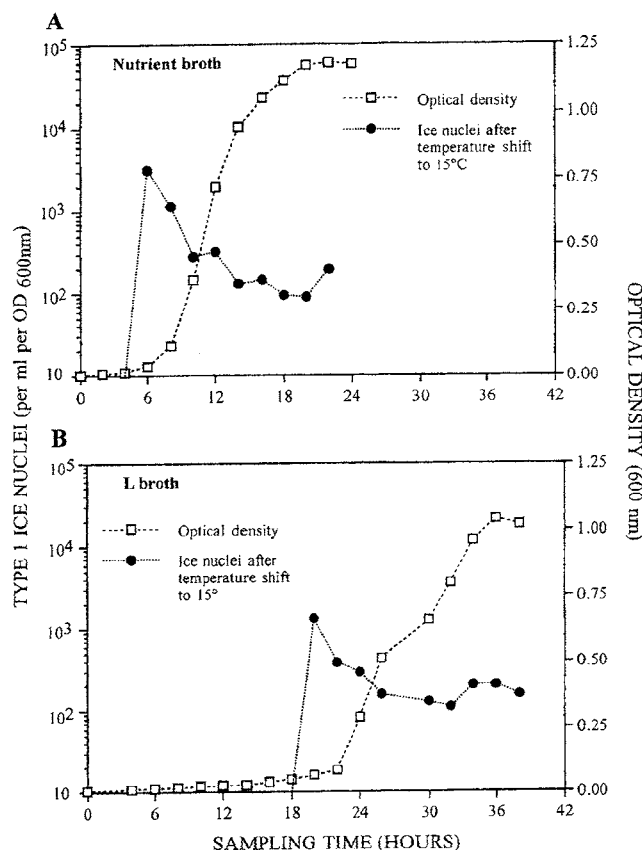
Differences in type 1 ice nuclei expression among various culture conditions tested were analyzed by use of analysis of variance (ANOVA). For experiments on the effects of induction time and temperature, a two-way ANOVA was used with time and temperature as factors. For cultures in nutrient broth, type 1 ice nuclei at ~23, 15, and 4°C were compared; whereas in L broth only temperatures 15 and 4°C were compared because no type 1 ice nuclei were present at ~23°C. For experiments on the effects of different carbon sources in the culture medium, a two-way ANOVA was conducted with carbon source and carbon levels as factors. Beetle SCPs were analyzed by use of the same method with treatment and sampling time as factors. Means were separated at 5% level of significance by Fisher protected LSD test (SAS Institute, 1999).

## RESULTS AND DISCUSSION

### *Expression of Ice-Nucleating Activity*

*P. fluorescens* F26-4C grew faster in nutrient broth, a complex medium consisting of beef extract and peptone, compared to L broth, a defined medium. The stationary phase of the growth cycle was reached in 20 h in nutrient broth, compared to 36 h in L broth (Fig. 1).

The ice-nucleating frequency of *P. fluorescens* F26-4C varied during its growth cycle in the two liquid media. Bacterial cells in nutrient broth at 23°C showed type 1 ice nuclei only during the stationary phase (data not shown). In contrast, cells in L broth did not exhibit type 1 ice nuclei during growth at ~23°C. Both type 2 and type 3 ice nuclei were present in both media from



**FIG. 1.** Expression of type 1 ice nuclei in *Pseudomonas fluorescens* F26-4C after a temperature shift from 23 to 15°C for 2 h during its growth phase. Bacteria were grown in (A) nutrient broth or in (B) L broth at room temperature (~23°C). Aliquots were obtained every 2 h to measure cell density (OD<sub>600</sub>) and the number of ice nuclei present. At each sampling time, an aliquot was also incubated at 15°C for 2 h to induce expression of type 1 ice nuclei. Type 1 ice nuclei were not observed in L broth at 23°C and are not shown for simplicity. These data represent results of typical experiments from three separate experiments per growth medium.

the early exponential into the stationary phase (data not shown).

Shifting of the growing cells in either nutrient broth or L broth from 23 to 15°C induced the expression of type 1 ice nuclei during the early exponential phase and continued into the stationary phase (Fig. 1). After only 2 h of incubation at 15°C, the level of type 1 ice nuclei increased to 10<sup>2</sup>–10<sup>3</sup>/ml/OD<sub>600</sub> or approximately 1 per 10<sup>6</sup> to 10<sup>7</sup> cells. In either nutrient or L broth, bacterial cells in the early exponential phase had a 10-fold higher level of type 1 nuclei than bacterial cells in the mid-exponential to the stationary phase (Fig. 1). In all ensuing experiments, cells were grown to the stationary phase to minimize the effect of growth stage on induction of type 1 ice nuclei using different culture conditions.

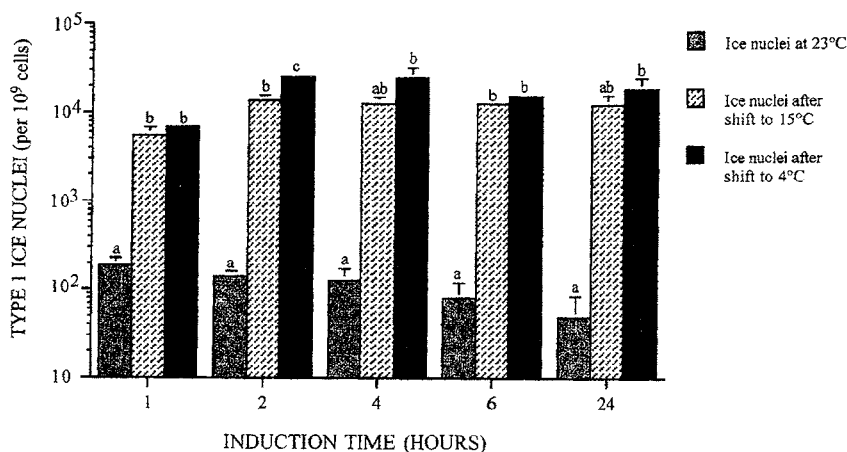
#### Effect of Induction Time and Temperature on Expression of Type 1 Ice Nuclei

In nutrient broth, the level of type 1 ice nuclei was significantly affected by induction temperature ( $F = 46.6$ ;  $df = 2, 30$ ;  $P < 0.001$ ), by length of induction ( $F = 4.21$ ;  $df = 4, 30$ ;  $P = 0.008$ ), but not by interaction between temperature and time ( $F = 1.76$ ;  $df = 8, 30$ ;  $P = 0.12$ ). After 1 h of incubation at either 4 or 15°C, a marked increase in type 1 ice nuclei was observed in cells at the stationary phase in nutrient broth; ice nuclei levels increased more than 10-fold from values observed at 23°C (Fig. 2). After another hour of incubation, the level increased to almost 100-fold and to 180-fold for induction at 15°C and at 4°C, respectively (Fig. 2). No further increases were observed with longer incubation.

Bacterial cells in L broth, which had no type 1 ice nuclei at 23°C, also showed induction at either 4 or 15°C (data not shown). The level of type 1 activity induced in this medium was much less than that observed in nutrient broth. Significant differences were observed in the induction of type 1 nuclei in L broth due to length of induction ( $F = 16.1$ ;  $df = 4, 20$ ;  $P < 0.001$ ). No significant differences were observed between induction at 4 or 15°C ( $F = 0.34$ ;  $df = 1, 20$ ;  $P = 0.58$ ) nor in the interaction between temperature and time ( $F = 2.93$ ;  $df = 4, 20$ ;  $P = 0.07$ ). At 4°C comparable values were observed from 1 to 6 h of induction, but after 24 h the level of type 1 nuclei declined by half (data not shown). At 15°C the level of type 1 nuclei was highest after 2 h and started to decline after 4 h (data not shown). For all subsequent induction of type 1 ice nuclei, a low temperature shift to 4°C for 2 h was adopted. Even though the level of type 1 ice nuclei in cells grown in L broth was lower than that in nutrient broth, we used L broth for further studies because its components can be individually manipulated to test the effects of different nutrient sources and nutrient levels on ice nuclei induction.

#### Effect of Growth Media on Expression of Type 1 Ice Nuclei

Bacterial cells in any of the L media with a limiting level of various nutrients had type 2 and type 3, but not type 1 ice nuclei at ~23°C (data not shown). When transferred to 4°C for 2 h, type 1 ice nuclei appeared, and the number of ice nuclei differed significantly among nutrient treatments ( $F = 4.12$ ;  $df = 8, 9$ ;  $P = 0.02$ ). Only the reduction of nitrogen and the combined reduction of nitrogen and phosphorus resulted in higher levels of type 1 ice nuclei, 72 and 54%, respectively, compared to values observed in regular L broth (Fig. 3). The 10-fold reduction of other nutrients or combination of nutrients did not result in any significant difference in the expression of type 1 ice nuclei (Fig. 3).



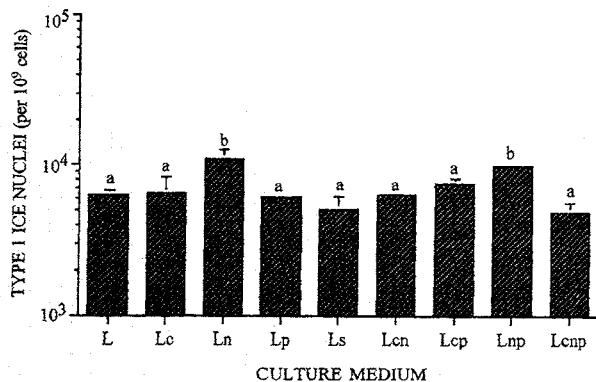
**FIG. 2.** Effect of induction time and temperature on expression of type 1 ice nuclei in *Pseudomonas fluorescens* F26-4C grown in nutrient broth. For each replicate, bacteria were grown in a single flask to the early stationary phase at room temperature (~23°C) and then transferred to three separate flasks held at ~23°C or transferred to 4 or 15°C before measurement of type 1 ice nuclei. Bars (mean ± SE) with the same letter for a given sampling time are not significantly different at  $\alpha = 0.05$  (Fisher protected LSD test).

We then tested whether different carbon sources could significantly increase the level of inducible type 1 ice nuclei. Sorbitol in the regular L broth was replaced with the same amount (0.4%) of dextrose, galactose, glycerol, inositol, mannose, or sucrose. Except for sucrose, all carbon sources tested resulted in growth of *P. fluorescens* at ~23°C. Expression of type 1 ice nuclei cells at the stationary phase was observed only after induction at 4°C. Comparison of type 1 nuclei expression in L broth with various carbon sources (0.4% of growth medium) showed differences among the various carbons tested ( $F = 3.91$ ;  $df = 5, 12$ ;  $P = 0.02$ ). Only

dextrose and galactose, compared to sorbitol, showed higher levels of ice nuclei, 59 and 64%, respectively (data not shown). The other tested carbon sources resulted in values comparable to (glycerol and inositol) or lower than (mannose) those in sorbitol.

Because we routinely use NAG for maintaining our cultures of ice-nucleating bacteria, we wanted to determine whether higher carbon levels in L broth would result in higher levels of inducible type 1 ice nuclei. For this study we used dextrose, galactose, glycerol, and sorbitol and increased carbon levels in the media from 0.4 to 1, 2.5, or 5%. The results showed significant differences in the level of type 1 ice nuclei due to carbon source ( $F = 2.91$ ;  $df = 3, 32$ ;  $P = 0.05$ ) and carbon levels ( $F = 5.6$ ;  $df = 3, 32$ ;  $P = 0.003$ ), but not due to the interaction between carbon source and level ( $F = 1.77$ ;  $df = 9, 32$ ;  $P = 0.11$ ). Greater expression of type 1 ice nuclei were observed in cultures with 5% galactose ( $F = 18.8$ ;  $df = 3, 8$ ;  $P < 0.001$ ) (Fig. 4). Nearly a fourfold increase in expression of type 1 ice nuclei was induced by the increase of galactose level from 0.4 to 5%. No significant differences between use of 5 and 0.4% dextrose were observed ( $F = 1.85$ ;  $df = 3, 8$ ;  $P = 0.21$ ). Increase in levels of glycerol ( $F = 0.04$ ;  $df = 3, 8$ ;  $P = 0.98$ ) or sorbitol ( $F = 0.60$ ;  $df = 3, 8$ ;  $P = 0.63$ ) also did not change the level of type 1 ice nuclei induction (Fig. 4).

The level of expression of type 1 ice nuclei in *P. fluorescens* F26-4C cells grown in liquid medium with limiting nutrients and transferred to lower temperature appeared low (approximately one ice nucleus per  $10^5$  cells) compared to reported values approaching one active nucleus in 10 cells to one in every cell in the population (Nemecek-Marshall *et al.*, 1993; Fall and Fall, 1998). However, as was also shown in those stud-



**FIG. 3.** Effect of nutrient starvation on type 1 ice nuclei expression in *Pseudomonas fluorescens* F26-4C. Bacteria were grown in L broth with limiting nutrient or combination of limiting nutrients designated as follows: Lc, carbon; Ln, nitrogen; Lp, phosphorus; Ls, sulfur; Lcn, carbon and nitrogen; Lcp, carbon and phosphorus; Lnp, nitrogen and phosphorus; Lcnp, carbon, nitrogen, and phosphorus. Bacterial cultures were grown to the early stationary phase at room temperature (~23°C) before being transferred to 4°C for 2 h. Bars (mean ± SE) with the same letter are not significantly different at  $\alpha = 0.05$  (Fisher protected LSD test).

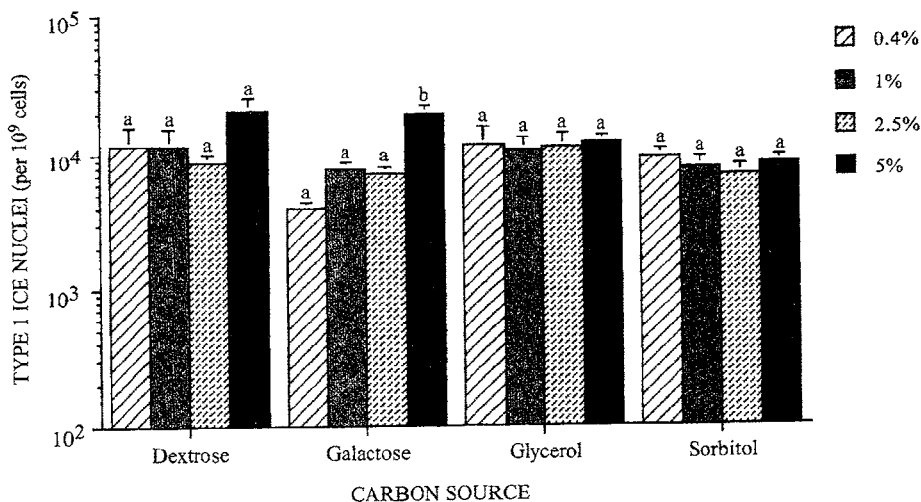


FIG. 4. Effect of different carbon sources and levels on expression of type 1 ice nuclei in *Pseudomonas fluorescens* F26-4C. Bacteria were grown in L broth with dextrose, galactose, glycerol, or sorbitol as carbon source. Carbon level was increased from 0.4 to 1, 2.5, and 5%. Bacterial cells were grown to early stationary phase at room temperature (~23°C) before being shifted to 4°C for 2 h prior to ice nuclei assay at -5°C. Bars (mean ± SE) with the same letter for a given carbon source are not significantly different at  $\alpha = 0.05$  (Fisher protected LSD test).

ies, there is great variability in the range of maximum levels of inducible type 1 ice nuclei among different strains under a given set of conditions. For example, the conditions optimal for expression in *Pseudomonas syringae* T1 did not elicit the same level of expression in other *P. syringae* strains (Nemecek-Marshall *et al.*, 1993). Moreover, because the conditions that we used were based on studies that used epiphytic bacterial strains, culture conditions may not have been optimal for our gut-derived *P. fluorescens* strain.

It has been suggested that the low nutrient levels reflect conditions on the leaf surfaces where epiphytic bacteria are found. This environment plus the shift to low temperature may trigger ice nucleation as a survival mechanism, composed of initial freezing damage in susceptible plants and the resulting release of plant nutrients to epiphytic bacteria (Buttner and Amy, 1989; Nemecek-Marshall *et al.*, 1993). How conditions that induce activity in epiphytic strains relate to the biology of *P. fluorescens* F26-4C and other nonepiphytic ice-nucleating bacteria is not clear. Induction of type 1 ice nuclei in *P. fluorescens* F26-4C during the exponential stage, however, suggests that nutritional deprivation may not be as critical for induction in this species as in some epiphytic species.

Ice-nucleating bacteria are normal constituents of the microflora in a variety of insects and other freeze-tolerant animals (Lee, 1991; Lee *et al.*, 1991, 1995). It has been proposed that these microorganisms enhance the survival of freeze-tolerant organisms in winter by triggering freezing at relatively high subzero temperatures, a strategy that decreases the chance of osmotic shock and intracellular freezing (Lee, 1991). Thus,

these animal-derived microorganisms naturally occur in conditions markedly different from those found on leaf surfaces. Their growth and activity inside the insect depend on nutrient levels and physiological conditions in the gut and on the environmental temperatures.

#### *Effect of Culture Conditions on the Persistence and Efficacy of the Bacterium against Beetles*

The beetle SCP was significantly affected by treatment ( $F = 41.7$ ;  $df = 4, 20$ ;  $P < 0.001$ ), but not by sampling time ( $F = 2.86$ ;  $df = 1, 20$ ;  $P = 0.11$ ) nor the interaction between treatment and sampling time ( $F = 0.86$ ;  $df = 4, 20$ ;  $P = 0.51$ ) (Table 1). *P. fluorescens* cells grown in liquid media transferred to 4°C expressed as much activity as cells grown on NAG at ~23°C (data not shown). Beetles fed bacterial cells grown in nutrient broth, in L broth with limited nitrogen and 5% dextrose, and on NAG had elevated SCPs compared to control beetles (Table 1).

As the beetles were slowly cooled, approximately 50% of all treated beetles had frozen by the time they reached -4°C (Fig. 5). In contrast, untreated adults required a temperature of -7°C to freeze the same proportion of beetles (Fig. 5). These data indicated that cold tolerance of Colorado potato beetles was markedly reduced by all of the bacterial treatments.

Even after 2 weeks, significant differences in SCPs in treated and control beetles were maintained (Table 1), indicating that culture in liquid media did not affect efficacy or persistence of the bacterium in the beetle gut. The ranges of SCPs for individual beetles fed *P.*

TABLE 1

Efficacy and Persistence of Ice-Nucleating *Pseudomonas fluorescens* F26-4C with Induced Type 1 Ice Nuclei against Overwintering Colorado Potato Beetles

Bacterial culture conditions			Beetle supercooling point (°C) <sup>a</sup>	
Medium	Phase	Induction <sup>b</sup>	1.5 h	2 weeks
Nutrient agar with 2.5% glycerol	Solid	No	-4.2 ± 0.2a	-4.5 ± 0.3a
Nutrient agar with 2.5% glycerol	Solid	Yes	-3.8 ± 0.3a	-4.6 ± 0.6a
Nutrient broth with 2.5% glycerol	Liquid	Yes	-4.1 ± 0.3a	-4.3 ± 0.1a
L broth with limiting N and P and with 5% dextrose	Liquid	Yes	-4.3 ± 0.1a	-4.1 ± 0.3a
Control			-6.9 ± 0.9b	-7.5 ± 0.3b

<sup>a</sup> Mean values ± SE ( $N = 30$ ) within a column followed by the same letter are not significantly different at  $\alpha = 0.05$  (Fisher protected LSD test).

<sup>b</sup> Type 1 ice nuclei expression in *P. fluorescens* F26-4C was induced at 4°C for 2 h.

*fluorescens* grown on NAG at ~23°C and on NAG transferred to 4°C were -3.1 to -7.5°C ( $N = 30$ ) and -3.0 to -6.8°C ( $N = 30$ ), respectively. In beetles that were fed cells from liquid media, the ranges of individual SCPs were -3.4 to -6.0°C ( $N = 30$ ) and -3.1 to -5.4°C ( $N = 30$ ) for NB and Lnp with 5% dextrose, respectively. The range of individual SCPs in control beetles was -4.2 to -9.0°C ( $N = 30$ ).

Results of this study suggest that the shift to low temperature may not be necessary prior to field application of ice-nucleating bacteria. The low temperature experienced by overwintering beetles may likely promote ice-nucleating activity. By midwinter, when soil temperatures drop low enough to initiate freezing in treated beetles, *P. fluorescens* would be growing under conditions prevailing in the beetle gut. Elevated beetle SCPs maintained up to 2 weeks, and projected to persist throughout winter (L. A. Castrillo *et al.*, unpub-

lished data), indicated that gut conditions permit growth and expression of ice-nucleating activity. Therefore, the low temperature shift required for enhancement of type 1 activity in *P. fluorescens* will be provided naturally as the soil temperatures decrease in winter.

Our study has shown that *P. fluorescens* F26-4C grown in liquid media is as persistent and as effective in elevating beetle SCPs as cultures grown on solid NAG. This would facilitate the development of a mass production system for *P. fluorescens* for field applications. Furthermore, our results indicate that the conditions in the gut of overwintering beetles are conducive to expression of bacterial ice-nucleating activity. The stimulus provided by decreasing soil temperatures in winter will not only enhance activity but will also increase the likelihood of mortality in beetles fed this ice-nucleating bacterium.

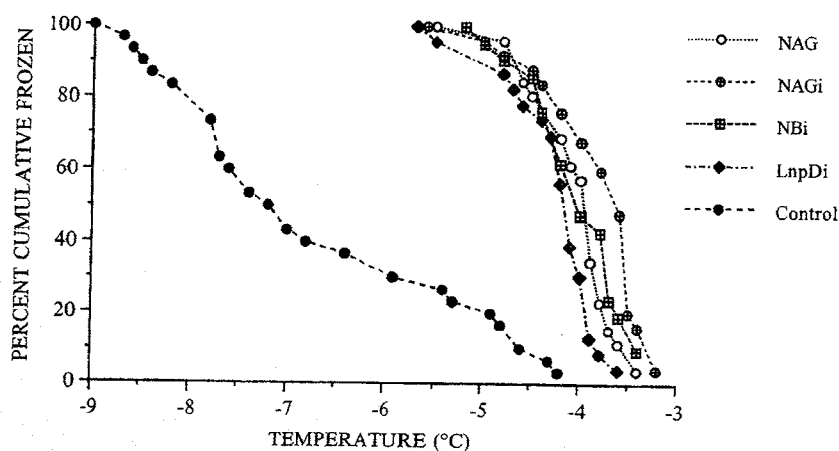


FIG. 5. Cumulative supercooling points for individual Colorado potato beetles ( $N = 30$ , with 10 beetles sampled/replicate/treatment) 1.5 h after being fed *Pseudomonas fluorescens* F26-4C. Bacteria were grown on different media and induced to express type 1 ice nuclei by transfer to 4°C, except for one treatment (NAG). Bacterial cells cultured on solid media (NAG and NAGi) were grown for 4 days; those cultured in liquid media (NBi, nutrient broth; LnpDi, L broth with limiting levels of nitrogen and phosphorus and with 5% dextrose) were grown to the early stationary phase at ~23°C.

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