Xanthomonas campestris pv. *translucens* Strains Active in Ice Nucleation

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ABSTRACT

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Xanthomonas campestris pv. *translucens* strains isolated from diseased barley plants expressed ice nucleation activity at temperatures from −2 to −8 C. Strains from 12 other *Xanthomonas* species or *X. campestris* pathovars did not exhibit ice nucleation activity at temperatures above −8 C. The ice nucleation activity of six strains of *X. c.* pv. *translucens* varied both quantitatively and qualitatively. The −5 C ice nucleation activity of strains of *X. c.* pv. *translucens* was correlated significantly with extent of frost injury at −5 C to corn seedlings inoculated with these strains.

Additional key words: bacterial diseases of plants

The geographic extent of many vegetable, fruit, and agronomic crops is limited by their level of cold hardiness. Frost-sensitive plant species have low tolerance for intracellular ice formation in their tissues (4,14). The water in flowers and immature fruit tissue of several frost-sensitive crops has been shown to supercool under field conditions (13). The degree of supercooling normally observed in the field, however, is insufficient to avoid intracellular ice formation in tissues (4,14).

The presence of certain epiphytic bacteria on frost-sensitive plants limits the supercooling ability of water in these plants (1,11,16,18–22). These bacteria limit the ability of plants to avoid ice formation and thus predispose them to subsequent frost injury. In the absence of these bacteria, ice formation does not occur in many nonwoody frost-sensitive plants at temperatures above −5 C (11,21).

Many pathovars of *Pseudomonas syringae* van Hall (1,9,11,12,20,21,23,29), some strains of *Erwinia herbicola* (Löhnis) Dye (19,25,32), *P. fluorescens* Migula (24), and *P. viridiflava* (Burkholder) Dawson (27) are active as ice nuclei at temperatures above −5 C. These bacteria function as heterogeneous ice nuclei in supercooled water at temperatures between −1 and −10 C. Thus, they are able to increase the nucleation temperature of water in or on leaves (11,21).

The population size of ice-nucleation-active bacteria on frost-sensitive plants has been correlated with the incidence of frost injury to plants cooled to between −2 and −5 C both in laboratory studies (1,18,19,21,22,27) and in the field (15,16,18). Some woody plant parts also may contain ice nuclei that are not produced by these bacteria (2). Ice nucleation-active strains of *P. syringae* and *E. herbicola* are found as epiphytes on a diversity of plant species from widely separated areas in the United States (11,15–17,20) and other parts of the world (25,32).

In this report, we establish that strains of a pathovar of a fifth bacterial species, the phytopathogen *Xanthomonas campestris* pv. *translucens* (*X. c.* pv. *translucens*), are active in ice nucleation, both in culture and on plants.

MATERIALS AND METHODS

Bacterial strains. All strains of *X. c.* pv. *translucens* were isolated by D. Sands from typical bacterial streak lesions on barley (*Hordeum vulgare* 'Klages') from several locations in Montana (10). All strains produced typical bacterial streak lesions on barley when inoculated under greenhouse conditions (10). Cell suspensions in distilled water (10^7/ml) were injected into the tips of hypocotyls of greenhouse-grown barley (Klages) seedlings in the two-leaf stage with a syringe fitted with a 26-gauge needle. Water-soaked streaks appeared after 10–14 days at a temperature of 20 C. Biochemical tests to confirm the identity of *X. c.* pv. *translucens* were also performed (7,8). All strains were oxidase-positive, gram-negative motile rods that produced a characteristic orange pigment but did not ferment glucose or reduce nitrate. Other pathovars of *X. campestris* and species of *Xanthomonas* used in this study are described in Table 1. *P. syringae* strains S-2, 31R1, and Cit7 from wheat, corn, and citrus leaves, respectively (1), were used as positive controls for ice nucleation activity.

Culture of bacteria. All strains of *X. c.* pv. *translucens* assayed qualitatively for ice nucleation activity or tested for pathogenicity were grown at 28 C for 4 days on Wilbrink's agar (5) containing sucrose, 10 g; Bacto peptone (Difco), 5 g; K_2HPO_4, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.25 g; Na_2SO_3, 0.05 g; and Bacto agar, 15 g per liter of distilled water. *P. syringae* strain S-2 and *P. fluorescens* strain Pf5 were grown at 28 C for 2 days on King's medium B (13). All other strains of *Xanthomonas* spp. were grown at 28 C for 4 days on a medium containing glucose, 10 g; nutrient broth, 8 g; casein hydrolysate, 5 g; yeast extract, 1 g; K_2HPO_4, 0.5 g; and Bacto agar, 15 g per liter of distilled water. Bacterial strains used in quantitative measurements of ice nucleation were grown for 5 days at 21 C on Bacto nutrient agar (Difco) amended to contain 2.5% glycerol. Bacteria used to inoculate corn seedlings for frost sensitivity tests were harvested after 3 days' growth at 22 C from plates of nutrient glycerol agar, suspended in distilled water, and adjusted to 10^7 cfu/ml.

Measurement of bacterial ice nucleation activity. Aqueous suspensions of bacterial strains were diluted to about 10^6 cfu/ml based on initial population estimates made with a Klett-Summerson photometric colorimeter with a green filter for qualitative ice nucleation assays. Three milliliters of each bacterial suspension were placed in each of 12 sterile test tubes and cooled to −8 C in a freezer for 3 hr. A bacterial strain was determined to have ice nucleation activity if one or more tubes of bacterial suspension froze. Uninoculated tubes containing only water did not freeze.

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The quantitative expression of ice nucleation activity by bacterial strains at different temperatures was determined as described previously (18,21). A collection of 40 10-μl drops from each of seven 10-fold serial dilutions of bacterial suspensions was cooled slowly (0.2 C/min) on the surface of a paraffin-coated aluminum block. The freezing of individual drops was determined visually. The temperature of the block was measured with an imbedded thermistor thermometer with a precision of ±0.01 C. The cumulative ice nucleus content of bacterial suspensions was calculated according to Vali (30) as modified by Lindow et al (21). The ice nucleus content was normalized for the number of cells present in the bacterial suspension (as determined by turbidity measurements).

Effects of bacteria on plant frost injury. The frost sensitivity of corn treated with several ice-nucleation-active bacterial species was determined by the methods of Arny et al (1) and Lindow et al (21). Greenhouse-grown three-leaf-stage corn seedlings were sprayed (about 0.5 ml/plant) with an aqueous suspension of about 1×10^7 cfu/ml of each bacterial strain. Ten replicate pots, each containing 10 seedlings, were used for each strain. All strains were compared in the same experiment. After inoculation, plants were placed in a mist chamber at 24 C for 48 hr. Plants were then allowed to dry for 1 hr, randomized with relation to position in a controlled-environment chamber, cooled to −5 C, held at this temperature for 1 hr, and then warmed to 24 C. The fraction of injured leaves in each pot of plants was determined 24 hr after exposure to cold temperatures. The mean fraction of leaves injured for each treatment was obtained by averaging the 10 replicates of each treatment. The average number of ice nuclei per leaf active at −5 C ($N(T)$) was calculated from the fraction of leaves damaged by freezing at −5 C (f) by the procedure of Lindow et al (22) as: $N(T) = -\ln(1 - f)$.

RESULTS

Ice nucleation activity of *Xanthomonas* species. All strains of *X. c.* pv. *translucens* were active in ice nucleation when tested by a tube-freezing technique (Table 1). The qualitative ice nucleation test was verified by the observation of this phenotype in authentic ice-nucleation-active strains of *P. syringae, E. herbicola,* and *P. fluorescens* (Table 1). Ice nucleation activity was not detected in *Xanthomonas* species (other than *X. c.* pv. *translucens*) that were examined, even when tested at concentrations greater than 10^8 cfu/ml. The expression of ice nucleation activity by xanthomonads other than *X. campestris* pv. *translucens*, if present, must be extremely low (i.e., less than 10^{-5} that of active strains).

The in vitro ice nucleation activity of six strains of *X. c.* pv. *translucens* differed greatly when quantified with a droplet-freezing assay (Figs. 1 and 2). All strains except X-56 had threshold ice nucleation temperatures of about −2 C. Only a very low fraction of cells (one cell in 10^7–10^8) expressed ice nucleation activity at these temperatures. A great increase in the fraction of cells that expressed ice nucleation activity was observed with decreasing temperature for strains X-34, X-40, and X-87 (Fig. 1), whereas only a small increase was observed in strains X-99s, X-58, and X-56 (Fig. 2). The fraction of cells active in ice nucleation at −5 C varied from less than one cell in 10^8 for strain X-56 to about one cell in 1,000 for strains X-40 and X-34.

All strains except X-56 exhibited two temperature regions where the ice nucleation activity of cells increased appreciably with decreasing temperature. Ice nucleation activity of all strains except X-56 increased in the temperature ranges of −2 to −4 C and −7 to −10 C. Strain X-56 exhibited detectable ice nucleation activity only at colder temperatures. All strains except X-56 exhibited a relatively high frequency of ice nucleation (one cell in 100–1,000 active in ice nucleation) at temperatures lower than −10 C.

Effects of *Xanthomonas* strains on plant supercooling. All ice-nucleation-active strains of *P. syringae, E. herbicola, P. fluorescens,* and *X. c.* pv. *translucens* significantly reduced the supercooling ability of corn seedlings when inoculated onto the surfaces of healthy plants (Table 2). Some *X. c.* pv. *translucens* strains were as active as ice-nucleation-active strains of *P. syringae* in causing frost damage to corn seedlings at −5 C. Significant differences in the incidence of frost injury to corn treated with different strains of *X. c.* pv. *translucens* were observed (Table 2).

The incidence of frost injury to corn at −5 C was correlated signficantly with the −5 C in vitro ice nucleation activity of *X. c.* pv. *translucens* strains ($r = 0.885$, $P < 0.05$). A highly significant linear relationship ($y = 0.829X + 8.14$, $r = 0.878$, $P < 0.05$) was also found when the number of ice nuclei per leaf was regressed against the nucleation frequency (22) of *X. c.* pv. *translucens* (expressed as log [ice nuclei per cell active at −5 C]).

Table 1. Bacterial strains tested for ice nucleation activity

Bacterial species	Strain	Source[a]	Ice nucleation activity
Pseudomonas syringae	S-2	1	+
	31R1	2	+
	Cit7	2	+
P. fluorescens	F-8	3	+
	Pf5	4	−
Erwinia herbicola	26SR6-2	2	+
Xanthomonas albilineans	29184	5	−
X. axonopodis	19312d	5	−
X. fragariae	NCPP2473	5	−
X. campestris pv. *begoniae*	077-3382	5	−
X. c. pv. *carotae*	Floral-1	5	−
X. c. pv. *campestris*	β73	5	−
X. c. pv. *manihotis*	Xm6	5	−
X. c. pv. *pelargonii*	078-1100	5	−
X. c. pv. *phaseoli*	86	5	−
X. c. pv. *pruni*	8D51	6	−
X. c. pv. *translucens*	14 field isolates	1	+[b]
X. c. pv. *vesicatoria*	β26	5	−
X. c. pv. *vitians*	068-790M-7D51	6	−
	068-1406-7D5	6	−
	069-561-7D52	6	−

[a] Source of strains 1 = D. Sands, Montana State University, Bozeman; 2 = S. E. Lindow, University of California, Berkeley; 3 = L. Maki, University of Wyoming, Laramie; 4 = C. Howell, University of Texas; 5 = M. Sasser, University of Delaware; and 6 = C. Kado, University of California, Davis.
[b] All 14 *X. c.* pv. *translucens* strains tested were active in ice nucleation.

DISCUSSION

Until this report, the only bacteria documented to be active in ice nucleation were three species in the genus *Pseudomonas*, some strains of *E. herbicola*, and possibly *E. stewartii* (31). However, this last report has not been substantiated by other workers including ourselves. Some strains of *X. c.* pv. *translucens* reported here were as active as members of these other genera in this phenotype. A brief report of the qualitative ice nucleation activity of *X. c.* pv. *translucens* has also recently appeared (3). Other pathovars of *X. campestris* tested did not exhibit ice nucleation activity, confirming the results of Paulin and Luisetti (27).

The ice nucleation activity of different *X. c.* pv. *translucens* strains, like that of *P. syringae* strains (11,12,21,22), was shown to vary both quantitatively and qualitatively (Figs. 1 and 2). Nearly all

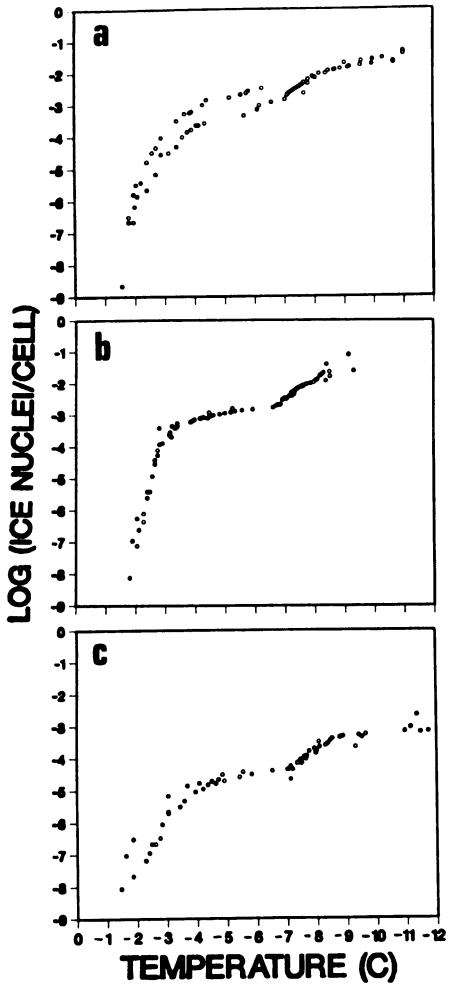

Fig. 1. Cumulative ice nucleation activity of *Xanthomonas campestris* pv. *translucens* strains **(A)** X-34, **(B)** X-40, and **(C)** X-87 as a function of decreasing temperature.

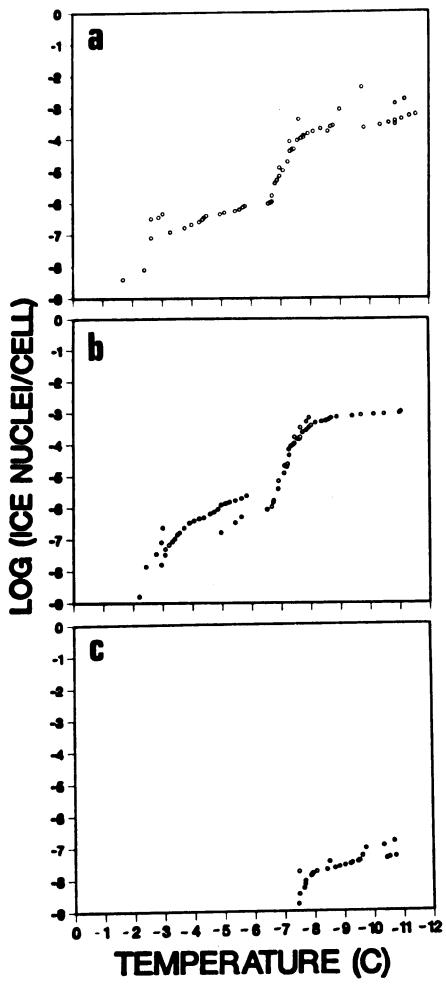

Fig. 2. Cumulative ice nucleation activity of *Xanthomonas campestris* pv. *translucens* strains **(A)** X-99s, **(B)** X-58, and **(C)** X-56 as a function of decreasing temperature.

strains, however, produced ice nuclei that were active at relatively warm subfreezing temperatures (>−5 C). Moreover, the efficiency of ice nucleation by these strains on plant surfaces was highly correlated with that in culture. If we assume the population sizes achieved by the different *X. c.* pv. *translucens* strains on corn were similar, then the relative efficiency of expression of ice nuclei by these strains in vitro and on plants was similar. Thus, although cultural parameters can greatly influence the expression of bacterial ice nucleation (22), the relative efficiency of expression of ice nucleation by different *X. c.* pv. *translucens* strains in a given environment appears constant and probably reflects differences in the genetic determinants for this phenotype.

The large *translucens* group of *Xanthomonas* apparently comprises a number of pathotypes, some with overlapping host ranges of cereals and grasses (8). Recently, the barley pathogen (culturally, physiologically, and biochemically indistinguishable from *X. campestris* except by host range) has been named a pathovar of *X. campestris*, as *X. c.* pv. *translucens* (6,7,33). Because all 14 strains of *X. c.* pv. *translucens* tested exhibited ice nucleation activity, and other strains of *X. campestris* were not ice-nucleation-active (Table 1), there may be justification for efforts to differentiate this taxon from other members of *X. campestris*.

The ice nucleation phenotype of *X. c.* pv. *translucens* may be an important trait determining the epidemiology of this pathogen. Considerable anecdotal evidence has linked epidemics of bacterial leaf streak on barley with frost injury. Black chaff disease recently was reported to be increased by freezing plants inoculated with ice-nucleation-active strains of *X. c.* pv. *translucens* (3). Because of the observation of ice nucleation activity of *X. c.* pv. *translucens*, more detailed work on the possible predisposition of barley to bacterial leaf streak disease by freezing injury, as in other diseases (26,28), should be initiated.

Table 2. Frost damage at −5 C to corn seedlings inoculated with various ice-nucleation-active bacteria 2 days before cold treatment

Bacterial species	Strain	Frost injury[y] (fraction of leaves)
Pseudomonas syringae	Cit 7	0.98 a
Xanthomonas campestris pv. *translucens*	X-34	0.95 a
X. c. pv. *translucens*	X-87	0.92 ab
Erwinia herbicola	26SR6-2	0.88 b
X. c. pv. *translucens*	X-40	0.87 b
P. syringae	31R1	0.79 c
X. c. pv. *translucens*	X-58	0.76 c
X. c. pv. *translucens*	X-99s	0.55 d
X. c. pv. *translucens*	X-56	0.53 d
P. fluorescens	F-8	0.44 e
Control[z]	...	0.01 f

[y] Means followed by the same letter do not differ ($P = 0.05$) according to Duncan's multiple range test.

[z] Plants sprayed only with sterile distilled water before moist incubation and cold treatment.

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