Survival of Ice Nucleation-Active and Genetically Engineered Non-Ice-Nucleating *Pseudomonas syringae* Strains after Freezing

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The survival after freezing of ice nucleation-active (INA) and genetically engineered non-INA strains of *Pseudomonas syringae* was compared. Each strain was applied to oat seedlings and allowed to colonize for 3 days, and the plants were subjected to various freezing temperatures. Plant leaves were harvested before and after freezing on two consecutive days, and bacterial populations were determined. Populations of the INA wild-type strain increased 15-fold in the 18 h after the oat plants incurred frost damage at −5 and −12°C. Plants colonized by the non-INA strain were undamaged at −5°C and exhibited no changes in population size after two freeze trials. As freezing temperatures were lowered (−7, −9, and −12°C), oat plants colonized by the non-INA strain suffered increased frost damage concomitant with bacterial population increases following 18 h. At −12°C, both strains behaved identically. The data show a relationship between frost damage to plants and increased bacterial population size during the following 18 h, indicating a potential competitive advantage of INA strains of *P. syringae* over non-INA strains in mild freezing environments.

The bacterium *Pseudomonas syringae* is a common epiphyte on a large variety of plant species from widespread geographical locations (5, 8, 12, 16–18, 21, 24). Many pathovars (4) of *P. syringae* are ice nucleation active (INA) and cause frost damage to sensitive plants through the initiation of ice crystal formation at temperatures as high as −1.5°C (1, 2, 12–14, 16, 18–21). The damage is caused by ice formation in plant cells which spreads both inter- and intracellularly, resulting in the disruption of cell membranes (3). The extent of freeze injury to plant leaves is directly related to the log of the number of INA bacteria on leaf surfaces (7, 13, 15, 16, 18, 21, 22). Reduction of INA bacteria on leaf surfaces decreases plant frost damage at a given temperature (1, 9, 11–13, 15, 16, 19, 21, 25). In the absence of INA bacteria, herbaceous plant tissues can supercool to temperatures below −5°C before suffering freeze injury (2, 7, 12, 14, 16, 18).

Ice nucleation-deficient strains of *P. syringae* have been constructed from INA strains by deletion of a portion of the chromosomal DNA sequence which codes for a membrane protein essential for ice nucleation (14). These constructed non-INA mutant strains are being evaluated for use in frost control on agricultural crops (9, 11, 14, 15, 19, 23). Reduction of INA bacteria on leaf surfaces, and therefore reduction of frost damage to plants by the application of non-INA strains, has been shown in several studies (1, 11–13, 15, 19, 20). Non-INA strains serving as antagonists are most effective in exclusion of INA strains when applied to plants prior to colonization by the INA bacteria (11, 14, 15). Neither strain appears capable of displacing preexisting populations of the other.

The mechanisms of competition between INA and non-INA strains on leaf surfaces are poorly understood. Knowledge of a potential competitive advantage of one strain over the other would be useful in predicting the outcome of large-scale releases of the non-INA strains for frost control on crops. No differences have been found between the wild-type and deletion mutant strains except for the temperature at which they freeze (11, 14). However, within the INA pathovars of *P. syringae* examined, naturally occurring non-INA strains are relatively low in abundance (5, 18, 24). The prevalence of INA strains when both strains behave identically is unexplained. The purpose of this experiment was to test the survival of INA and non-INA strains of *P. syringae* at various freezing temperatures to determine if there were differences in their survival under conditions similar to those found in nature.

**MATERIALS AND METHODS**

**Bacterial strains.** *P. syringae* Cit7 and *P. syringae* Cit7del1b were obtained from S. E. Lindow, University of California-Berkeley. INA *P. syringae* Cit7 was originally isolated from a healthy navel orange leaf near Exeter, Calif. (14). Strain Cit7del1b is a deletion mutant constructed from Cit7 and is no longer INA. Both strains are resistant to 100 μg of rifampin per ml.

**Culture preparation.** Both *P. syringae* strains were cultured on King medium B (9) supplemented with 100 μg of rifampin and 100 μg of cycloheximide per ml (KBRC). All broth cultures were incubated by shaking at 90 rpm and 28°C. Filter-sterilized antibiotic stock solutions contained 20 μg of rifampin per ml in 100% methanol, 20 μg of cycloheximide per ml in distilled water, and 100% ethanol (1:1, vol/vol). Stock cultures were grown in KBRC broth, diluted by half with 20% sterile glycerol, and stored at −80°C.

Bacteria were prepared for application to plants as follows. A 5-ml volume of an overnight culture in KBRC was used to inoculate 250 ml of KBRC broth and was incubated by shaking at 28°C until the optical density at 600 nm was 0.6 (Beckmann DU65 spectrophotometer). This culture was harvested by centrifugation at 13,700 × g for 5 min at 4°C. A 250-ml volume of wash buffer (0.01 M sterile potassium phosphate) was used to wash cells. This was followed by centrifugation under the same conditions. After two washes the bacteria were suspended in 225 ml of sterile 0.01 M potassium phosphate buffer and diluted with sterile distilled water (1:1, vol/vol) to a final estimated concentration of 5.5 × 10⁸ CFU/ml. Actual determination of CFU per milliliter was made by spread plating serial dilutions of culture in 0.01...
M potassium phosphate buffer onto KBRC agar. Triplicate plates were incubated for 48 to 72 h at 28°C, and colonies were counted.

**Plant propagation.** Common oat (*Avena sativa*) seeds, obtained from a local feed store, were germinated and grown in 15-cm-diameter pots containing a peat-perlite (1:2, vol/vol) potting mixture. Approximately 18 g of seeds were planted per pot. Pots were placed in a growth bay with a 12-h photoperiod. Illumination was provided by two fluorescent lamps with an average intensity of 75 microeinsteins. Temperature and humidity were recorded continuously with a Weather Measure hygrothermograph. The mean maximum and minimum temperatures were 27 and 17°C, respectively, and the average relative humidity was 35%. The soil was watered and seedlings were misted daily with distilled water. For experiments with cold-acclimated plants, pots of seedlings were grown in an environmental chamber under illumination of 375 microeinsteins with 25°C days, 5°C nights, and average relative humidity of 70%.

**Application of bacteria to plants.** A single strain of *P. syringae*, Cit7 or Cit7del1b, was applied to 9- to 11-day-old oat plants. Individual pots of oat plants were spray inoculated by a hand-held plant mister with bacterial suspensions of 5.5 × 10^5 CFU/ml just until runoff. Bacteria were allowed to colonize plants for 3 days. Plants were maintained as described above.

**Plant treatments.** Survival of *P. syringae* Cit7 was measured at temperatures of −5 and −12°C. Strain Cit7del1b was tested at −5, −7, −9, and −12°C. Five replicate pots of colonized oat plants were subjected to freezing on two consecutive days in the −5 and −7°C experiments. For tests at −9 and −12°C, three replicate pots of colonized plants were the maximum number of test plants which could be lowered to the desired temperature because of the cooling limitations of the incubator. Three grams of plant leaves was harvested from each pot of oat plants before the first freeze. Leaves were clipped from plants along a line from the edge of the pot to the center approximately the same height above the soil. After freezing 3 g of leaf material from each pot of oat plants (a pot of oat plants will hereafter be designated a plant), the plants were placed in a Lab Line incubator and the temperature was gradually lowered to the desired value and held for 1 h. Plants were warmed to room temperature and removed from the incubator, and the extent of damage was estimated. Three grams of leaf material was again harvested from each plant immediately after freezing. Damaged and undamaged leaves were harvested from plants partially injured by freezing in proportion to the estimated percentage of injury. The procedure was repeated after 24 h.

Control plants consisted of two types: colonized plants which were not subjected to freezing and frozen noninoculated plants. In each freeze trial, one control plant colonized by the test strain was maintained without freezing and sampled at the same times as frozen plants. One noninoculated control sprayed with sterile distilled water was subjected to the same freezing conditions as test plants. Leaf material was harvested four times from all test and control plants both before and after freezing on two consecutive days.

**Recovery of bacteria from plant surfaces.** Three days after inoculation with bacteria, plant material was harvested before and after freeze treatments as described above. Harvested plant material was immediately placed in 150 ml of sterile peptone water wash (0.01 M potassium phosphate containing 0.1% peptone) (Difco Laboratories) in 250-ml flasks. Flasks were shaken at 260 rpm and 25°C for 2 h to remove bacteria from leaf surfaces. Serial dilutions of the leaf washings were spread plated onto KBRC agar and incubated for 48 to 72 h at 28°C. Triplicate plates were counted to determine CFU/gram (fresh weight) of plant material for each replicate pot. Population sizes from each replicate pot were averaged to determine the mean population size. The limit of detection (30 colonies per plate at the lowest dilution) in these experiments was 1.5 × 10^4 CFU/g of plant material. Selected colonies recovered from each plant replicate in all trials were tested for ice nucleation activity by using a modification of the replica-freezing technique developed by Lindow et al. (17). Colonies were picked from plates and suspended in 0.25 ml of buffer (0.01 M potassium phosphate). Of each cell suspension, 100 µl was placed on a paraffin-coated aluminum foil “boat” floated on the surface of an ethanol bath cooled to −6°C. After 2 min, numbers of frozen and unfrozen droplets were recorded.

**RESULTS**

**Temperature effect on plants.** Oat plants colonized by *P. syringae* Cit7 suffered severe freeze damage after exposure to −5°C, whereas *P. syringae* Cit7del1b-colonized plants were undamaged (Fig. 1A and B). Damage was manifested as flaccidity or discoloration of the leaves upon rewarming of the plants (13). Although plant 4 in Fig. 1B appears droopy and possibly damaged, the leaves showed no signs characteristic of freeze damage, so the appearance was attributed to placement next to an incubator fan which blew air directly onto the plant. Control plants sprayed with sterile distilled water in place of the bacterial solution also appeared undamaged after freezing. Plants colonized by strain Cit7 which were cold acclimated were slightly less damaged at −5°C than plants grown at higher temperatures. At −7°C, oat plants inoculated with strain Cit7del1b suffered visible injury to ca. 20% of the leaves. Approximately 33% of the leaves exhibited damage when Cit7del1b-colonized plants were exposed to −9°C. Extent of damage was determined by estimating the percentage of oat leaves in all frozen repli-
cates showing discoloration or flaccidity. At freezing temperatures of \(-12^\circ C\), Cit7dellb-treated plants and controls sprayed with sterile distilled water in place of the bacterial solution suffered severe damage comparable to that suffered by Cit7-colonized plants frozen at \(-5^\circ C\).

**Survival of bacteria.** Bacterial population sizes on oat leaves were determined with KBRC medium on two consecutive days both before and after freezing. In repeated experiments, the initial mean population size varied between \(10^7\) and \(10^6\) CFU/g of plant material for each strain. This variation was due to the approximate nature of the optical density method used to determine the bacterial numbers in the spray solution and variability in the process of spraying bacteria onto the plants. After cooling plants to \(-5^\circ C\) (Fig. 2A) and maintaining the temperature for 1 h, tests showed no significant difference \((P > 0.05)\) in strain between the means before freezing and after freezing when a paired \(t\) test was used. However, the mean population size of Cit7 before the second freeze (BF2, 18 h later) was significantly larger \((P < 0.05)\) than the population mean recovered immediately after the first freeze (AF1). Strain Cit7 showed a 14.2-fold increase in number on damaged plants in 18 h and an increase of 8.9-fold (data not shown; \(P < 0.01\)) on cold-acclimated plants. The non-INA strain Cit7dellb showed no significant difference in the population means AF1 and BF2 or between any pairs of means from the four treatments.

At freezing temperatures of \(-12^\circ C\) (Fig. 2B), strain Cit7 populations were similar to those in the \(-5^\circ C\) trial. There was no significant difference \((P > 0.05)\) in mean numbers recovered BF1 and AF1. In the interval AF1 to BF2, bacterial numbers increased by 15.5-fold on the damaged plant leaves, but the paired \(t\) test found no significant difference between the means in this case \((P > 0.05)\). Populations of strain Cit7dellb recovered from plants exposed to \(-12^\circ C\), unlike populations in the \(-5^\circ C\) trial, increased by 24.3-fold in the interval AF1 to BF2 \((P < 0.05)\). Oat plants colonized by Cit7dellb, which were partially damaged at temperatures of \(-7^\circ C\) and \(-9^\circ C\), supported bacterial population increases of 6.6-fold \((P < 0.01)\) between AF1 and BF2 in the \(-7^\circ C\) trials (Fig. 3A) and 3.1-fold \((P > 0.05)\) in the \(-9^\circ C\) tests (Fig. 3B). The smaller three-replicate plant sample sizes required in the \(-9^\circ C\) trials resulted in population increases 18 h after freezing considered insignificant in two cases in which populations had increased by 3- and 15-fold (Cit7dellb, \(-9^\circ C\); Cit7, \(-12^\circ C\)), respectively. These apparent contradictions were not observed in trials with five replicate plants.

Freezing had no deleterious effect on the survival of either bacterial strain at any of the temperatures tested. The differences in mean numbers of bacteria recovered BF2 and AF2 were insignificant for both strains in all experiments. Likewise, the population differences BF1 and AF1 were insignificant for all freeze temperatures with the exception of Cit7dellb at \(-12^\circ C\) \((P < 0.01)\).

**Experimental controls.** Nonfrozen controls consisted of oat plants treated with the appropriate \(P.\ syringae\) strain but not subjected to freezing temperatures. One nonfrozen control plant was sampled at the same times as test plants in each temperature trial. Nonfrozen control data from all trials \((n = 6;\ data\ not\ shown)\) were pooled and analyzed. These
control data showed no significant differences between any pairs of population means in the four treatments.

A second type of control plant, which was sprayed with sterile distilled water in place of the bacterial solution, was also maintained in all temperature trials. Leaf washings from one noninoculated control plant were plated on nonselective King medium B. Bacterial populations varied considerably between trials, with an average of $5 \times 10^4$ CFU/g of plant material BF1 and $3 \times 10^5$ CFU/g of plant material BF2. Dilutions of leaf washings from noninoculated controls were also plated onto selective media (KBRC agar). Four rifampin-resistant colonies were recovered from the six trials.

Colonies recovered from test plants and nonfrozen controls were freeze tested (17) at $-6^\circ C$ to confirm the presence of the desired strain of P. syringae. Colonies recovered from plants sprayed with the non-INA strain, Cit7dellb, were negative for 60 of the 60 colonies tested. From plants sprayed with INA strain Cit7, 58 of 60 colonies tested positive for ice nucleation activity. Of the 20 colonies recovered from noninoculated controls, 16 tested negative for ice nucleation activity.

DISCUSSION

Previous work on freeze survival of wild-type and deletion mutant strains of P. syringae involved freezing bacterial suspensions (14) or individual plant leaves in test tubes containing buffer (S. E. Lindow, unpublished data). Experiments with bacterial suspensions in test tubes subjected to repeated freeze-thaw cycles resulted in a decrease in bacterial numbers of approximately 50% after each freeze cycle. In contrast, in test tubes containing individual potato leaves in buffer which were frozen at $-5^\circ C$ for 1 h, there was no effect of freezing conditions on the survival of both strains of bacteria on potato leaves.

The approach taken in these experiments was designed to more closely approximate natural conditions by measuring survival of each strain on oat plant leaves before and after freezing of the entire plant without submersion in water or buffer. Freezing temperatures alone had no effect on survival of either strain for the four temperatures tested, a result in agreement with the $-5^\circ C$ freeze data from potato leaf experiments (14). However, these experiments demonstrated a difference in behavior between the wild-type and deletion mutant strains in response to mild freezing temperatures. The INA strain Cit7 caused freeze damage to oat plants at $-5^\circ C$ and colder by disruption of the plant cells during freezing (3). The release of plant cell contents after disruption due to freezing presumably made nutrients available to bacteria present on the leaf surface and allowed INA bacterial populations to increase as much as 15-fold during the 18 h following the freeze. Gross et al. (6) reported a 10-fold increase in an INA P. syringae strain on apricot flowers following a $-4.7^\circ C$ frost in which flowers were damaged, and higher rates of multiplication have been observed for P. syringae on bark tissue of apricot trees subjected to $-10^\circ C$ (10). Data reported here from strain Cit7 out leaf trials at $-5$ and $-12^\circ C$ agree with these observations.

Populations of strain Cit7dellb on oat plants cooled to $-5^\circ C$ were unaffected. Freezing of plant leaves colonized by non-INA strain Cit7dellb began at lower temperatures, resulting in population increases which approached those of strain Cit7 as the temperature decreased. Partially damaged plants supported intermediate increases in bacterial populations compared with populations on severely damaged plants, although a linear relationship between extent of plant damage and magnitude of bacterial population increase was not observed. The percentage of oat leaf damage sustained at various freezing temperatures was similar to results obtained by Hirano et al. (7) with oat leaves with no detectable INA bacteria and was supported by the observation of Lindow (14) that strain Cit7dellb does not raise the freezing temperatures of plants above those of plants with no bacteria.

The data presented demonstrate that plant leaf damage allows for population increase of P. syringae strains on oat plants under laboratory conditions. Similar population growth may result under natural conditions in mild freezing climates: populations of INA strains of P. syringae increase after plants are freeze damaged, while on plants colonized by non-INA bacteria and undamaged by freezing temperatures, there is no increase in population size. Studies of the effect of mixed populations of INA and non-INA strains on plants subjected to freezing are needed to further address the selective role of the INA phenotype. Phenotypic differences demonstrated here suggest that INA strains may have a competitive advantage over non-INA strains under natural conditions.

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LITERATURE CITED


