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Isolation of Ice-Nucleating Active Bacteria from the Freeze-Tolerant Frog, *Rana sylvatica*

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Ice-nucleating active (INA) bacteria were isolated from the gut of field-collected freeze-tolerant wood frogs (*Rana sylvatica*) collected in winter. Thirteen strains of *Pseudomonas fluorescens,* four strains of *Pseudomonas putida,* and two strains of *Enterobacter agglomerans* had ice-nucleating activity. Each of the INA pseudomonad strains was psychrophilic. *P. putida* strains were differentiated from *P. fluorescens* strains by gelatinase, lecithinase, and lipase production. The maximum nucleation temperatures (T_{max}) of aqueous suspensions (10^9 bacteria/ml) of the four INA *P. putida* strains ranged from −1.6 to −3.0°C, which places this INA species among the most potent known biological nucleators. Ingestion of INA *P. putida* isolated from *R. sylvatica* by another freeze-tolerant frog, *Pseudacris crucifer,* decreased the capacity of this frog to supercool and remain unfrozen at −2°C. This is the first report of INA bacteria isolated from a vertebrate, and suggests that, as part of the gut flora in some posthibernation freeze-tolerant wood frogs, these bacteria may play a role in enhancing winter survival by promoting ice nucleation at high subzero temperatures (ca. −2°C).

Ice-nucleating active (INA) bacteria are defined as those bacteria that can initiate ice nucleation in water at temperatures above −10°C (23). Bacterial nucleation is attributed to the presence of a protein, comprised of highly repetitive sequences of amino acids, in the bacterial outer membrane that serves as a template for ice crystallization (9, 30). Although the broad distribution of INA bacteria in soils, in leaf mulch, and on plants, as well as their significance in promoting frost damage in crops is well documented, the number of known INA bacterial species is relatively small (20, 35). Only recently has the presence and identity of INA bacteria and fungi been reported in the gut of insects (10, 15).

Wood frogs (*Rana sylvatica*) are among the few vertebrates that naturally tolerate freezing of their body tissues (13, 29, 31, 32). These frogs hibernate in terrestrial burrows under leaf litter and snow where subzero microhabitat temperatures may occur (13, 29, 31, 32). During this time and their subsequent migration to breeding ponds, the frogs do not eat (J. P. Costanzo, personal communication). Freeze-tolerance in frogs is promoted by the initiation of ice formation at high subzero temperatures which allows ice to form gradually (29); rapid freezing is lethal (5).

The presence of INA bacteria in vertebrates has not been reported. However, if INA bacteria are present in wood frogs (and other freeze-tolerant vertebrates), they may play an important role in initiating ice nucleation at high subzero temperatures. To investigate this possibility, we examined the normal flora of the wood frog's gastrointestinal tract and posed the following questions: (1) Are INA bacteria present in the gut? and (2) If so, do these INA bacteria possess ice nucleation activity sufficient to explain the supercooling point (crystallization temperature), the temperature at which ice forms spontaneously, which for this species is near −2°C (13, 29)? In this report, we document that INA strains of *Pseudomonas fluorescens, Pseudomonas putida,* and *Enterobacter agglomerans* occur naturally in the gut of *R. sylvatica,* and that these strains have ice-nucleating

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activity at high subzero temperatures that closely corresponds to whole animal supercooling points. Additionally, we report that ingestion of INA *P. putida* significantly decreases the supercooling capacity of the freeze-tolerant spring peeper, *Pseudacris crucifer,* increasing the occurrence and promoting the onset of ice nucleation.

MATERIALS AND METHODS

Frog Sampling and Isolation of Bacteria

Sixty male wood frogs (*R. sylvatica*) were collected from breeding ponds in Adams County, southcentral Ohio, during February 1989. Twenty male spring peepers (*P. crucifer*) were collected from wetlands in Butler County, southwest Ohio, during March 1990. The intestines of wood frogs were removed aseptically. Scrapings from the intestinal wall, along with the negligible intestinal contents, were cultured on Difco nutrient agar with 2.5% glycerol (NAG) and in Difco nutrient broth with 2.5% glycerol (NBG) to enhance the phenotypic expression of ice nucleation activity, and 0.5% cycloheximide to inhibit fungal growth. All plates and broths were incubated aerobically at 20°C unless otherwise stated. Following incubation for 7 days, selected morphologically distinct colonies on the NAG plates were isolated in pure culture and reincubated for 7 days. Each culture tube containing NBG was incubated for 48 h, and subsequently tested for ice nucleation activity (see below). Bacteria growing in NBG, detected by gram staining the NBG, were subcultured onto NAG. Morphologically distinct colonies growing on NAG, isolated either directly from the gut samples or indirectly from the nutrient broths, were checked for ice nucleation activity following 7 days of aerobic incubation. Only those strains with ice nucleating activity following three subcultures were scored as positive.

Detection and Identification of INA Bacteria

Suspensions of each bacterial isolate, ca. 10^9 organisms/ml sterile distilled water, from 7-day, aerobic NAG plates were made by diluting 0.80 A_{540nm} (Gilford 250 spectrophotometer) bacterial suspensions, following direct bacterial counts using a custom-made hemocytometer with a reduced cell depth of 0.02 mm and double Neubauer ruling (Hausser Scientific Co.). Aqueous bacterial suspensions and nutrient broths were tested for ice nucleating activity by placing 40 10-μl drops on aluminum boats floating on a refrigerated, circulating ethanol bath and determining visually whether drops froze within 3 min at −10°C. *Pseudomonas syringae* cit 7 and *Erwinia herbicola* Am 3000 (provided by S. E. Lindow) served as INA controls, while *Escherichia coli* ATCC 35421 served as a non-INA control. Taxonomic identifications of INA bacteria were made phenotypically, based upon colonial morphology and pigmentation, gram reaction, somatic shape, number of flagella, API 20E biochemical tests, Rapid NFT (Rapid Nonfermenters Test; Analytab Products, Inc.) biochemical profiles, production of pyoverdin, pyocyanin, lecithinase, and lipase, and utilization of kynurenine, anthranilate, L-tryptophan, and histamine.

Characterization of Bacterial Ice-Nucleating Activity

Cumulative concentrations of ice nuclei per cell were established *in vitro* according to the methods of Vali (34) as modified by Lindow *et al.* (19). Ice-nucleating determinations of each bacterial suspension were established at progressively decreasing temperatures (ca. −0.3°C/min).

In Vivo Test of Ice-Nucleating Activity

Forty microliters of an aqueous suspension of 1×10^9 INA *P. putida*/ml, isolated from the intestinal tract of *R. sylvatica,* was directly fed to each of 10 spring peepers. Ten additional individuals, directly fed 40 μl of sterile distilled water, served as controls. Each of the 20 frogs had a thermocouple positioned against their abdomens, was placed in a plastic tube, and exposed to −2°C in a refrigerated bath. A data logger recorded body temperature continuously over the 24-h test period, and the records were examined for the release of the latent heat of

fusion, indicative of ice nucleation within body fluids (5).

RESULTS

Our screening procedure for INA bacteria was not designed to be a comprehensive characterization of INA bacteria, rather only selected aerobic colonies which macroscopically resembled Pseudomonadaceae or Enterobacteriaceae were checked for ice-nucleating activity. INA bacteria were isolated from the gut of 15% (9/60) of the wood frogs. Four of the nine frogs harbored two INA bacterial species, *P. fluorescens* and *P. putida.* Since these frogs had not ingested any food for approximately 6 months, the presence of INA bacteria in any of their guts is significant. Additionally, these INA bacteria belong to genera representative of normal gut flora in hibernating frogs (2). One of these four additionally harbored two strains of INA *Enterobacter agglomerans.* Owing to the limited number of colonies tested the frequency of occurrence (15%) of frogs harboring intestinal INA bacteria is likely to be a highly conservative estimate of the actual proportion of frogs harboring INA bacteria.

The phenotypic characteristics of INA *P. fluorescens* and *P. putida* isolated from frogs closely correspond to the standard reference values for these species (7) (Table 1). *P. putida* isolated from frogs was differentiated from *P. fluorescens* strains based on established species' characteristics (7, 8, 16), with a lack of gelatinase (0/4), lecithinase (0/4), and lipase (0/4) production, inability to reduce nitrate (0/4), and the capacity for growth at 37°C by *P. putida* strains compared to production of gelatinase (13/13), lecithinase (7/13), and lipase (10/13) by a majority of *P. fluorescens* strains, and production of N_2 gas following nitrate reduction by some *P. fluorescens* strains (2/13). Identifying traits of the two *E. agglomerans* strains were yellow pigmented colonies, positive tests for fermentation of glucose, motility by peritrichous flagella, utilization of the carbon of sodium citrate as the sole source of carbon for growth, and butylene glycol type of fermention (Voges–Proskauer test), production of in-

TABLE 1
Phenotypic Reactions of 13 INA *Pseudomonas fluorescens* and 4 *Pseudomonas putida* Strains Isolated from the Wood Frog, *Rana sylvatica*

Characteristic	Percentage of strains with characteristic	
	P. fluorescens	*P. putida*
Production of		
Indophenol oxidase	100 (100)	100 (100)
Pycocyanin (Flo agar)	0 (0)	0 (0)
Pyoverdin (Flo agar)	100 (95)	100 (84)
Arginine dihydrolase	92 (99)	100 (99)
Gelatinase	100 (100)	0 (0)
Lecithinase (hydrolysis of phosphatidylcholine)	54 (90)	0 (0)
Lipase (Tween 80)	77 (63)	0 (0)
Nitrate reduction	0 (19)	0 (0)
Gas from nitrate	14 (5)	0 (0)
Motility	100 (100)	100 (100)
Polar tuft of three or more flagella per pole (gray flagellar stain)	100 (100)	100 (100)
Growth at 42°C	0 (0)	0 (0)
Mannitol fermentation	0 (93)	0 (18)

Note. The columns indicate percentage of our strains that were positive for the indicated characteristics followed, parenthetically, by reference values from the "Manual of Clinical Microbiology" (8). Cultures were grown at 20°C on nutrient agar with 2.5% glycerol for 48 h prior to biochemical tests that were performed at 25°C.

dole from tryptophane, and negative for the production of lysine decarboxylase, arginine dihydrolase, and ornithine decarboxylase.

These two INA strains belonged phenotypically to the large, heterogeneous *E. herbicola–E. agglomerans* complex. *E. agglomerans* and *E. herbicola* are synonyms historically, and, in general, isolates phenotypically placed into this complex are named *E. agglomerans* if isolated from animals and *E. herbicola* if isolated from plants (3, 4). Although one DNA hybridization group of *E. agglomerans* is established to be *Pantoea agglomerans* comb. nov. based upon DNA–DNA hybridization (6), we identified our INA frog-origin isolates as *E. agglomerans* based upon phenotypic characteristics, pending DNA relatedness studies.

Each of the INA pseudomonad strains from *R. sylvatica* grew at both 25 and 4°C. However, unlike typical strains of *P. fluorescens,* the INA *P. fluorescens* strains of frog origin grew poorly, if at all, at 37°C, which is a lethal temperature for the frog. Thus, all plates and biochemical tests were run at 25°C, unless otherwise indicated. The majority of INA *P. fluorescens* strains (10/13) were identified as biovar I and the remainder as biovar II, based upon lipase and lecithinase production, ability to reduce nitrate, production of levan from sucrose, and utilization of rhamnose and isobutanol (25). Each of the INA *P. fluorescens* strains was atypical in their inability to utilize mannitol. All four INA *P. putida* strains were biovar A based upon their inability to utilize kynurenine (1/4), anthranilate (1/4), L-tryptophan (0/4), sucrose (0/4), L-arabinose (0/4), and histamine (1/4), and their production of pyoverdin (4/4), compared to utilization of each of these substrates by >90% of biovar B strains and inconsistent production of pyoverdin (10 to 90%) by B strains (25).

The nucleation characteristics of INA bacterial strains isolated from frogs are documented in Table 2 and Fig. 1. Aqueous suspensions (ca. 10^9 bacteria/ml) of *P. putida* strains exhibited a T_{max} (mean threshold temperature of nucleation), T_{50} (mean temperature at which 50% of droplets froze), and T_{90} (mean temperature at which 90% of droplets froze) of −2.4, −3.6, and −4.3°C, compared to −4.2, −6.4, and −8.2°C for INA *P. fluorescens* strains, and −2.8, −4.7, and −5.8°C for *E. agglomerans. P. putida* ATCC type strain 12633 was shown to be INA positive, although a weaker ice nucleator than the INA *P. putida* strains isolated from *R. sylvatica.* A 1-week incubation of *P. putida* biotype A strain ATCC 12633 at 4°C compared to its activity at 25°C on NAG for 1 week increased the T_{max} for ice nucleation from −8.0 to −5.0°C, suggesting Group II nucleating structures (37). No enhancement in the T_{max}, T_{50}, or T_{90} of INA *P. putida* strains from frogs was induced by culturing on Koser's citrate agar, by holding bacterial suspensions at 4 or 15°C for 5 h prior to testing for ice-nucleating activity, or by incubating the INA organisms at 15°C for 48 h, as documented with *P. syringae* (26), *E. herbicola* (28), and recombinant ina$^+$ *E. coli* (24). The presence of class A nucleating structures, the most potent and uncommon of nucleating

TABLE 2

Comparison of T_{max}, T_{50}, and T_{90} for INA *Pseudomonas fluorescens, Pseudomonas putida,* and *Enterobacter agglomerans* Isolated from Frogs (1×10^9 Bacteria/ml Sterile Distilled Water) and the Type Strain *P. putida* Biotype A ATCC 12633 (American Type Culture Collection) Following 48-h Incubation Aerobically at 20°C on Nutrient Agar with 2.5% Glycerol

INA bacteria (*n* strains)	Temperature (°C): mean (range)		
	T_{max}	T_{50}	T_{90}
P. putida (4)	−2.4 (−1.5 to −3.0)	−3.6 (−2.6 to −4.1)	−4.3 (−3.1 to −5.1)
P. fluorescens (13)	−4.2 (−2.5 to −5.9)	−6.4 (−4.6 to −8.4)	−8.2 (−5.2 to −11.8)
E. agglomerans (2)	−2.8 (−1.3 to −4.6)	−4.7 (−2.5 to −8.0)	−5.8 (−3.4 to −9.5)
P. putida (1) (biotype A ATCC)	−8.0	−11.2	−15.2

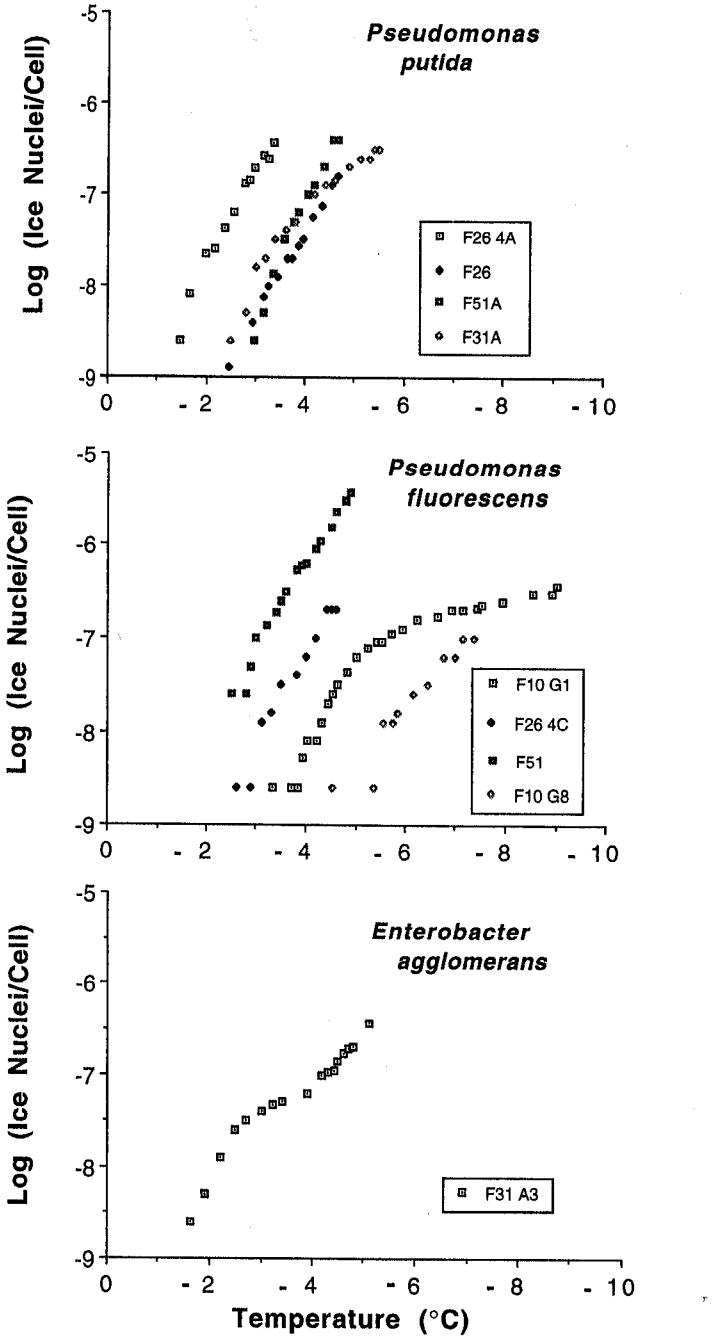

FIG. 1. Ice-nucleating activity, expressed as log (ice nuclei/cell) as a function of temperature, for frog-origin INA *Pseudomonas putida, P. fluorescens,* and *Enterobacter agglomerans* strains. Data, plotted for frog-origin INA bacterial strains, are cumulative ice nucleation spectra determined in duplicate on 80 10-μl drops of aqueous suspensions, ca. 1×10^9 bacteria/ml, harvested from 7-day NAG plates incubated at 20°C.

classes described by Turner *et al.* (33), on all four frog-origin INA *P. putida* strains was evidenced by the ability of each of these cultures to nucleate water at temperatures above −4.4°C and their effectiveness at nucleating D_2O, in which the T_{max} was increased from 2.4 to 4.5°C.

Ingestion of INA *P. putida* by the freeze-tolerant spring peeper, *P. crucifer,* caused a significant increase (χ^2, $P < 0.001$) in the number of frogs (10/10) freezing during a 24-h exposure at −2°C compared to the number of control frogs (2/10) which ingested water. In the control group, 2 frogs spontaneously froze at 120 and 888 min after being placed at −2°C, whereas the remaining 8 individuals remained supercooled for the 24-h test period. In contrast, all 10 frogs fed *P. putida* froze within 24 h. Five of these froze within 10 min with a mean (±SEM) time to the onset of freezing equal to 5.0 ± 2.2 h. Each of the frogs in the control group and test group survived. The presence of INA *P. putida* in the gut limited the frogs' ability to remain supercooled at −2°C.

DISCUSSION

The INA phenotype is known in four bacterial genera, *Erwinia* (1, 2, 19), *Pseudomonas* (20, 22, 27), *Xanthomonas* (12), and *Enterobacter* (16). Within the genus *Pseudomonas* both INA and non-INA bacteria exist (17). INA pseudomonads have been isolated from the surface of plants (18, 22), roots of alfalfa plants (27), soil (18), water from streams and lakes (23), snow and rain (23), and from insects (15). The identification of INA *P. putida* in the frog, *R. sylvatica,* extends the range of documented hosts for reported INA bacteria to vertebrates. A diversity of ice nucleating ability among *P. putida* strains is evidenced by the potent ice nucleating activity of the *P. putida* strains isolated from frogs, the comparatively weak ice-nucleating activity of *P. putida* biotype A type strain ATCC 12633, and the lack of ice nucleating activity in certain *P. putida* strains (18). The *P. putida* strains from wood frogs demonstrate INA phenotypes that include Class I nucleating structures comparable to the most potent previously reported INA bacterium, *P. syringae* (33).

Each of the frog-origin INA isolates was able to grow at 4°C, but not at 37°C. The presence of psychrophiles among the frogs' large intestinal flora was reported by Banas *et al.* (2). The ice nucleation phenotype of INA bacteria, characterized by "ice nucleation frequency" or the logarithm of the number of cells per ice nucleus in a given population of cells at a given temperature, is highly variable and greatly influenced by both physical and biological factors (21). Thus, the effects of natural habitats, host species, and climatic conditions on the ice nucleation frequency or expression of the ice nucleation phenotype need to be investigated.

Freeze tolerance in the wood frog, which has the northernmost distribution of any North American amphibian, persists from autumn until spring (29). The INA bacteria harbored by our wood frogs, sampled in February, may play an important role in this species' freeze tolerance, since it is generally believed that ice nucleation at high subzero temperatures promotes survival. Interestingly, the activity temperature for these bacteria (−2 to −3°C) is in close accord with whole-animal supercooling points determined in the laboratory (13, 31, 32). Therefore, INA bacteria may serve to induce freezing at physiologically safe temperatures, particularly when internal freezing has not been initiated inoculatively by contact with external ice (14). Future study is needed to determine quantitatively whether a significant proportion of frogs collected in late autumn and early winter contain these bacteria.

Other mechanisms for ice nucleation in *R. sylvatica* have been proposed, including the action of a proteinaceous ice nucleator in the blood (36). However, the activity of this agent, ca. −7°C, is substantially lower than both the whole-animal supercooling point and minimum survival temperature for this species (32). A more plausible mechanism for the onset of ice formation was proposed by Layne *et al.* (14), who demonstrated that contact of *R. sylvatica* skin with environmental ice rapidly seeded the freezing of internal water at ca. −0.5°C. Al-

though this "inoculative freezing" may be a primary stimulus for ice nucleation in moist microenvironments, it would be ineffectual should frogs become physically isolated from contact with external ice crystals or overwinter in dry hibernacula. Under these circumstances, the presence of INA bacteria in the gut may induce freezing at high subzero temperatures, and thereby promote freeze tolerance.

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REFERENCES

1. Arai, S., and Watanabe, M. Freeze texturing of food materials by ice-nucleation with the bacterium *Erwinia ananas*. *Agric. Biol. Chem.* **50,** 169–175 (1986).
2. Banas, J. A., Loesche, W. J., and Nace, G. W. Classification and distribution of large intestinal bacteria in nonhibernating and hibernating leopard frogs (*Rana pipiens*). *Appl. Environ. Microbiol.* **54,** 2305–2310 (1988).
3. Beji, A., Mergaert, J., Gavini, F., Izard, D., Kersters, K., Leclerc, H., and DeLey, J. Subjective synonymy of *Erwinia herbicola, Erwinia milletiae* and *Enterobacter agglomerans* and redefinition of the taxon by genotypic and phenotypic data. *Int. J. Syst. Bacteriol.* **38,** 77–88 (1988).
4. Brenner, D. J., Fanning, G. R., Knutson, J. K. Leete, Steigerwalt, A. G., and Krichevsky, M. I. Attempts to classify Herbicola group–*Enterobacter agglomerans* strains by deoxyribonucleic acid hybridization and phenotypic tests. *Int. J. Syst. Bacteriol.* **34,** 45–55 (1984).
5. Costanzo, J. P., Lee, R. E., and Wright, M. F. Effect of cooling rate on the survival of frozen wood frogs. *J. Comp. Physiol.* **161,** 225–229 (1991).
6. Gavini, F., Mergaert, J., Beji, A., Mielcarek, C., Izard, D., Kersters, K., and DeLey, J. Transfer of *Enterobacter agglomerans* (Beijerinck 1988) Ewing and Fife 1972 to *Pantoea* gen. nov. as *Pantoea agglomerans* comb. nov. and description of *Pantoea dispersa* sp. nov. *Int. J. Syst. Bacteriol.* **39,** 337–345 (1989).
7. Gilardi, G. L. Pseudomonas. *In* "Manual of Clinical Microbiology" (E. H. Lennette, A. Ballows, W. J. Hausler, Jr., and H. J. Shadomy, Eds.) pp. 350–359. American Society of Microbiology, Washington, DC (1985).
8. Gilardi, G. L. *Pseudomonas* and related genera. *In* "Manual of Clinical Microbiology" (A Balows, W. J. Housler, Jr., K. L. Herrmann, H. D. Isenberg, and H. J. Shadomy, Eds.), pp. 429–441. American Society of Microbiology, Washington, DC (1991).
9. Green, R. L., and Warren, G. J. Physical and functional repetition in a bacterial ice nucleation gene. *Nature* **316,** 645–468 (1985).
10. Kaneko, J. T., Yoshida, T., Owada, T., Kita, K., and Tanno, T. *Erwinia herbicola:* Ice nucleation active bacteria isolated from diamondback moth, *Plutella xylostella* L. pupae. *Jpn. J. Appl. Ent. Zool.* **35,** 247–251 (1991).
11. Kim, H. K., Orser, C., Lindow, S. E., and Sands, D. C. *Xanthomonas campestris pv. translucens* strains active in ice nucleation. *Plant Dis.* **71,** 994–997 (1987).
12. Kozloff, L. M., Schofield, M. A., and Lute, M. Ice nucleating activity of *Pseudomonas syringae* and *Erwinia herbicola. J. Bacteriol.* **153,** 222–231 (1983).
13. Layne, J. R., and Lee, R. E. Freeze tolerance and the dynamics of ice formation in wood frogs (*Rana sylvatica*) from southern Ohio. *Can. J. Zool.* **65,** 2062–2065 (1987).
14. Layne, J. R., Lee, R. E., and Huang, J. L. Inoculation triggers freezing at high subzero temperatures in a freeze-tolerant frog (*Rana sylvatica*) and insect (*Eurosta solidaginis*). *Can. J. Zool.* **68,** 506–510 (1990).
15. Lee, R. E., Strong-Gunderson, J. M., Lee, M. R., Grove, K. S., and Riga, T. J. Isolation of ice nucleating active bacteria from insects. *J. Exp. Zool.* **257,** 124–127 (1991).
16. Lelliott, R. A., Billing, E., and Hayward, A. C. A determinative scheme for the fluorescent plant pathogenic pseudomonads. *J. Appl. Bacteriol.* **29,** 470–489 (1966).
17. Lindow, S. E. Epiphytic ice nucleation-active bacteria. *In* Phytopathogenic Prokaryotes (M. S. Mount and G. H. Lacy, Eds.) pp. 335–362. Academic Press, Inc., New York (1982).
18. Lindow, S. E. Competitive exclusion of epiphytic bacteria by ice—*Pseudomonas syringae* mutants. *Appl. Environ. Microbiol.* **53,** 2520–2527 (1987).
19. Lindow, S. E., Arny, D. C., and Upper, C. D. *Erwinia herbicola:* A bacterial ice nucleus active in increasing frost injury to corn. *Phytopathology* **68,** 523–527 (1978).
20. Lindow, S. E., Arny, D. C., and Upper, C. D. Distribution of ice nucleation-active bacteria on plants in nature. *Appl. Environ. Microbiol.* **36,** 831–838 (1978).
21. Lindow, S. E., Hirano, S. S., Barchet, W. R., Arny, D. C., and Upper, C. D. Relationship between ice nucleation frequency of bacteria and frost injury. *Plant Physiol.* **70,** 1090–1093 (1982).
22. Maki, L., Galyan, E. L., Chang-Chien, M. M., and Caldwell, D. R. Ice nucleation induced by

Pseudomonas syringae. Appl. Microbiol. **28,** 456–459 (1974).

23. Maki, L. R., and Willoughby, K. J. Bacteria as biogenic sources of freezing nuclei. *J. Appl. Microbiol.* **17,** 1049–1053 (1978).
24. Mueller, G. M., Wolber, P. K., and Warren, G. J. Clustering of ice nucleation protein correlates with ice nucleation activity. *Cryobiology* **27,** 416–422 (1990).
25. Palleroni, N. J. Pseudomonadaceae. *In* Bergey's Manual of Systematic Bacteriology (N. R. Krieg and J. G. Holt, Eds.) pp. 141–168. Williams and Wilkins, Baltimore (1984).
26. Pooley, L. and Brown, T. A. Effects of culture conditions on expression of the ice nucleation phenotype of *Pseudomonas syringae. Microbiol. Lett.* **77,** 229–232 (1991).
27. Richard, C., Gagne, S., and Antoun, H. A note on the presence of ice nucleation-active bacteria in roots of alfalfa grown in Quebec. *Phytoprotection* **68,** 127–129 (1987).
28. Rogers, J. S., Stall, R. E., and Burke, M. J. Low-temperature conditioning of the ice nucleation active bacterium, *Erwinia herbicola. Cryobiology* **24,** 270–279 (1987).
29. Schmid, W. D. Survival of frogs in low temperature. *Science* **215,** 697–698 (1982).
30. Sprang, M. L., and Lindow, S. E. Subcellular localization and partial characterization of ice nucleating activity of *Pseudomonas syringae* and *Erwinia herbicola. Phytopathology* **71,** 256 (1981).
31. Storey, K. B. Life in a frozen state: Adaptive strategies for natural freeze tolerance in amphibians and reptiles. *Am. J. Physiol.* **258,** R559–R568 (1990).
32. Storey, K. B. and Storey, J. M. Freeze-tolerance in animals. *Physiol. Rev.* **68,** 27–84 (1988).
33. Turner, M. A., Arellano, F., and Kozloff, L. M. Three separate classes of bacterial ice nucleation structures. *J. Bacteriol.* **172,** 2521–2526 (1990).
34. Vali, G. Quantitative evaluation of experimental results on the heterogeneous freezing nucleation of supercooled liquids. *J. Atmos. Sci.* **28,** 401–409 (1971).
35. Warren, G. J., and Wolber, P. K. Heterogeneous ice nucleation by bacteria. *Cryo-Letters* **8,** 204–215 (1987).
36. Wolanczyk, J. P., Baust, J. G., and Storey, K. B. Seasonal ice nucleating activity in the freeze-tolerant frog, *Rana sylvatica. Cryo-Letters* **11,** 143–150 (1990).
37. Yankofsky, S. A., Levin, Z., Bertold, T., and Sandlerman, N. Some basic characteristics of bacterial freezing nuclei. *J. Appl. Meterol.* **20,** 1013–1019 (1981).