Identification of Ice-Nucleating Active *Pseudomonas fluorescens* Strains for Biological Control of Overwintering Colorado Potato Beetles (Coleoptera: Chrysomelidae)

LOUELA A. CASTRILLO,[1] RICHARD E. LEE, JR.,[1] MARCIA R. LEE,[2] AND STEVEN T. RUTHERFORD[2]

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ABSTRACT Laboratory studies were conducted to identify ice-nucleating active bacterial strains able to elevate the supercooling point, the temperature at which freezing is initiated in body fluids, of Colorado potato beetles, *Leptinotarsa decemlineata* (Say), and to persist in their gut. Adult beetles fed ice-nucleating active strains of *Pseudomonas fluorescens*, *P. putida*, or *P. syringae* at 10^6 or 10^3 bacterial cells per beetle had significantly elevated supercooling points, from –4.5 to –5.7°C and from –5.2 to –6.6°C, respectively, immediately after ingestion. In contrast, mean supercooling point of untreated control beetles was –9.2°C. When sampled at 2 and 12 wk after ingestion, only beetles fed *P. fluorescens* F26-4C and 88–335 still had significantly elevated supercooling points, indicating that these strains of bacteria were retained. Furthermore, beetle supercooling points were comparable to those observed immediately after ingestion, suggesting that beetle gut conditions were favorable not only for colonization but also for expression of ice-nucleating activity by these two strains. The results obtained from exposure to a single, low dose of either bacterial strain also show that a minimum amount of inoculum is sufficient for establishment of the bacterium in the gut. Persistence of these bacteria in Colorado potato beetles long after ingestion was also confirmed using a polymerase chain reaction technique that detected ice-nucleating active bacteria by virtue of their *ina* genes. Application of these ice-nucleating active bacteria to elevate the supercooling point of this freeze-intolerant insect pest could significantly reduce their winter survival, thereby reducing local populations and, consequently, crop damage.

KEY WORDS *Leptinotarsa decemlineata*, biological control, cold-hardiness, ice-nucleating active bacteria, overwintering insect pests

PEST MANAGEMENT STRATEGIES for the Colorado potato beetle, *Leptinotarsa decemlineata* (Say), the major arthropod pest of potatoes, have focused primarily on adult and larval populations during the crop-growing season. In the northern potato growing areas, this season lasts about 5 mo, with beetles infesting potatoes in the spring and dispersing in autumn (Wyman et al. 1994). During the rest of the year, adults are found in habitats surrounding the cultivated potato field. In autumn, after vine senescence and crop harvest, adult Colorado potato beetles disperse and locate protected sites where they burrow into the ground for winter diapause. This burrowing behavior minimizes exposure to extreme temperatures and promotes survival during the coldest part of winter (Lashomb et al. 1984, Boiteau and Coleman 1996). Survival is also enhanced by the adult's capacity to supercool (e.g., remain unfrozen below the equilibrium freezing point of their body fluids) (Lee et al. 1994). Because their cold-hardiness is an important factor determining winter survival, it provides an avenue for the development of pest control strategies targeting the overwintering populations (Lee et al. 1998).

The cold-hardiness of adult Colorado potato beetles can be reduced by exposure to ice-nucleating active bacteria, either by ingestion or topical application (Lee et al. 1994, Lee et al. 1996, Costanzo et al. 1998). These bacteria are efficient ice nucleators and can be used to raise the beetle's supercooling point, the temperature at which ice crystal formation is initiated in their body fluids. Because Colorado potato beetles are intolerant of internal freezing and their supercooling points represent their lower lethal temperature, application of ice-nucleating bacteria has the potential to significantly reduce the survival of overwintering beetles. These observations are the basis of a novel strategy using ice-nucleating active bacteria to reduce the winter survival of freeze-intolerant Colorado potato beetles and other insect pests (Lee et al. 1998).

The development of ice-nucleating bacteria as a biological control agent for overwintering Colorado potato beetles, however, requires not only their ability to elevate the beetle's supercooling point, but also their persistence in the digestive tract of these beetles during the winter season. This is necessary because Colorado potato beetles must be exposed to ice-nucleating active bacteria in late summer to early au-

[1] Department of Zoology, Miami University, Oxford, OH 45056.
[2] Department of Microbiology, Miami University, Oxford, OH 45056.

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Table 1. Species, strain, host source, and activity of ice-nucleating active *Pseudomonas* spp. assayed for biocontrol of overwintering Colorado potato beetles

Species	Strain	Host	T_{max}, °C
P. fluorescens	F26-4C[a]	*Rana sylvatica* (wood frog)	−2.6 ± 0.1
	88-335	*Cerotoma trifurcata* (Coleoptera: Chrysomelidae)	−2.8 ± 0.2
P. putida	Hr6-1	*Cerotoma trifurcata*	−2.0 ± 0.0
P. syringae	Hr6-3B	*Cerotoma trifurcata*	−2.0 ± 0.1
	cit 7[b]	Epiphyte	−3.0 ± 0.0

T_{max} is the highest temperature at which freezing occurred based on exotherm produced by 10 μl of aqueous bacterial suspension (10^8 bacterial cells/ml), with 5 replicates per bacterial strain.

[a] (Lee et al. 1995).

[b] Provided courtesy of S. E. Lindow (University of California, Berkeley).

tumn, before burrowing commences. The ingested ice-nucleating bacteria must then persist in the beetle's digestive tract into winter, until soil temperature decreases sufficiently to initiate freezing of its body fluids. Recent field (Castrillo et al. 1999) and laboratory (Costanzo et al. 1998) studies showed that a few ice-nucleating active bacterial strains not only elevate the supercooling point of these beetles but also persist in their gut through winter. However, variability observed in the number of beetles retaining the ice-nucleating active bacteria and in the activity of the bacteria necessitates that these factors be addressed specifically.

The ability of ice-nucleating active bacteria to catalyze ice crystal formation is based on the presence of *ina* genes (also called *ice* genes) that code for ice-nucleating proteins located on the bacteria's outer membrane (Warren 1995). These proteins may aggregate and serve as templates promoting ice crystal formation (Warren and Corotto 1989). The potency of ice-nucleating active bacteria varies with bacterial species and strain, genotype, expression of the *ina* gene, and the type of ice nuclei they produce. Ice nuclei are classified by the range of temperatures in which they initiate ice crystal formation. A type 1 nucleus catalyzes freezing at temperatures between −2 and −5°C; type 2 between −5 and −7°C; and type 3 between −7 and −10°C (Turner et al. 1990). An ideal ice-nucleating active bacterial strain for use in biological control of Colorado potato beetles would be a potent catalyst for type one nuclei, colonize and persist in the beetle digestive tract, and maintain its ice-nucleating activity for extended periods. To identify ice-nucleating bacterial strains useful for development as biological control agents, this study was conducted comparing the activity and retention of different strains in adult Colorado potato beetles under simulated overwintering conditions.

Materials and Methods

Bacterial Strains and Ice-Nucleating Activity. Ice-nucleating active bacterial strains capable of initiating freezing of aqueous solutions at temperatures greater than or equal to −4.0°C were chosen for this study (Table 1). Bacterial strains used were frog-derived *Pseudomonas fluorescens* F26-4C (Lee et al. 1995); insect-derived *P. fluorescens* 88–335, *P. putida* Hr6–1, and *P. syringae* Hr6–3B (M.R.L., unpublished data); and epiphytic *P. syringae* cit 7 (provided courtesy of S. E. Lindow, University of California, Berkeley). Bacteria were identified using API 20E biochemical tests (BioMerieux Vitek, Hazelwood, MO) and confirmed by Analytab Products, Plainview, NY. Bacterial strains were cultured on nutrient agar with 2.5% glycerol for 4 d at 22°C, a regimen known to enhance expression of ice-nucleating proteins (Lindow et al. 1982). Aqueous suspensions were prepared and bacterial cell concentrations were determined using a hemocytometer and by plating serial dilutions on nutrient agar plates with 2.5% glycerol and incubating at 22°C for 2 d.

Ice-nucleating activity was determined by placing 10 μl aqueous suspension of 10^8 bacterial cells per milliliter in a 20-μl capillary tube to which a 30-gauge copper-constantan thermocouple was attached. The capillary tube was placed inside a glass test tube that was plugged with plastic 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) that recorded the temperature of the capillary tube at 30-s intervals. The highest temperature at which freezing of the sample occurred was indicated by an exotherm (i.e., release of latent heat of crystallization) and was recorded as T_{max}. Ice-nucleating activity was determined from five replicates per bacterial strain.

Test Insects. Experiments were conducted using field collected adult Colorado potato beetles that were conditioned to enter diapause for simulated overwintering in the laboratory (Costanzo et al. 1998). Beetles were collected from recently harvested potato fields using potato tuber baits at Hancock Agricultural Station in central Wisconsin in early August 1998. Beetles were then shipped overnight to Miami University, where groups of 200 beetles were transferred to ventilated plastic shoe boxes containing 400 g of autoclaved sand moistened with 40 ml of sterile deionized water. Beetles were fed slices of potato tubers and were placed in an incubator at 15°C with a photoperiod of 12:12 (L:D) h.

Persistence Studies. To study the persistence of ice-nucleating active bacteria in the gut of adult Colorado potato beetles, each bacterial strain was intro-

duced to beetles via feeding. Their presence in the beetle digestive tract was assayed three times: immediately after a 22-h feeding period, 2 wk after ingestion, and 12 wk after ingestion.

The desired bacterial concentration was applied as a 10-μl drop on a small potato cube (≈5 by 5 mm) inside an individual well in a 24-well tissue culture plate. Because feeding is normally concentrated on the edges and surface of potato slices (Costanzo et al. 1998), bacteria were applied as evenly as possible, coating all exposed surfaces of the potato cube. For controls, a 10-μl drop of sterile distilled water was applied. A single beetle, starved for 24 h at ≈23°C and a photoperiod of 12:12 (L:D) h before the test, was introduced into each well and allowed to feed for 22 h. There were 20 beetles per replicate with three replicates per bacterial dose for each sampling time; however, only 10 beetles from each replicate were sampled for their supercooling points. Beetles to be assayed immediately after feeding were kept in the tissue culture plate until measurement of their supercooling points. Beetles to be sampled after 2 and 12 wk were transferred to small deli cups with ≈70 g of moist sand (10 ml of deionized water per 100 g of autoclaved sand). Each deli cup held 20 beetles representative of one replicate. Deli cups were kept in a 15°C incubator with a photoperiod of 10:14 (L:D) h to induce diapause (Costanzo et al. 1998). After the 2-wk incubation, most of the beetles had burrowed into the sand. From each replicate, 10 beetles were retrieved by gentle sieving and were assayed for their supercooling points. Beetles to be sampled 10 wk later were transferred to a cold room (4°C) and covered with a black cloth to simulate winter conditions.

During the long holding period, deli cups were examined regularly to monitor sand moisture. Moisture was added, as needed, by misting the surface of the sand without disturbing the beetles. Dead beetles found on the surface were removed to prevent possible cross infection between those that died of fungal infection and the live beetles.

The effect of dose on the persistence of ice-nucleating active bacteria in Colorado potato beetle digestive tract was assessed using two concentrations of each bacterial strain (10^6 and 10^3 bacterial cells in 10 μl of sterile distilled water). Presence of ice-nucleating bacteria in the gut was detected by measuring the supercooling points of treated beetles versus control beetles, by culture of their digestive tract contents on selective media, and by polymerase chain reaction (PCR) technique.

Measurement of Beetle Supercooling Capacity. Beetle supercooling points were determined as previously described above, except the thermocouple was in contact with the beetle inside a 1.5-ml polyethylene tube (Lee et al. 1994). Data were analyzed using analysis of variance PROC (ANOVA, SAS Institute 1996) to determine effects of bacterial strain, bacterial dose, sampling time, and interactions between these factors on the supercooling point of Colorado potato beetles. Because the interaction between bacterial strain and sampling time was statistically significant, a separate analysis was generated for each strain with sampling time as the main effect. Differences among sample means were separated by Fisher protected least significant difference (LSD) (SAS Institute 1996). Because the main goal of this study was to identify bacterial strains able to influence supercooling capacity of the beetles, supercooling point means from different bacterial treatments were compared with the untreated control mean within each sampling time using Dunnett *t*-test (SAS Institute 1996).

Culture of Gut Bacteria. After measuring their supercooling points, three beetles from one replicate of each dose and bacterial strain were aseptically dissected and their digestive tracts triturated in 200 μl of sterile deionized water in 1.5-ml polyethylene tubes using sterile wooden sticks. Serial dilutions (1:10, 1:100, and 1:1000) of a 50-μl aliquot from each beetle gut suspension were plated on *Pseudomonas* isolation agar (Fisher, Pittsburgh, PA). Culture plates from beetles treated with *P. fluorescens* strains and the corresponding control beetles were incubated at 4°C. This species can be differentiated from the two other *Pseudomonas* spp. based on its ability to grow at this temperature (Gilligan 1995). Bacterial colonies observed after five to 6 d of incubation were recorded. Gut cultures from beetles treated with *P. putida* and *P. syringae* strains, incubated at 22°C, were detectable after 48 h.

Extraction of Bacterial Genomic DNA. The presence of ice-nucleating bacteria in treated beetles was also detected by PCR amplifications using DNA extracted from bacterial cultures isolated from the digestive tract of treated and control beetles. A 100-μl aliquot of the triturated gut suspension was used to inoculate 10 ml of nutrient broth with 2.5% glycerol in 125-ml flasks and cultured overnight in an incubator shaker set at 150 rpm at 24°C. Bacterial cells from 1 ml of broth were used for DNA extraction using the QIAamp tissue kit (Quiagen, Santa Clarita, CA).

PCR Assays. Ice-nucleating *P. fluorescens* and *P. syringae* strains were detected using primers based on published sequences of the ice-nucleating genes *inaW* and *inaZ* of these species, respectively (Warren et al. 1986): INA-W3 (5'-GCGGTCTGGTATGGCCTATT-3') and INA-W4 (5'-CCGGCGTATCGCTATTGTCC-3'); and INA-Z1 (5'-ATCCAGTCATCGTCCTCGTC-3') and INA-Z2 (5'-CAAGTGTCACGTTACCGGTG-3'). Because the *ina* gene for *P. putida* has not been sequenced, possible homology with either *inaW* or *inaZ* was tested. A PCR product > 4.5 Kb in *P. putida* screened with INA-Z primers similar to the product seen in preliminary PCR assays using the two *P. syringae* strains, Hr6–3B and cit 7, was observed. Consequently, assays for *P. putida* Hr6–1 were conducted using the INA-Z primer pair. PCR reaction mixture consisted of PCR buffer (100 mM Tris-HCl, pH 8.3; 1×10^{-3} M EDTA, pH 8.0), 1.5 mM $MgCl_2$, 200 μM each of ATP, CTP, GTP, and TTP, 0.5 μM of each primer, 100 ng of template DNA, and 0.5 U of *Taq* polymerase (Quiagen, Santa Clarita, CA) in a 50-μl volume and overlaid with mineral oil. Amplifications were performed in a Perkin-Elmer Cetus thermal cycler (mod-

Table 2. Mean supercooling points (°C) of Colorado potato beetles measured at three sampling times after ingestion of ice-nucleating active *Pseudomonas* spp. applied at two concentrations

Bacterial strain	1.5 h	2 wk	12 wk	*F*	df	*P*
		Fed 10^6 bacterial cells				
F26-4C	−4.8 ± 0.2a	−5.3 ± 0.3a	−5.5 ± 0.5a	0.84	2, 87	0.43
88-335	−4.7 ± 0.3a	−5.0 ± 0.4a	−5.8 ± 0.5a	2.31	2, 82	0.10
Hr6-1	−4.5 ± 0.2a	−9.6 ± 0.4b	−12.4 ± 0.6c	74.95	2, 86	<0.01
Hr6-3B	−5.7 ± 0.4a	−9.7 ± 0.5b	−11.6 ± 0.6c	33.14	2, 85	<0.01
cit 7	−5.7 ± 0.3a	−10.2 ± 0.5b	−13.1 ± 0.5c	56.08	2, 86	<0.01
		Fed 10^3 bacterial cells				
F26-4C	−5.2 ± 0.4a	−6.9 ± 0.4b	−6.7 ± 0.6b	3.54	2, 82	0.03
88-335	−5.6 ± 0.3a	−5.2 ± 0.4a	−7.2 ± 0.5b	5.16	2, 87	0.01
Hr6-1	−5.6 ± 0.4a	−9.7 ± 0.5b	−11.3 ± 0.6c	32.49	2, 85	<0.01
Hr6-3B	−6.6 ± 0.3a	−10.4 ± 0.6b	−12.5 ± 0.6c	28.16	2, 86	<0.01
cit 7	−5.9 ± 0.3a	−9.8 ± 0.4b	−12.3 ± 0.6c	47.29	2, 84	<0.01
Control	−9.2 ± 0.5a	−8.6 ± 1.0a	−10.1 ± 0.3a	1.71	2, 86	0.18

Means ± SEM within a row followed by the same letter are not significantly different at $\alpha = 0.05$ (Fisher protected LSD test).

el Gene Amp PCR system 2400, Branchburg, NJ) programmed for an initial denaturation at 94°C for 4 min, followed by 35 cycles of 1 min at 94°C, 1 min at 55°C, 3 min at 72°C, and a final extension of 10 min at 72°C for the INA-Z primer pair. For the INA-W primer pair, the same conditions were used, except for an annealing temperature of 47°C. Amplification products were visualized in 1.0% agarose gels stained with ethidium bromide. For each PCR run, positive controls using pure bacterial DNA of the tested strains and a negative control using sterile water were used. Amplifications were replicated thrice per sample.

Results

Effect of Ice-Nucleating Active Bacteria on Beetle Supercooling Capacity. Supercooling points of treated Colorado potato beetles varied throughout the three sampling times ($F = 111.1$; df = 2, 60; $P < 0.01$). Immediately after feeding, the range of mean supercooling points was −4.5 to −6.6°C (Table 2). After 2 and 12 wk, mean supercooling values ranged from −5.0 to −10.4 and −5.5 to −13.1°C, respectively. Also, highly significant were the effects of bacterial strain ($F = 28.2$; df = 4, 60; $P < 0.01$) and interaction between bacterial strain and sampling time ($F = 6.01$; df = 8, 60; $P < 0.01$) on beetle supercooling points, indicating significant changes in the effects of bacterial strain at different sampling times. Posthoc analysis showed that supercooling values of beetles treated with either F26-4C or 88–335 were not significantly different across the three sampling times (Table 2). In strains Hr6–1, Hr6–3B, and cit 7, however, there was a significant decrease in supercooling values 2 and 12 wk after ingestion (Table 2). The effect of bacterial dose was not significant ($F = 1.76$; df = 1, 60; $P = 0.35$), indicating comparable results whether beetles were fed a dose of 10^6 or 10^3 bacterial cells.

The ranges of individual supercooling points of treated beetles at 1.5 h after feeding was wide (−2.6 to −10.0°C), suggesting that beetles ingested variable numbers of bacteria. In untreated beetles, the range of observed values, −5.8 to −16.0°C, was also highly variable. Among the treated beetles, 50% froze near −5.0°C, whereas for control beetles a lower temperature of approximately −9.0°C was required to freeze the same percentage (Fig. 1A). The highest supercooling points observed at 1.5 h for different bacterial strains ranged from −2.6 to −3.4°C. After 2 and 12 wk, the highest values observed for strains Hr6–1, Hr6–3B and cit 7 decreased to −4.3, −4.2, and −6.8, and −5.0, −6.6 and −6.4°C, respectively (Fig. 1 B and C). The highest supercooling points observed in beetles treated with either F26-4C or 88–335 at 2 and 12 wk after ingestion were close to values observed at 1.5 h after feeding (Fig. 1). The highest observed supercooling values at 1.5 h, 2, and 12 wk after ingestion for F26-4C treatments were −3.4, −3.4, and −2.8°C, respectively; and for 88–335 they were −3.0, −3.2, and −2.8°C, respectively.

Supercooling points of treated beetles were compared with untreated controls to determine if the differences observed among bacterial strains across sampling times significantly affected beetle supercooling capacity. Immediately after feeding, all bacterial strains tested significantly elevated the supercooling point of treated beetles compared with untreated controls ($F = 9.83$; df = 10, 22; $P < 0.01$). Significant differences between treated and untreated beetles persisted after 2 wk ($F = 11.2$; df = 10, 22; $P < 0.01$) and 12 wk ($F = 17.1$; df = 10, 22; $P < 0.01$) after ingestion, but only for strains F26-4C and 88–335. Supercooling points of beetles treated with Hr6–1, Hr6–3B, or cit 7 after 2 and 12 wk were comparable to supercooling points of control beetles. Thus, in treatments Hr6–1, Hr6–3B, or cit 7, the highly significant decrease in supercooling values after 2 and 12 wk correlated to a loss of significant effect on beetle supercooling capacity. Overall, bacterial strains F26-4C and 88–335 were effective at either dose (10^6 or 10^3 bacterial cells per beetle) in elevating the supercooling points of adult Colorado potato beetles up to 12 wk after ingestion.

Detection Methods. In addition to measuring beetle supercooling points, we sought a direct method to detect ice-nucleating active bacteria in the gut. Our first approach was to isolate and culture bacteria from triturated gut suspensions using selective media. Re-

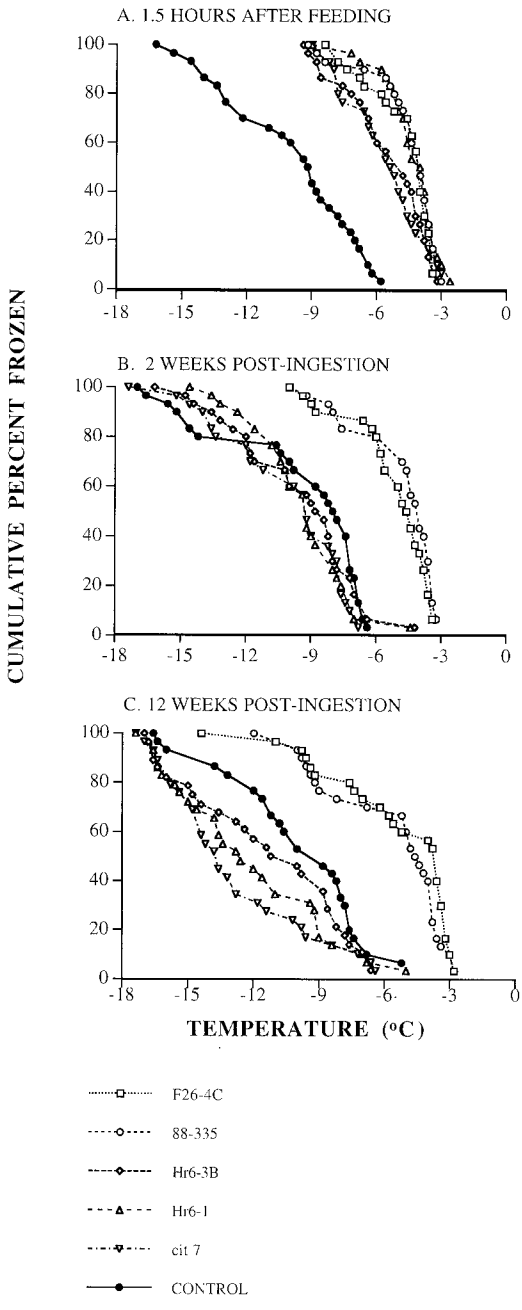

Fig. 1. Cumulative freezing profiles based on individual supercooling points of Colorado potato beetles fed potato slices coated with ice-nucleating active bacteria (10^6 bacterial cells in 10 μl of sterile distilled water). Supercooling point values of beetles treated with each bacterial strain, along with control beetles, were measured 1. 5 h (A), 2 wk (B), and 12 wk (C) after ingestion. Values represent beetles from three replicates (n = 27–30).

sults (data not shown) however showed variable numbers of bacterial colonies from treated beetles that did not reliably correlate with observed supercooling points. The presence of *Pseudomonas* colonies, likely non-ice-nucleating strains, in control beetles indicated that this genus is a common gut flora in *L. decemlineata*, which complicates detection based on cultural methods. Consequently, we used another method of detection, PCR amplification of the *ina* gene of the bacteria. This method provided molecular evidence of the presence or absence of ice-nucleating active bacteria fed to beetles, and allowed correlation of the presence of these bacteria with the beetle's elevated supercooling point. In adult beetles fed the two *P. fluorescens* strains, a band corresponding to the *inaW* gene was observed in beetles with elevated supercooling points even 12 wk after ingestion (Fig. 2A). Of 21 treated beetles exhibiting supercooling points above −5.4°C, only one did not show the band despite a relatively high reading of −4.6°C. Treated beetles having supercooling points below −9.4°C (n = 5) and sampled 2 and 12 wk after ingestion did not exhibit the band.

Colorado potato beetles fed with *P. syringae* Hr6–3B and cit 7 showed a band, >4.5 Kb corresponding to the *inaZ* gene when sampled immediately after feeding (Fig. 2B). Beetles fed with *P. putida* Hr6–1 showed a similar band 1.5 h after feeding (data not shown). Only one out of 11 beetles with supercooling values above −5.8°C did not exhibit the band. When the supercooling points of treated beetles were comparable to control beetles 2 and 12 wk after ingestion, the band was not detected and confirmed the absence of either *P. putida* or *P. syringae*, suggesting loss of INA bacteria. None of the control beetles exhibited a PCR product when screened with either the INA-W or the INA-Z primer pairs.

Discussion

To further develop the potential of ice-nucleating active bacteria for biological control, the current study was conducted to screen insect-derived ice-nucleating *Pseudomonas* strains for ones that are better retained by overwintering Colorado potato beetles. These bacterial strains were compared with frog-derived *P. fluorescens* F26-4C, which was observed to persist in overwintering Colorado potato beetles in the field through the course of winter (Castrillo et al. 1999). Our results confirmed the persistence of *P. fluorescens* F26-4C in the beetle digestive tract and showed that the insect-derived strain 88–335 was also retained. The two other insect-derived strains, *P. putida* Hr6–1 and *P. syringae* Hr6–3B, along with the epiphyte cit 7, were lost by 2 wk after ingestion.

Although the use of insect-derived ice-nucleating *Pseudomonas* strains may facilitate retention in the beetle digestive tract, given that this genus is common in the gut flora of insects (Steinhaus 1941, Bucher and Stephens 1959, Charpentier et al. 1978, Mead et al. 1988), other factors may influence their persistence. Costanzo et al. (1998) suggested that host source may be an important factor determining which bacterial strains are retained by overwintering Colorado potato beetles. They found that frog-derived strains of both *P. fluorescens* and *P. putida* were retained for 10 wk,

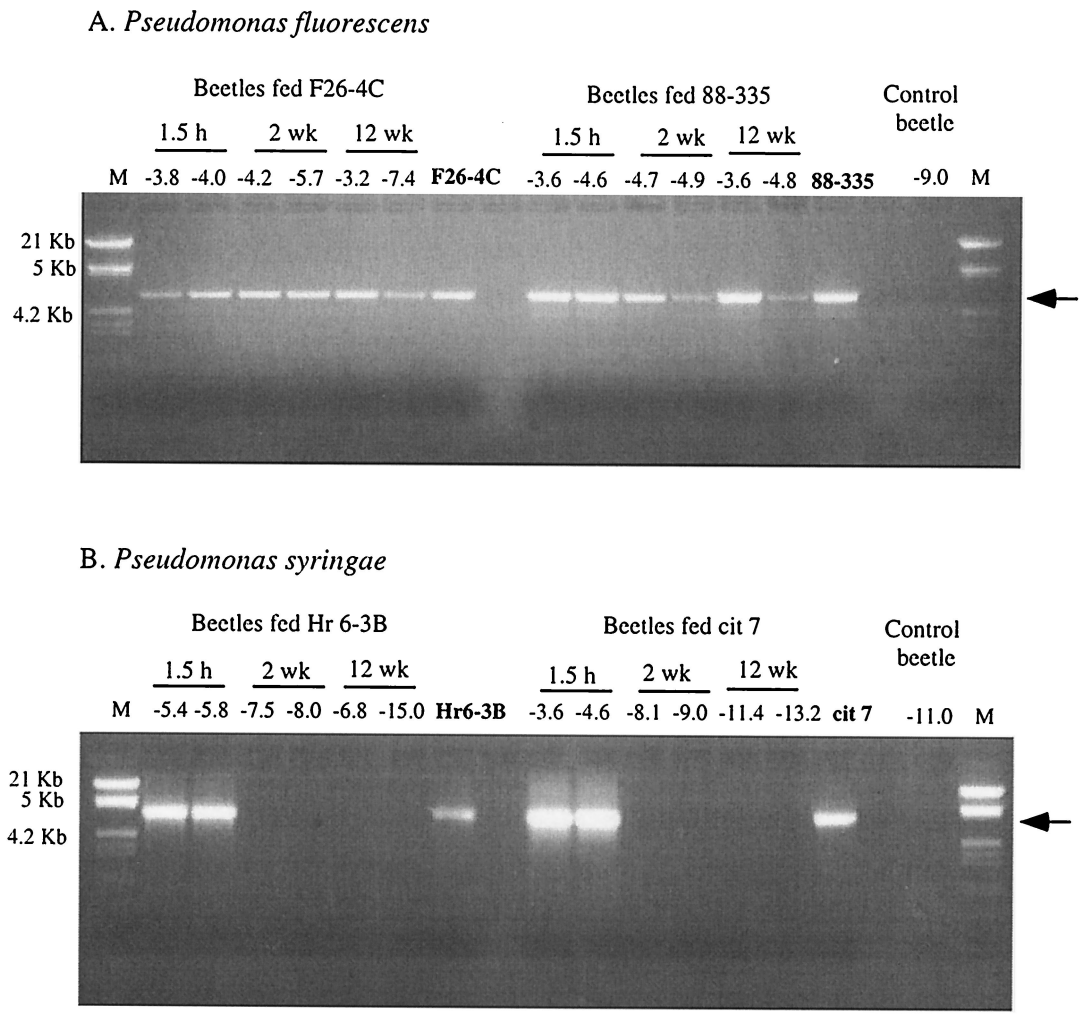

Fig. 2. Detection of ice-nucleating active bacteria in the digestive tract of Colorado potato beetles at different sampling times after feeding: 1. 5 h, 2 wk, and 12 wk. *P. fluorescens* strains, F26-4C and 88–335, were detected using primer pair based on the *inaW* gene from *P. fluorescens; P. syringae* strains, Hr6–3B and cit 7, were detected using primer pair based on the *inaZ* gene from *P. syringae.* Values above each sample indicate individual beetle's supercooling point (°C), sampling time, and the ice-nucleating active bacterial strain (10^6 bacterial cells per beetle). Supercooling point values for control beetles were measured 1. 5 h after feeding. DNA from the ice-nucleating active bacterial strains were used as positive controls. Molecular marker (M) used was lambda restricted with *Hin*dIII and *Eco*RI. Arrows indicate bands corresponding to the *ina* gene.

whereas the epiphyte *P. syringae* was not, suggesting that animal-derived strains are better retained than those from plants. Surprisingly, the insect-derived *Enterobacter agglomerans* was also lost by 12 d after ingestion (Costanzo et al. 1998). Similar results were obtained in this study, as the insect-derived *P. putida* and *P. syringae* strains were as readily lost as the epiphytic *P. syringae* cit 7. However, the isolation of a given bacterial species from an insect gut, hence classified as insect-derived, does not positively indicate that the bacterial species was not from other sources such as soil or plant foliage. Isolates obtained from the digestive tract may be transient floras that are taken in while feeding (Mead et al. 1988) and thus may not reflect native gut floras. Because *P. syringae* is a common epiphytic bacterium (i.e., capable of living on plant surfaces) (Hirano and Upper 1990), it may be readily ingested along with foliage and may be incidentally isolated when culturing beetle gut flora. Nonetheless, it has been shown that some epiphytic bacteria can colonize the gut of some insects (Takahashi et al. 1995; Watanabe et al. 1996, 1998), suggesting that establishment of an introduced bacteria in an insect gut varies with the species and strain of bacterium and the insect host. Insect gut conditions (i.e., pH, enzymes present, native bacterial flora) vary between species and may limit the growth of an introduced bacterial strain. The purging behavior of some insects, including adult Colorado potato beetles, to remove gut contents before overwintering also re-

moves bacterial flora ingested with food material. Establishment of transient bacteria in overwintering Colorado potato beetles would require not only utilization of available nutrients in the beetle gut but also adherence to gut tissues.

A significant outcome of this study was the close correlation between elevated supercooling points of treated beetles with the presence of ice-nucleating bacteria in their gut as evidenced by PCR assays. Previous observations (Castrillo et al. 1999) showed that presence of INA bacteria in the beetle gut was not always manifested by an increase in the supercooling point. Variability in the number of ice-nucleating bacterial cells ingested and in the ice-nucleating activity of these cells are factors that influence supercooling point elevation. PCR proved to be a good indicator of the presence of ice-nucleating bacteria because this technique detects the bacteria by virtue of their *ina* gene and not their protein products. Ice-nucleating bacteria can be detected even in treated beetles that had low supercooling points caused, possibly, by low bacterial numbers.

Because of their ability to persist in the beetle digestive tract, the two ice-nucleating *P. fluorescens* strains F26-4C and 88–335 are excellent candidates for further development as biological control agents for overwintering Colorado potato beetles. Elevated supercooling points observed in overwintering beetles 12 wk after initial exposure indicate that the introduced ice-nucleating bacteria colonized the beetle gut. Furthermore, supercooling point values as high or even higher than those observed after immediate exposure to these strains strongly suggest that beetle gut conditions are favorable for expression of ice-nucleating activity. Efficacy of the *P. fluorescens* strains to elevate beetle supercooling points even with a single, low dose also suggests that a minimum inoculum is sufficient for establishment in the gut despite the beetle's purging of its gut contents before overwintering.

By manipulating the beetle's cold-tolerance using ice-nucleating bacteria, it may be possible to reduce survival of the overwintering population, and thereby delay beetle population build-up the following summer. We envision the application of ice-nucleating bacteria to potato trap crops in late summer before beetles burrow into the soil to overwinter. In late summer, before potatoes are harvested, foliage is killed mechanically or chemically using vine desiccants leading to beetle congregation upon any remaining pockets of foliage. Thus, one can spray these trap crops with ice-nucleating bacteria. When beetles burrow into the ground after feeding, they will have ice-nucleating bacteria in their gut that will reduce survival when temperatures decrease to subzero levels several months later.

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