Fate of Ice Nucleation-Active *Pseudomonas syringae* Strains in Alpine Soils and Waters and in Synthetic Snow Samples

R. A. GOODNOW, M. D. HARRISON, J. D. MORRIS, K. B. SWEETING, and R. J. LADUCA

Genencor International, 1870 Winton Road, South, Rochester, New York 14618; Department of Plant Pathology and Weed Science, Colorado State University, Fort Collins, Colorado 80523; and Eastman Kodak Company, Rochester, New York 14650

Received 20 February 1990/Accepted 8 May 1990

The stability of the ice nucleation activity (INA) and viability of INA *Pseudomonas syringae* 31a, used as an ice nucleator in the manufacture of synthetic snow, was determined in snow. The viability of *P. syringae* 1-2b, a rifampin-resistant mutant selected from strain 31a to improve recovery from test samples, was determined in laboratory tests of three alpine soil and water samples from three different sources. Snow samples were exposed to environmental conditions or held in darkness at −20°C. Samples of soil and water were maintained in darkness at 0, 7.5, or 15°C. Parent strain 31a INA decreased significantly (>99.0%) in snow exposed to sunlight and freeze-thaw, while the INA of the cell population in snow held in darkness at −20°C remained essentially unchanged. No viable strain 31a was detected in snow exposed to the environment after 7 days, while the viability of strain 31a in snow held in darkness at −20°C decreased to <3% of the original inoculation at the test conclusion. Mutant strain 1-2b viability was undetectable or had decreased significantly 19 days postinoculation in soil samples held at 0 or 15°C. In contrast, 1-2b viability remained detectable at low levels for the duration of the test in soils held at 7.5°C. The 1-2b population demonstrated a significantly longer half-life in peatlike soil than in the loam soils tested. The rate of decrease in 1-2b viability was essentially the same in the three alpine water samples tested with respect to water temperature and sample location.

Maki et al. (10) discovered that a broth culture of *Pseudomonas syringae*, isolated from decaying alder leaves, froze at warmer than expected temperatures. This finding has since been extended to provide the biotechnical basis for using ice nucleation-active (INA) *P. syringae* in manufacturing synthetic snow. In the presence of *P. syringae* INA strains, ice crystal formation, which normally occurs at −8 to −10°C, can be stimulated at −3 to −5°C.

The release of *P. syringae* as an ice nucleator to induce snow has prompted concern by environmental officials about the fate of the organism in snow, alpine soils, and waters. Concerns include (i) that this release will result in an increase of *P. syringae* in the environment where it already exists naturally or an introduction of the organism in areas formerly free of INA *P. syringae*, (ii) that the *P. syringae* INA will accumulate in soil, causing early season frost zones which may shorten the growth period of area vegetation, and (iii) that possible survival of this organism in snow-melt water runoff will affect natural surface waters.

*P. syringae*, a member of the phytopathogenic group of fluorescent pseudomonads, is primarily a foliar pathogen which survives between growing seasons in association with the host plant or host plant residues (1, 4, 8, 11, 14). These organisms have been reported to have no extended soil phase (2). Moreover, Lindow et al. (9) reconfirmed this report by observing that the survival of aerially dispersed *P. syringae* in contact with upper soil layers decreased to an undetectable level after an average of 2 days.

There are few reports concerning the fate of *P. syringae* in water environments or snow. However, in an attempt to preserve *P. syringae* for the long term, Iacobellis and DeVay (7) reported that various pathogenic isolates of *P. syringae* grown on nutrient agar and then inoculated into distilled water (10⁷ to 10⁹ CFU/ml) survived for 24 years, while undergoing an average decrease in viability of only 2 orders of magnitude.

Microbes in the upper layers of snow are confronted with stresses associated with ambient environmental conditions. Grigsby and Calkins (6) reported that only 0.01% of organisms in wastewater survived when exposed to sunlight for a 24-h period. Although not necessarily applicable to *P. syringae*, Morley et al. (13) did demonstrate that a single freeze-thaw cycle (−27 to 23°C) resulted in 40 to 60% mortality of *Pseudomonas paucimobilis* inoculated in soil.

The objective of this study was to determine the effect on viability of a mutant INA strain, 1-2b, of *P. syringae* when added to field samples of alpine soils and waters in the laboratory. Further, the study evaluated the viability and stability of the INA of the parent INA *P. syringae*, strain 31a, in snow.

MATERIALS AND METHODS

Microorganisms. *P. syringae* 31a was isolated from a healthy corn plant and was selected for its positive INA. The isolate has been demonstrated to be nonpathogenic for 82 agriculturally important or alpine plants and is considered nonphytopathogenic (personal communication). Strain 1-2b was selected to be rifampin resistant from a population of strain 31a grown on Flo-agar (BBL Microbiology Systems, Cockeysville, Md.) containing (grams per liter) pancreatic digest (10.0), peptic digest of animal tissue (10.0), dipotassium phosphate (1.5), magnesium sulfate (1.5), agar (4.0), and rifampin (150 ppm [150 µg/g]) to improve recovery of the strain from alpine soil and water samples. Strains 1-2b and 31a possess similar INA, colony type, growth rate, and desiccation survival properties.

*P. syringae* 31a was grown aerobically in computer-
controlled fermentation vessels containing 10 to 15,000 liters of culture broth.

Dry *P. syringae* 31a cell mass was obtained by first concentrating the fermentation cell broth by cross-flow ultrafiltration, followed by pelletization in liquid nitrogen and then freeze-drying. Dried cell pellets were stored at −20°C. A $10^6$-CFU/ml suspension of strain 1-2b was prepared by rinsing a 24- to 36-h-old growth off Flo-agar plates containing 150 μg of rifampin per g. This suspension was diluted with sterile distilled water and mixed with alpine soils or waters to provide at least $10^2$ viable organisms per gram of test soil or milliliter of test water.

**Snow preparation.** A suspension of desiccated *P. syringae* 31a (0.57 g/liter of H$_2$O) was prepared and injected through a commercial snow gun system (Ratnik Industries, Victor, N.Y.) at a final concentration of 0.08 mg/liter of water. The ambient temperature during snow preparation was −11° to −9°C.

**Viability in snow.** Samples (50) of prepared snow, collected with a sterile spatula from the formed snow, were placed in sterile 50-ml glass tubes (29 by 120 mm, Cortex tubes; Fisher Scientific Co., Pittsburgh, Pa.) which permitted up to 80% transmission of incident UV light present in natural sunlight. Twenty-five of the samples were maintained out of doors and exposed to natural fluctuations in temperature and sunlight for 21 days. During this time interval, outside temperatures varied between −14 and 34°C. The other 25 samples were held in darkness in a −20°C freezer.

Snow samples were thawed on ice and diluted in sterile water, and 0.1 ml of an appropriate dilution was plated on Flo-agar plates in triplicate to determine cell viability (CFU per milliliter).

**INA.** INA was determined by a droplet-freezing method described by Vali (17). A total of 120 10-μl droplets from serial dilutions of each snow sample in 0.01 M potassium phosphate buffer (pH 7.0) were placed on the surface of a paraffin-coated aluminum foil sheet floating on a glycol (60%) bath at −5°C. After 5 min, droplet freeze was determined visually. Total INA per gram of Snowmax powder was calculated by using the following equation:

$$\text{Log INA per gram of dry powder} = \log \left[ \frac{N_o}{N(O)} \cdot \frac{C}{V} \cdot 10^{-D} \right]$$

where $N_o$ is the total number of drops analyzed, $N(O)$ is the total number of drops unfrozen at −5°C, $V$ is the volume of drops (milliliters), $C$ is the concentration of powder (milliliters per gram of powder), and $D$ is the number of 1:10 serial dilutions of sample. Analysis for INA was performed in triplicate, and averages were recorded.

**Statistical analysis.** Survival of *P. syringae* 1-2b in inoculated soil was evaluated by plotting the log CFU per gram of soil against time in days. For the water samples, the log CFU per milliliter was plotted against time in hours. To model the survival rates of *P. syringae*, a logistic function was fit to the data.

Survival rates were compared by using half-life, defined as the time it takes for a 50% reduction in CFU. The logistic function allows for extrapolating survival for those populations that failed to achieve a 50% reduction before the study was terminated. To determine the fate of strain 31a in synthetic snow samples, the rate of decrease in *P. syringae* viability was estimated by fitting an exponential decay model to the CFU versus log day (3). The rate of decline in INA was estimated by using a straight-line linear regression of INA versus log days (3).

**Alpine soils.** Samples of alpine soil were collected from the Nakiska ski area, Calgary, Alberta, Canada; from Rabbit Ears Pass, near the Steamboat Springs, Colo., ski area; and near the Breckenridge Colo., ski area. Soils were stored at 7.5°C in watertight containers until used.

Each soil sample was thoroughly mixed, and five subsamples were taken from each. Four of these samples were oven dried at 110°C until their weight became constant. Moisture content was calculated on an oven-dried weight basis. Pressure plate analysis was performed on the fifth sample to determine water holding capacity at a pressure of 3.3 kPa (field capacity) and 1,500 kPa (permanent wilting point).

Weighted amounts of soil were mixed with sufficient sterile distilled water containing about $3.0 \times 10^4$ to $1 \times 10^5$ CFU of *P. syringae* 1-2b per gram of oven-dried soil and allowed to achieve 100% field capacity (3.3 kPa of tension). The soil was then placed into 12 10.2-cm-diameter plastic pots. Each pot of soil was weighed periodically, and sufficient water was added to return soil to its original weight and moisture level. Control soil was prepared the same way, except that sterile distilled water without *P. syringae* was added. Four replicate pots per treatment, including controls, were placed in each of three incubators maintained at 0, 7.5, and 15°C.

Immediately after addition of *P. syringae* 1-2b and on days 2, 4, 8, 16, and 19 (days 2, 6, 14, 17, and 23 for the Canadian soil), samples of soil equivalent to 1.0 gram of oven-dried soil were taken from each of the four pots in each treatment. After being sampled, the pots were returned to the controlled temperatures and held until the next sampling. Samples were suspended in sterile distilled water and mixed with a vortex mixer to disperse soil particles and bacteria. One 0.2-ml portion of the suspension was spread plated onto each of four Flo-agar plates containing 150 ppm of rifampin (150 μg/g) to reduce background populations of aerobic bacteria and 30 ppm of the fungicide 2,6 dichloro-4-nitroaniline (Botran; Nor-Am Chemical Co., Wilmington, Del.) and 100 ppm of cycloheximide (100 μg/g) (Acti-dione; The Upjohn Co., Kalamazoo, Mich.) to inhibit fungal growth. The Flo-agar plates were incubated at room temperature (22 to 25°C) for 3 days, and colonies of fluorescent bacteria were
from each four Flo-agar samples from Walton Creek at water sample milliliter per pendulum counted. Counts were converted to CFU per gram of oven-dried soil and averaged for the four replicates.

Alpine waters. Samples of alpine water were collected from Walton Creek near Rabbit Ears Pass, Colo., the Elk River near Steamboat Springs, Colo., and the Blue River near Breckenridge, Colo. A sample was collected from each stream and returned to the laboratory on the day of collection. Samples were stored at 7.5°C until used. Water (150 ml) from each source was added to each of 24 presterilized 600-ml Erlenmeyer flasks. Flasks were plugged with sterile cotton stops, placed randomly into wooden racks, suspended in water bath tanks in a greenhouse, and held at constant temperatures of 0, 7.5, and 15°C. Flasks were shaded to prevent exposure to sunlight.

Four replicate flasks, containing strain 1-2b and four controls, for each of the three water sources were included at each temperature. Test flasks and water were allowed to equilibrate to the desired temperatures before addition of \textit{P. syringae}. The strain 1-2b culture was prepared in the same manner as described for the mixture with soil. Immediately after inoculation (time zero), one 0.2-ml portion of inoculated control water from each flask was spread on each of four Flo-agar plates containing microbial inhibitors similar to those used in the soil studies to reduce growth of background organisms. The plates were incubated at room temperature (22 to 25°C) for 3 days. The number of fluorescent colonies per milliliter of test water was determined. Sampling was repeated at 1, 2, 4, 8, 16, 32, 64, 128, 360, and 604 h after inoculation. The number of viable \textit{P. syringae} 1-2b in each water sample at various temperatures and time intervals was calculated by averaging CFU per milliliter from four replicate samplings.

**TABLE 1.** Soil characteristics

<table>
<thead>
<tr>
<th>Source</th>
<th>Soil type</th>
<th>Water-holding capacity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nakiska, Alberta, Canada</td>
<td>Gray clay loam</td>
<td>18.3 6.5</td>
</tr>
<tr>
<td>Rabbit Ears Pass, Steamboat Springs, Colo.</td>
<td>Brown clay loam</td>
<td>23.4 12.7</td>
</tr>
<tr>
<td>Breckenridge, Colo.</td>
<td>Peatlike</td>
<td>41.1 29.8</td>
</tr>
</tbody>
</table>

FIG. 2. Viability of \textit{P. syringae} 31a (△) and INA (■) in snow samples exposed to the environment.

FIG. 3. Viability of \textit{P. syringae} 1-2b in Nakiska, Alberta, clay loam soil. ○, 0°C; □, 7.5°C; △, 15°C.

**RESULTS**

Strain 31a viability in snow. During the 21-day test period, snow samples exposed to the environment were subjected to 20 freeze-thaw cycles due to daily temperature fluctuations. The viability of strain 31a in snow samples stored in the dark at −20°C decreased over time to where <3.0% of the initial inoculum was detectable at day 21 at test termination (Fig. 1). In contrast, the viability of strain 31a in snow samples exposed to sunlight and freeze-thaw decreased rapidly and they were undetectable at day 7 (Fig. 2). However, there was no significant difference between the exponential rate of decrease of strain 31a viability in snow held in darkness at −20°C (1.13 with a standard error of 0.163; Fig. 1) and samples exposed to sunlight and freeze-thaw (1.43 with a standard error of 0.256; Fig. 2).

INA. The INA of strain 31a in snow held in darkness at −20°C demonstrated essentially no decrease over the 21-day test period (Fig. 1). In contrast, the INA of strain 31a in snow exposed to the environment decreased by >99.0% over a 21-day period (Fig. 2).

Strain 1-2b viability in alpine soils. The general characteristics of the three soils are shown in Table 1. At 19 days postinoculation of soil samples held at 0 or 15°C, strain 1-2b viability was either undetectable or had decreased significantly (Fig. 3, 4, and 5). In contrast, the viability level of strain 1-2b remained at a significantly greater level \((P < 0.05)\) in the three soil samples held at 7.5°C.

FIG. 4. Viability of \textit{P. syringae} 1-2b in Steamboat Springs clay loam soil. ○, 0°C; □, 7.5°C; △, 15°C.
Further, the half-life of the 1-2b population inoculated in peatlike soil was significantly longer (P < 0.05) than the 1-2b population half-life in either of the loam soils collected near Steamboat Springs or Nakiska (Table 2).

Strain 1-2b survival in alpine waters. The viability of strain 1-2b remained relatively constant in each of three water samples (Elk River, Walton Creek, and Blue River) for 32, 32, and 128 h, respectively. This was followed by a steady decline in viability (Fig. 6, 7, and 8). There was no significant difference detected in the change in viability of the strain 1-2b population due to varying water temperatures or due to selecting water samples from different sources; however, there was a slight reduction in the 1-2b population half-life in waters held at 15°C (Table 2).

DISCUSSION

The biotechnical application of P. syringae (INA) as an ice nucleator in the manufacture of synthetic snow has raised questions concerning the release of this type of organism into alpine environments. The P. syringae (INA) cell, primarily encapsulated in ice-snow crystals, contacts soil and natural waters from snow melt runoff. However, most snow is eventually exposed to sunlight and freeze-thaw prior to soil or natural water contact. This study demonstrated the detrimental effects of ambient environmental conditions on both the INA and the viability of P. syringae 31a in snow samples held in glass collection tubes. Similar strain 31a INA decay and lack of viable strain 31a buildup were demonstrated in synthetic snow samples taken directly from a Dillon, Colo., and a Nakiska, Alberta, Canada, ski area (unpublished reports). Further, the present study suggests that only one to two freeze-thaw cycles (frozen prepared snow to snow sample thaw) are necessary to significantly decrease strain 31a viability (Fig. 1). It also suggests that INA would be maintained in deep synthetic snow until subjected to sunlight and freeze-thaw exposure (Fig. 1).

Further, due to the demonstrated instability of INA in exposed snow, it is unlikely that frost zones would develop in areas where P. syringae INA is released.

This study complements the work of others reporting that P. syringae has no extended soil phase (2, 16). The viability of the mutant 1-2b population inoculated in soil was eventually undetectable in all three soil samples held at 0°C and was undetectable at the study conclusion in the two loam soils held at 0 and 15°C. However, 1-2b was detectable at low levels and decreased during the duration of the test in all three soil types held at 7.5°C.

The extended half-life of the 1-2b strain population may have been due in part to the peatlike soil having the greater water-holding capacity of the three soil types tested (Table 1). Testing at somewhat higher temperatures, McCarter et al. (11) reported that P. syringae was undetected in soil held at 18 to 38°C for 7 days post-soil inoculation. These findings suggest that when laboratory-selected P. syringae strains are released into soil environments, as an essentially pure culture free from specific host tissue, the organism would not be expected to utilize most soil environments as part of its propagative habitat.

Similarly, this study indicates that the 1-2b population was

![FIG. 5. Viability of P. syringae 1-2b in Breckenridge peatlike soil. ○, 0°C; ■, 7.5°C; Δ, 15°C.](image)

![FIG. 6. Viability of P. syringae 1-2b in Elk River water. ○, 0°C; ■, 7.5°C; Δ, 15°C.](image)

![FIG. 7. Viability of P. syringae 1-2b in Walton Creek water. ○, 0°C; ■, 7.5°C; Δ, 15°C.](image)
stressed when inoculated into alpine water samples, as the viability decreased to either undetectable levels (samples held at 15°C) or to low levels (<10%) in samples held at 0 to 7.5°C over a relatively short period of time.

The report of survival of numerous pathogenic P. syringae kept in sterile distilled water under laboratory conditions for an average of 24 years (7) apparently reflects a vastly different water environment than that found in nonsterile alpine waters. The present study further suggests that the mutant P. syringae INA 1-2b tested was more hardy in alpine waters than reported for specific enteric bacteria in lake waters (5, 15), while significantly less capable of extended survival in natural waters than a sporeforming Bacillus thuringiensis (12).

ACKNOWLEDGMENTS
We thank James Pochodylo and Kris Carlberg for the manufacture of synthetic snow and G. Katz for review of the manuscript.

LITERATURE CITED