Survival of ice nucleation-active and genetically engineered inactive strains of Pseudomonas syringae

Mark P Buttner

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Survival of ice nucleation-active and genetically engineered inactive strains of *Pseudomonas syringae*

Buttner, Mark P., M.S.
University of Nevada, Las Vegas, 1989
SURVIVAL OF ICE NUCLEATION-ACTIVE AND GENETICALLY ENGINEERED INACTIVE STRAINS OF PSEUDOMONAS SYRINGAE

by

Mark P. Buttner

A thesis submitted in partial fulfillment of the requirements for the degree of

Master of Science

in

Biology

Biological Sciences Department
University of Nevada, Las Vegas
August, 1989
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August, 1989
ABSTRACT

The survival of ice nucleation-active (INA) and genetically engineered non-INa strains of *Pseudomonas syringae* was compared under starvation and freezing conditions. In the starvation experiments, both strains displayed an initial increase in viable numbers followed by a rapid decrease to less than ten percent of the initial culture. An increase in viable numbers of both strains after five weeks followed a decrease in total counts, suggesting cryptic growth of starved cells. Cells of both strains of *P. syringae* decreased in size, changing from rods to coccobacilli, but did not form ultramicrocells during the six week starvation.

Growth rates and length of the lag phase during recovery from starvation were nearly identical for both strains. The lag time increased with length of starvation until the third week, where a maximum lag of 28 hours was attained. Both strains of *P. syringae* demonstrated the ability to survive under starvation conditions, which may be an adaptation shared by other epiphytic bacteria. Both strains responded similarly in starvation and recovery experiments, indicating that the presence or absence of the ice nucleating protein does not affect survival of *P. syringae* under non-freezing conditions.

In the freezing experiments, each strain was applied to oat seedlings, allowed to colonize for three 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 hours 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 hours. 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 hours, indicating a potential competitive advantage of INA strains of P. syringae over non-INA strains in mild freezing environments.
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CHAPTER I

INTRODUCTION
BACKGROUND

The habitat of plant leaf surfaces, the phylloplane, is suitable for a variety of microorganisms, called epiphytes. The dominant microbial epiphytes on most terrestrial plants are bacteria, including both gram negative and gram positive species, although populations of yeast and fungi may exceed that of bacteria on some plant species (15). The size and makeup of epiphytic microbial populations at any given time depend on the chemical, biological and physical environment of the leaf surface (28). The sum of these factors influence the processes of growth, survival, death, emigration and immigration of microorganisms.

Bacteria on aerial plant surfaces may be referred to as pathogenic or saprophytic (non-pathogenic) (9). Phytopathogens have the potential to cause diseases or impairments which result in death or decreased growth yield of the plant. Bacterial species which are plant pathogens occur in the genera *Agrobacterium, Corynebacterium, Erwinia, Mycoplasma, Pseudomonas, Spiroplasma, Streptomyces and Xanthomonas* (7). Most of these bacterial phytopathogens are aerobic non-spore-forming rods. Many of these species belong to a class known as conditional, or opportunistic pathogens. Bacteria in this group are pathogenic only during appropriate environmental conditions and/or physiological states of the plant. Within this category are the bacteria which cause frost damage to plants by initiating ice crystal formation.

Frost damage to agricultural crops is a serious problem which results in production losses estimated at over $1 billion annually in the United States alone (43). Many plants, including herbaceous annuals, flowers of deciduous fruit trees,
and fruit of many plant species are frost-sensitive, meaning they cannot tolerate ice formation within their tissues (10, 13, 41). These plants must, therefore, avoid ice formation to escape frost injury. Ice formation in cells of these plants spreads both inter- and intracellularly, resulting in disruption of cell membranes (10). The damage is manifested as a flaccidity or discoloration of the plant leaves upon rewarming (41). Frost-hardy species are able to maintain cell integrity by restricting ice formation to the extracellular spaces, but suffer freeze-dehydration in the process (10).

Ice nuclei, which catalyze the transition from water to ice, are required for freezing to occur. There are two types of ice nuclei, heterogeneous and homogeneous. Heterogeneous ice nuclei are formed from materials which cause the ordering of water molecules into a lattice resembling ice, perhaps by aggregation of water molecules onto the solid face of the nucleus, as in the case of inorganic salts (11, 40). In the absence of heterogeneous ice nuclei, small volumes of pure water can supercool to as low as -40°C before homogeneous ice nucleation occurs due to random grouping of water molecules into an ice-like array (8). Several nonbiological sources of heterogeneous ice nuclei exist, the most common of which are minerals and inorganic materials such as dust. Most of these nuclei are efficient only at temperatures below -8°C (40). Plant materials alone are inefficient ice nuclei. Frost-sensitive herbaceous plant tissues can supercool to temperatures below -5°C before suffering freeze injury (5, 12, 26, 40, 43, 48, 50). However, frost damage most often occurs at temperatures between -2 and -5°C (48), warmer than the supercooling limits of plants. Therefore, heterogeneous ice nuclei in the form of atmospheric particles associated with the
plant surface were thought to be responsible. Recently, it was discovered that certain epiphytic bacteria are active in ice nucleation (57).

Five known bacterial species are ice nucleation-active (INA), having the ability to catalyze ice formation by serving as the site of heterogeneous ice nucleation. Many pathovars (17) of *Pseudomonas syringae*, and strains of *P. fluorescens, P. viridiflava, Erwinia herbicola* and *Xanthomonas campestris* pv. *translucens* catalyze ice formation at temperatures warmer than -5°C (42, 56). None of the hundreds of other bacterial species tested exhibit ice nucleation activity at temperatures warmer than -15°C (43, 49, 56). The most abundant INA bacterium is *Pseudomonas syringae* (39, 48, 49), which is a common epiphyte on a large variety of plant species from widespread geographical locations (24, 39, 40, 48, 49, 53, 62). Lindow et al. (49) found that 76% of over 1,000 INA isolates from 74 plant species were *P. syringae*. INA strains of *P. syringae* initiate ice crystal formation at temperatures as high as -1.5°C (5, 6, 12, 40, 41, 43, 48, 50-53), which places them among the most active naturally occurring ice nuclei known (41).

Not all cells of an INA bacterium are capable of forming ice nuclei at a given temperature. The nucleation frequency, or the fraction of cells active as ice nuclei, increases rapidly with decreasing temperature (39, 41, 48). For example, only 1 in $10^7$ to $10^8$ *P. syringae* cells is active as an ice nucleus at -1°C. Approximately 1 in 10 cells is active at -4°C and below (41). The nucleation frequency of an INA bacterium can be determined if both the number of ice nuclei and the total number of INA bacteria are known. Using the droplet-freezing procedure and equations of Vali (74), the number of ice nuclei in leaf-
washing liquid can be calculated by cooling many equal volumes of liquid and recording the number of frozen and unfrozen droplets at each temperature. The number of INA bacteria from leaf washings can be determined using the replica-freezing technique of Lindow (49). Colonies cultured from leaf washings are replica-plated (37) onto aluminum foil boats which are then cooled to -5°C. A fine mist of pure water is then sprayed onto the surface of the boat. INA colonies cause ice formation and are easily identifiable. By combining these two methods, the fraction of viable cells active as ice nuclei at a given temperature can be determined.

Populations of INA bacteria in nature vary both seasonally and with plant species (24, 28, 39, 49, 53). INA populations range from 10 to 10^7 bacteria per gram fresh weight of plant tissue and comprise from less than 0.01 to 10% of the total population of plants (56). Seasonally, INA bacterial populations are highest in the spring and fall, which coincides with the periods when frosts are most likely to occur and when frost-sensitive plant tissues such as buds, flowers, and fruit are present. Populations are lowest during the warm, dry summer months and on young vegetative tissues of plants.

The extent of freeze injury to many plant leaves is directly related to the logarithm of the number of INA bacteria or the log of ice nuclei on leaf surfaces; higher INA bacterial populations result in greater frost damage (26, 41, 45, 48, 50, 53, 54). Also, the supercooling temperature of plants is related directly to the log of the number of INA bacteria on leaves and increases with increasing INA populations (42, 46).
Traditional methods of frost control are based on maintaining the temperature of frost-sensitive plant tissues above the freezing temperature. These methods include the use of heaters, wind machines, sprinklers, insulation and artificial fogs (41). Limitations with these methods are combinations of high cost, low efficiency, and safety and environmental hazards. The discovery of epiphytic bacteria as the source of ice nuclei on plants, however, has made possible several alternate methods of frost protection.

Treatments which reduce the numbers and/or the ice nucleation activity of INA bacteria on leaf surfaces have potential as frost management tools. These methods of frost protection have been reviewed by Lindow (40, 41) and can be divided into three categories: bactericides, ice nucleation inhibitors and antagonistic bacteria. Bactericides have been successful in reducing INA populations when applied before bacterial populations develop, but have the potential for the selection of resistant strains. Ice nucleation inhibitors are compounds which inactivate the ice nucleating site of the bacteria without necessarily killing the cell. They have the advantage of fast action, within minutes to hours, but many are too phytotoxic for use on plants or are water soluble and likely to be washed from foliar surfaces. Much research needs to be done in these two areas in order for them to be effective as frost control agents. The remainder of this paper will be concerned with the potential use of antagonistic bacteria for biological frost control.

Natural competition by indigenous non-INA bacteria is usually inadequate to prevent a large population of INA bacteria from colonizing leaf surfaces. Therefore, in an effort to enhance competition and reduce INA populations,
various antagonistic bacteria have been applied to plant surfaces. Significant reductions in INA populations were obtained only when antagonistic non-INA bacteria were applied to plants when INA populations were low, for example on young vegetative tissues (39, 51). All successful antagonists acted by preventing population increases of INA bacteria and not by displacement of established populations (42, 43, 51). Preliminary studies indicated the establishment of non-INA antagonistic bacteria on plants resulted in reduction of INA bacterial population sizes from 10 to 100-fold, and reduction of frost injury from 30 to 95% compared with untreated plants (39, 44-46, 51, 52).

Only 58% of naturally occurring non-INA bacteria antagonistic to *P. syringae* on leaf surfaces produced antibiotics against this organism in vitro, and antibiosis-minus mutants of these bacteria were equally as antagonistic to *P. syringae* as their antibiotic-producing parental strains (44, 47). Therefore, competition for limited environmental resources appears sufficient to account for antagonism between epiphytic bacteria (42). However, these antagonistic non-INA strains differed in the degree to which they excluded INA *P. syringae* strains from leaf surfaces. It was hypothesized by Lindow (46) that "a similarity in ecological habitat preference and thus in genetic similarity is required to optimize competitive exclusion of *P. syringae* or other species from leaf surfaces by this or other related species". To test the specificity of competition, mutant strains lacking the INA phenotype were constructed from INA strains of *P. syringae*.

In order to construct non-INA strains, the DNA sequence conferring ice nucleation activity was first determined and characterized. DNA from *Pseudomonas syringae* Cit7 was purified, partially digested with restriction
endonucleases and ligated into a cosm I vector (67). The vector was then introduced into Escherichia coli cells by transduction. Only transduced E. coli cells were able to grow on a selective medium containing the antibiotic tetracycline, due to a tetracycline resistance gene carried by the cosmid. The colonies which carried the "ice" gene were easily identified by testing for ice nucleation activity using the replica freezing technique described earlier (49). E. coli cells that were normally non-INA were converted into the INA phenotype using this method (67).

The isolation of the ice region of the genome of P. syringae into E. coli allowed the construction of non-INA mutant strains. Deletions within the ice region were made by treatment with restriction endonucleases followed by re-ligation of the DNA (43, 67). Plasmids containing the ice region of P. syringae but lacking an internal restriction fragment were unable to confer the INA phenotype to transduced E. coli cells. Non-INA mutants of P. syringae Cit7 were then constructed using the method of Ruvkun and Ausubel (69), which results in a reciprocal exchange of the plasmid-borne deleted ice region with the chromosomal ice region. The product was a non-INA mutant strain, P. syringae Cit7del1b, which was obtained from the parental strain, Cit7.

The gene which determines ice nucleation in P. syringae is approximately 4.2 kilobases in length (66, 67). The gene sequence predicts a protein largely composed of a repeated 16 amino acid sequence (23), which is consistent with the idea that an ice nucleus must have the symmetry or regularity required to bind water molecules in an orderly manner resembling the lattice of ice. The ice protein has been isolated and purified, and the structure corresponds well with
that predicted from the gene sequence (75). Other INA bacteria exhibit some sequence homology with the *P. syringae* Cit7 ice gene (43), and it has been shown that antibodies raised against the ice protein of *Pseudomonas fluorescens* will cross-react with the ice proteins of *P. syringae* and *Erwinia herbicola* (14).

The ice nucleation site is localized in the outer membrane of the Gram negative cell wall (21), however the actual structure and organization of the nucleating site are unknown. Experiments indicate the site is variable in size and composed of between 1 and 53 ice protein subunits along with lipids and other membrane components (21, 22, 34, 35).

The genetically engineered deletion mutant strain Cit7del1b has been tested extensively in the laboratory. The bacterium does not cause frost damage to plants above -5°C, unlike the INA parental strain, and freeze injury to plants colonized by strain Cit7del1b is not significantly different from plants without INA bacteria at temperatures above -7°C (43). Strain Cit7del1b did not differ from the INA parental strain Cit7 in growth rate, survival or population size on any of 67 plant species tested. There was no difference in their survival in soil, or in any of 178 biochemical and physiological tests (43). The only observed difference between the two strains is the temperature at which they freeze.

The results of competition experiments between strains Cit7 and Cit7del1b and other strains were dependent on the relative initial population size and not the genotype of the bacterial strains (46, 56), contrary to Lindow’s hypothesis that genetic similarity optimizes competitive exclusion of *P. syringae*. When the two strains were co-inoculated onto plant leaf surfaces, the strain inoculated in fewer numbers exhibited little growth. Non-INA Cit7del1b, when applied before
inoculation with INA strains, reduced the population size of INA strains compared with plants not treated before inoculation with INA strains. The reverse was also true, however. Neither strain could displace pre-existing populations of the other.

These laboratory experiments concluded that: 1) neither strain exhibited a competitive advantage over the other, and 2) the most effective application of strain Cit7del1b as a biological frost control agent would be prior to colonization of leaf surfaces by large populations of INA bacteria. Field tests are ongoing to evaluate the behavior and frost control potential of genetically engineered strains of *Pseudomonas syringae* (55, 56), the first recombinant microorganisms approved for release into the environment.

**EXPERIMENTAL APPROACH**

The mechanisms of competition between INA and non-INA strains on leaf surfaces are poorly understood. Much of the current research is devoted to studying the competitive interactions of the two strains (38, 46, 55, 56). Knowledge of a potential competitive advantage of one strain over the other would be useful in predicting the outcome of large-scale releases of non-INA strains for frost control on crops. In addition, there is much concern over the possibility of displacement of INA strains by non-INA strains and the resulting ecological impact. For example, INA bacteria are possibly significant sources of atmospheric ice nuclei with importance in initiating precipitation (22, 40), and it
has been suggested that a large reduction in their number could affect rainfall and global climate.

No differences have been found between the two strains except for the temperature at which they freeze (38, 43). The prevalence of INA strains of *P. syringae* when both strains behave identically is unexplained. It has been speculated that the INA phenotype confers a selective advantage to those epiphytic bacteria which possess it, however this has not been demonstrated in the laboratory. Certainly the ubiquity of INA strains and the homology between the ice genes from different species raises some interesting questions about the origin and adaptive role of the INA phenotype.

The approach taken in these experiments was to examine two factors, nutrient deprivation (Chapter II) and freezing temperatures (Chapter III), which could contribute to a competitive advantage for either the INA strain or the non-INA mutant strain under natural conditions. The null hypothesis formed was that there is no significant difference in growth or survival between *Pseudomonas syringae* wild-type INA strain Cit7 and the deletion mutant non-INA strain Cit7del1b.

Testing the survival of the two bacterial strains under freezing conditions was chosen because freezing represented the only known phenotypic difference between the two strains and the most obvious environmental condition where a difference in growth or survival might be found. Although earlier work which had been performed on freeze survival of the wild-type and deletion mutant strains of *P. syringae* found no differences between the two strains, the approach taken in these experiments was designed to more closely approximate natural
conditions. The previous experiments involved freezing bacterial suspensions (43) or individual plant leaves (S. E. Lindow, unpublished data) in test tubes containing buffer which were then frozen at -5°C. Neither strain survived well in suspension after repeated freeze-thaw cycles and both strains were unaffected when frozen on potato leaves in buffer for 1 hour.

It was thought that test tube freezes represented an unrealistic and probably harsher condition than occurs in nature. Therefore, in these experiments, the survival of each strain was measured on oat (*Avena sativa*) leaves before and after freezing of the entire plant without submersion in water or buffer.

Survival of the two strains under starvation conditions was also measured. Starvation survival has been defined as a physiological state resulting from an insufficient level of nutrients, especially energy, to provide for growth and reproduction (60). The leaf surface can be viewed as a hostile environment for microorganisms which are exposed to extremes of temperature, solar radiation and desiccation (28). Epiphytic microorganisms compete for limiting nutrients and are dependent on water to leach inorganic and organic compounds from leaves (20). The successful epiphytic bacterium must also be able to survive discontinuities in growth caused by seasonal effects on either the bacterium or the plant host. Thus, both long- and short-term starvation occurs on plant leaf surfaces and the vegetative cells of INA bacteria must be able to withstand these conditions, since INA species are non-spore-formers.

The starvation survival process has been extensively studied in marine bacteria (1-4, 19, 32, 36, 61, 63-65, 71, 71) and in other aquatic and soil bacteria
(59, 60,), however the process has not been studied in epiphytic bacteria. The purpose of this study was to compare the starvation survival process of the INA strain *P. syringae* Cit7 and the non-INA genetically engineered strain Cit7del1b to determine if there were differences in their survival or recovery which could contribute to a competitive advantage of one strain over the other.
CHAPTER II

STARVATION SURVIVAL OF PSEUDOMONAS SYRINGAE
ABSTRACT

The starvation survival of a wild-type ice nucleation-active (INA) strain and a non-INA deletion mutant strain of *Pseudomonas syringae* were compared for six weeks. Both strains displayed an initial increase in viable numbers followed by a rapid decrease to less than ten percent of the initial culture. An increase in viable numbers of both strains after five weeks followed a decrease in total counts, suggesting cryptic growth of starved cells. Cells of both strains of *P. syringae* decreased in size, changing from rods to coccobacilli, but did not form ultramicrocells during the six week starvation. Growth rates and length of the lag phase during recovery from starvation were nearly identical for both strains. The lag time increased with length of starvation until the third week, where a maximum lag of 28 hours was attained. Both strains of *P. syringae* demonstrated the ability to survive under starvation conditions, which may be an adaptation shared by other epiphytic bacteria. That both the wild-type INA strain and the non-INA mutant strain responded similarly in starvation and recovery experiments indicates that the presence or absence of the ice nucleating protein does not affect survival of *P. syringae* under non-freezing conditions.

INTRODUCTION

Starvation survival has been defined as a physiological state resulting from an insufficient level of nutrients, especially energy, to provide for growth and reproduction (60). Most natural environments are oligotrophic, a situation
created in part by the microorganisms' efficient utilization of available substrates. In fact, starvation of many bacteria occurs in every environment because a given ecosystem does not provide substrates for all physiological types of microorganisms present. Therefore, in order for nonspore-forming bacterial species to survive, the vegetative cells must be able to withstand nutrient deprivation and be able to grow and reproduce when conditions again become favorable.

The ocean is an oligotrophic environment which is very low in available organic carbon (58) and the starvation survival of marine bacteria has been studied extensively (1, 30, 32, 36, 61, 71), particularly the psychrophilic vibrio ANT-300 (2-4, 19, 63-65, 73). The initial response of ANT-300 to starvation is a large increase in the number of viable cells which is dependent on the initial concentration of cells and length of time in exponential growth prior to starvation. This phase is followed by a decline to a constant level (64), a pattern which has been observed in other marine bacteria (1, 32, 36). The starved cells retain the ability to quickly metabolize substrates, however the longer the starvation period, the longer the lag phase of the cells when nutrients are reintroduced (3). ANT-300 has the ability to survive periods of starvation as long as 3.5 years (R. Y. Morita, unpublished data). Starved cells of ANT-300 also decrease in size, changing from rods to coccobacilli (63). The occurrence of cells less than 0.3 μm in diameter, termed ultramicrocells (72), has been known for some time, and has been associated with nutrient deprivation (63, 71). The presence of ultramicrobacteria in both aquatic and soil environments has been reviewed by Morita (59), however the number which have been formed as the
result of starvation is unknown. Decreased cell size provides an increased surface
to volume ratio to aid in uptake of substrates, an adaptation to low nutrient
collection, but may also arise in some environments as an avoidance to
bacteriovores.

The starvation survival process has not been studied in bacteria which
inhabit plant leaf surfaces. The successful epiphytic bacteria must be able to
survive discontinuities in growth caused by seasonal effects on either the bacteria
or the plant host. It has been demonstrated in several studies that
phytopathogens, which do not form resting spores, remain dormant during
quiescent periods in association with a variety of agents, including plant residues,
insects, seeds, and soil (70).

Many pathovars (17) of the common epiphytic bacterium *Pseudomonas*
syringae are ice nucleation-active (INA) and cause frost damage to plants at
temperatures as high as -1.5°C (48, 50, 53). 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 (67, 69). These constructed non-INA strains are being evaluated
for use in frost control on agricultural crops (56). The mechanisms of
competition between INA and non-INA strains on leaf surfaces are poorly
understood. The purpose of this study was to compare the starvation survival
process of a wild-type INA strain and a genetically engineered non-INA strain of
*P. syringae* to determine if there were differences in their survival or recovery
period which could contribute to a competitive advantage of one strain over the
other.
MATERIALS AND METHODS

Organisms and Media. *Pseudomonas syringae* Cit7 and *Pseudomonas syringae* Cit7del1b were obtained from Steve Lindow, University of California-Berkeley. Ice nucleation-active (INA) *P. syringae* Cit7 was originally isolated from a healthy navel orange leaf near Exeter, California (43). Strain Cit7del1b is a non-INA deletion mutant constructed from strain Cit7 (67, 69). Both strains are resistant to 100 µg/ml rifampicin.

Both *P. syringae* strains were cultured on liquid or solid (1.5% agar added) King's medium B (31) supplemented with 100 µg/ml rifampicin (Sigma) (KBR). Liquid cultures were incubated by shaking at 110 rpm and 28°C. Rifampicin stock solutions and *P. syringae* stock cultures were prepared as reported by Buttner and Amy (in press). The cells were placed in a salts buffer (SB) containing: K₂H₂P₂O₇, 0.56 g; KH₂PO₄, 0.56 g; MgSO₄·7H₂O, 0.13 g; and distilled water, 1 liter. The pH of both media was adjusted to 7.0 and SB was filter sterilized using a 0.45 µm membrane prior to autoclaving.

Growth and starvation conditions. Three replicate starvation cultures were maintained for each bacterial strain. Cells were prepared for starvation as follows: sterile 250 ml flasks containing 100 ml of KBR broth were inoculated with mid- to late-log phase *P. syringae* cells (1:1000) and incubated with shaking until an optical density at 600 nm of 0.5 was reached (Beckmann DU65 spectrophotometer). This culture was harvested by centrifugation (Sorvall RC2-B) at 13,700 x g for five minutes at 4°C. The cell pellet was washed in an equal volume of sterile SB, followed by centrifugation under the same conditions. After
two washes with SB, the final pellet was suspended in 100 ml SB. Replicate starvation cultures of each strain containing an initial density of $10^6$ to $10^7$ cells/ml were prepared by diluting aliquots of the final suspension in SB by 1:100. Starvation cultures were incubated by shaking at 110 rpm and 28°C. Recovery from starvation was initiated by the transfer of cells from each starvation culture to KBR broth at a 1:16 dilution and incubation under the same conditions as starvation cultures.

**Cell counts.** The number of viable cells in growing or starving cultures was determined from triplicate spread plates (KB agar) of starved cells diluted in sterile SB. Plates were incubated for 3-7 days at 28°C and colonies counted. Total counts were determined using the acridine orange direct-count (AODC) technique of Hobbie et al. (29). Starved cells were diluted with SB, fixed with 2% formaldehyde (final concentration) and stained with 0.01% acridine orange (final concentration) for five minutes. Acridine orange, formaldehyde and SB solutions were filter sterilized immediately prior to use. The stained cells were filtered onto 0.2 μm blackened polycarbonate membrane filters (Poretics Corp.) and rinsed with filter-sterilized distilled water. Total numbers of cells were determined using a Nikon Optiphot epifluorescence microscope fitted with a 100 watt mercury bulb and a B-2A filter block. Ten fields or a total of at least 200 bacteria were counted per sample. Only objects with distinct orange or green fluorescence and recognizable bacterial shape were counted as bacterial cells. The number of cells/ml in the original culture was determined by calculating an area conversion factor between the microscopic field and the working area of the filter.
Estimation of cell size. A size analysis of cells was conducted for each strain at selected times during starvation. Cells were stained with acridine orange as previously described and examined by fluorescence microscopy. The length and width to the nearest 0.2 μm was determined for at least 30 randomly selected cells of each strain. Assuming an approximately cylindrical shape for bacterial rods, the volume of the cells was calculated over the course of starvation.

RESULTS

Total and viable cell counts of *Pseudomonas syringae* cultures during starvation are shown in Fig. 1. Viable cell numbers of both strains Cit7 and Cit7del1b increased (Cit7, 1.5-fold; Cit7del1b, 1.4-fold) during the initial 24 hours of starvation, followed by a gradual decrease which stabilized temporarily between the third and fourth weeks of starvation. Viability of strains Cit7 and Cit7del1b decreased to ca. 8% and 4%, respectively, of the initial cultures. Viable cells of both strains began to increase in number after five weeks of starvation, presumably due to uptake of compounds released from lysed or leaking cells. Viability of strains Cit7 and Cit7del1b increased to 23% and 21%, respectively, of the initial cultures by the end of the six-week experiment. Viable counts from samples taken after 17 weeks were similar to the week six values. Total counts were consistent for both strains and followed the pattern of viable cells.

Changes in the cell volume of *P. syringae* during starvation are shown in Fig. 2. Before starvation, cells were rod-shaped with average dimensions of ca.
FIG. 1. Total (Cit7, ●; Cit7del1b, ▲) and viable (Cit7, ○; Cit7del1b, △) numbers of *P. syringae* during starvation (mean ± 1 standard error).
FIG. 2. Cell volume (mean + 1 standard error) of *P. syringae* strains Cit7 (O) and Cit7del1b (Δ) during starvation.
3.8 by 0.8 μm. After one week of starvation, a shortening of the cells of both strains to ca. 2.8 μm was observed. By the sixth week, the cells had undergone further miniaturization, averaging 1.7 by 0.8 μm. Less than one percent of the cells were able to pass through a 0.45 μm membrane filter. After six weeks of starvation, the mean cell volume of the wild-type strain Cit7 was significantly larger (p < 0.05) than that of strain Cit7del1b using the Student's t-test. No significant difference was determined between the two strains at any of the other starvation times. Cells measured after 17 weeks of starvation were slightly larger than at six weeks, ca. 2.0 by 0.9 μm. Cells of both strains became coccobacilli in response to starvation, but the cell diameter after 6 weeks of starvation was approximately the same as that in growing cells.

During recovery from starvation, P. syringae cultures displayed typical bacterial growth curves as indicated by turbidimetric readings (O.D.₆₀₀), with lag, log and stationary phases of growth clearly recognizable (Fig. 3). The two strains showed nearly identical growth rates during the recovery experiments. The length of the lag phase increased with the length of the starvation period (Fig. 4), and the relationship between the starvation period and the delay before cellular biomass increase was a linear function through three weeks. After the third week of starvation-survival, the length of starvation did not appreciably lengthen the lag time. The maximum lag phase of 28 hours observed for both strains at six weeks did not increase at 17 weeks of starvation.
FIG. 3. Growth curves of *P. syringae* strains Cit7 (○, •) and Cit7del1b (△, ▲) during recovery from starvation at time zero (initial) and after 3 weeks starvation.
FIG. 4. Length of lag phase before recovery from starvation vs. length of starvation for cells of *P. syringae* strains Cit7 (○) and Cit7del1b (△).
DISCUSSION

Both strains of Pseudomonas syringae demonstrated the ability to survive under starvation conditions. The pattern of rapid increase in viable cell numbers in response to starvation followed by a gradual decrease was observed by Novitsky and Morita, 1976, in a marine Vibrio species (64) and in other marine bacteria (1, 32, 36, 71). Viable populations of bacteria demonstrating this pattern of starvation survival then stabilized at 0.1% to 10% of the initial culture. P. syringae Cit7 and Cit7del1b stabilized at 8% and 4%, respectively, of the initial populations.

The increase in viable numbers beginning after five weeks of starvation may be the result of a phenomenon known as cryptic growth, or growth on nutrients released from dead cells. Cryptic growth has been demonstrated in starved bacterial populations (16, 68) by the addition of heat-killed cells to starvation cultures, resulting in estimates ranging from 6.7 to 50 cell deaths required to support the division of one starved bacterium. The magnitude of the increase in viable numbers reported here is ca. 1.3 x 10^6 CFU/ml for both strains, and when compared with the loss in viability of approximately 1 x 10^7 CFU/ml which had occurred before regrowth, an estimate of between seven and ten cell "deaths" per starved cell division was obtained (Cit7, 9.4 deaths/division; Cit7del1b, 7.9 deaths/division). These estimates, however, ignore the presence of viable but nonculturable bacteria. Some of the increase in viable cells may be due to resuscitation of viable but nonculturable cells. However, the accompanying increase in total counts indicates the occurrence of cell divisions,
and not merely resuscitation of cells. The increase in viable numbers was not due to concentration of the cultures by evaporation. Data from controls indicates only a 0.1%, or $10^3$ CFU/ml increase from week 3 to week 6 as a result of evaporation loss.

Information provided from total bacterial counts also supports the possibility of cryptic growth. The gradual decrease in total counts preceding regrowth suggests lysis of some cells, in contrast to previous reports where cell lysis has not been observed in starving populations (4, 36). Decreases in total counts over time have been observed previously during starvation of marine isolates (71) due to reduced intensity of fluorescence of starved cells, but the reduced counts were neither comparable in magnitude to that reported here nor followed by an increase in total counts. Additional experiments with radiolabelled substrates will be needed to determine the basis for the observed increase in viable cell numbers.

Reduction in size has been associated with starvation conditions in marine bacteria (63, 71), presumably due to endogenous respiration in response to a lack of exogenous substrates. In most cases, starved cells in the marine environment form ultramicrocells, defined by Torella and Morita (72) as bacteria having a diameter of less than 0.3 μm. Ultramicrocells have been observed in both marine and soil environments (59), however their presence among epiphytic bacteria has not been established, to our knowledge. While cells of both strains of P. syringae decreased in size in response to starvation, neither strain formed true ultramicrocells, evidenced by the observation that less than one percent of the
total bacteria were able to pass through a 0.45 µm membrane filter. The apparent larger size of strain Cit7 after six weeks starvation remains unexplained.

The lag phase of a bacterial growth curve is related to the acclimation of bacteria to a new environment and/or the synthesis of specific inducible enzymes. With starved cells, the lag phase has been observed to increase with the length of the starvation period (3). This pattern was observed in *P. syringae* only up to three weeks, at which point a maximum of 28 hours lag time was reached and continued starvation for up to 17 weeks did not result in an increase. It is interesting to note that the lag time did not decrease at six weeks of starvation despite an increase in viable cell numbers from five to six weeks due to cell division. Morita (60) has surmised that starved cells degrade many biosynthetic and catabolic enzymes not necessary for the purpose of obtaining energy from the environment, and when nutrients are again present, these enzymes are then resynthesized. The regrowth observed here after five weeks suggests that nutrients were present in concentrations which allowed synthesis of the biosynthetic enzymes necessary for growth and division. A shortened lag phase was expected for the six-week starvation cultures which contained cells that had recently undergone growth and division, however this was not observed. It may be that a necessity of long exposure to elevated nutrient levels has evolved as a survival mechanism for these bacteria when starved.

Epiphytic bacteria have evolved numerous mechanisms to survive through the winter and other periods of low nutrient availability (70). The demonstrated starvation survival of *Pseudomonas syringae* very likely contributes to its ubiquity in nature. Whether other epiphytic bacteria display a similar starvation response
remains to be answered. The wild-type INA strain and the non-INA deletion mutant responded similarly in starvation and recovery experiments, indicating that the presence or absence of the ice nucleating protein does not affect starvation survival of \textit{P. syringae} in the laboratory. Additional experiments detailing the physiological responses of this bacterium to starvation may answer some of the questions raised here and help to elucidate the process of survival on the phylloplane.
CHAPTER III

SURVIVAL OF PSEUDOMONAS SYRINGAE

ON OAT PLANTS AFTER FREEZING
ABSTRACT

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 then 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 hours 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 hours. 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 hours, indicating a potential competitive advantage of INA strains of *P. syringae* over non-INA strains in mild freezing environments.

INTRODUCTION

The bacterium *Pseudomonas syringae* is a common epiphyte on a large variety of plant species from widespread geographical locations (24, 27, 40, 48-50, 53, 62). Many pathovars (17) 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 (5, 6, 40, 41, 43, 48, 50-53). The damage is caused by ice formation in plant cells which spreads both inter- and intracellularly, resulting in the disruption of cell membranes (10). The extent of freeze injury to plant leaves is directly related to the log of the number of INA bacteria on leaf surfaces (26, 41, 45, 48, 50, 53, 54). Reduction of INA bacteria on leaf surfaces decreases plant frost damage at a given temperature (6, 38, 40, 41, 45, 48 51, 53, 76). In the absence of INA bacteria, herbaceous plant tissues can supercool to temperatures below -5°C before suffering freeze injury (5, 26, 40, 43, 48, 50).

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 (43). These constructed non-INA mutant strains are being evaluated for use in frost control on agricultural crops (38, 43, 45, 51, 55). 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 (6, 38, 40, 41, 45, 51, 52). 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 (38, 43, 45). 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 (38, 43). However, within the INA pathovars of P. syringae examined, naturally occurring non-INA strains are relatively low in abundance (24, 50, 62). 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. (43). Strain Cit7del1b is a deletion mutant constructed from Cit7 and is no longer INA. Both strains are resistant to 100 \( \mu \text{g} \) of rifampicin per ml.

Culture preparation. Both P. syringae strains were cultured on King medium B (31) supplemented with 100 \( \mu \text{g} \) of rifampicin and 100 \( \mu \text{g} \) 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 mg of rifampicin per ml in 100% methanol, 20 mg 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 \( x \ g \) for 5 minutes 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 \( \times \) 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 hours 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-hour 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 ca. $5.5 \times 10^8$ 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 were 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 at approximately the same height above the soil. After harvesting 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 hour. Plants were warmed to room temperature and removed from the incubator, and the extent of damage was estimated. Three grams of leaf material were 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 hours.

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 hours to remove bacteria from leaf surfaces. Serial dilutions from the leaf washings were spread plated onto KBRC agar and incubated for 48 to 72 hours at 28°C. Triplicate plates were counted to determine CFU per 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 $1.5 \times 10^4$ 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 minutes, numbers of frozen and unfrozen droplets were recorded.

RESULTS

Temperature effects 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. 5A and B). Damage was manifested as flaccidity or discoloration of the leaves upon rewarming of the plants (41). Although plant 4 in Fig. 5B 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 replicates showing discoloration of flaccidity. At freezing temperatures of -12°C, Cit7del1b-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°C.
FIG. 5. Oat plants before (A) and after (B) freezing at -5°C. Plants: 1, unfrozen control colonized by strain Cit7; 2, treated with strain Cit7; 3, cold-acclimated, treated with strain Cit7; 4, treated with strain Cit7del1b; 5, control sprayed with sterile water in place of bacteria. Plant 4 showed no characteristic signs of freeze damage. Its appearance after freezing was due to placement next to the incubator fan.
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 $10^5$ 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°C (Fig. 6A) and maintaining the temperature for 1 hour, tests showed no significant difference ($P > 0.05$) in either 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 hours 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 hours and an increase of 8.9-fold (data not shown; $P < 0.01$) on cold-acclimated plants. The non-INA strain Cit7del1b 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°C (Fig. 6B), strain Cit7 populations were similar to those in the -5°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 Cit7del1b recovered from plants exposed to -12°C, unlike populations in the -5°C trial, increased by 24.3-fold in the interval AF1 to BF2 ($P < 0.05$). Oat plants colonized by Cit7del1b, which were partially damaged at temperatures of
FIG. 6. Population sizes (mean + 1 standard error) of *P. syringae* CIt 7 (■) and CIt7del1b (□) recovered from oat plants subjected to temperatures of -5°C (n=5) (A) and -12°C (n=3) (B). Freeze 2 occurred 24 hours after freeze 1.
-7 and -9°C, supported bacterial population increases of 6.6-fold \( (P < 0.01) \) between AF1 and BF2 in the -7°C tests (Fig. 7A) and 3.1-fold \( (P > 0.05) \) in the -9°C tests (Fig. 7B). The smaller three-replicate plant sample sizes required in the -9 and -12°C trials resulted in population increases 18 hours after freezing considered insignificant in two cases in which populations had increased by 3- and 15-fold (Cit7del1b, -9°C; Cit7, -12°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 Cit7del1b at -12°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 \( (n = 6; \text{data not shown}) \) 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
FIG. 7. Population sizes (mean ± 1 standard error) of *P. syringae* Cit7del1b recovered from oat plants subjected to temperatures of -7°C (n=5) (A) and -9°C (n=3) (B). Freeze 2 occurred 24 hours after freeze 1.
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 (49) at -6°C to confirm the presence of the desired strain of \textit{P. syringae}. Colonies recovered from plants sprayed with the non-INA strain, Cit7del1b, 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 colonies recovered from noninoculated controls, 16 of 20 tested negative for ice nucleation activity.

\textbf{DISCUSSION}

Previous work on freeze survival of wild-type and deletion mutant strains of \textit{P. syringae} involved freezing bacterial suspensions (43) 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°C for 1 hour, 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°C freeze data from potato leaf experiments (43). 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°C and colder by disruption of the plant cells during freezing (10). 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 hours following the freeze. Gross et al. (25) reported a 10-fold increase in an INA *P. syringae* strain on apricot flowers following a -4.7°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°C (33). Data reported here from strain Cit7 oat leaf trials at -5 and -12°C agree with these observations.

Populations of strain Cit7del1b on oat plants cooled to -5°C were unaffected. Freezing of plant leaves colonized by non-INA strain Cit7del1b 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. (26) with oat leaves with no detectable INA bacteria and was supported by the observation of Lindow
(43) that strain Cit7del1b 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 \textit{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 \textit{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.
CHAPTER IV

DISCUSSION
There is speculation that expressing the INA phenotype somehow confers a selective advantage to epiphytic bacteria. This speculation is based on the occurrence of the trait in five different bacterial species and the ubiquity of INA species on plant leaf surfaces (24, 40, 48-50, 53, 56, 62). Within the species *Pseudomonas syringae*, for example, over half of the pathovars are INA (40). On the other hand, there are concerns that the genetically engineered strain may have an as yet unknown competitive advantage under a particular set of conditions in nature, and have the ability to competitively exclude INA wild-type strains from plant leaf surfaces. Because INA bacteria occur in the atmosphere and may be important in initiating precipitation by causing ice formation in atmospheric water droplets (40), exclusion by non-INA strains could theoretically result in a reduction in the numbers of atmospheric ice nuclei, which could affect the climate.

The hypothesis tested in these experiments was that there is no significant difference in growth or survival between the INA strain, *P. syringae* Cit7, and the non-INA deletion mutant strain, Cit7del1b. All previous work (43) and the results obtained here from starvation studies support the experimental hypothesis. However, the data presented here from freezing experiments refute the hypothesis and indicate a potential competitive advantage for wild-type strains expressing the INA phenotype.

The results from starvation experiments were nearly identical for the two strains. Both the INA and the non-INA strains demonstrated similar survivability, miniaturization, and recovery during starvation. These results suggest that the presence or absence of the ice gene does not affect the starvation survival of
P. syringae in the laboratory.

Although the starvation process has not been studied in epiphytic bacteria, it was not surprising that P. syringae was able to survive starvation, since many pseudomonads and other species have been shown to survive through the winter in association with seeds or dry plant debris (70). Epiphytic bacteria in nature face the additional problem of desiccation during starvation, a factor which was not addressed in these laboratory studies. Desiccation is closely related to starvation, because water is important in the accumulation of organic and inorganic nutrients from both the atmosphere and leachates from plant tissues (20). Many plant pathogens, although nonspore-forming, are resistant to desiccation, possibly due to the production of a glycocalyx composed primarily of exopolysaccharides (EPS), which surrounds the cell (18, 70). EPS are thought to aid in bacterial survival during starvation due to their high water holding capacity, and have also been reported to act in adhesion to leaf surfaces and as determinants of pathogenicity (18, 70). Both strains of P. syringae used in these experiments are able to produce the EPS, levan (43).

Additional starvation experiments are needed to address the role of desiccation in starvation survival of P. syringae. In these experiments, the two strains will be applied to leaf surfaces, subjected to desiccation, and their survival over time measured. These results could be compared with the starvation results obtained in buffer to determine the similarities and differences between strains or between methods of starvation. If EPS are involved in starvation survival, perhaps mutants which overproduce EPS (18) may be constructed which are
better able to survive starvation and have a greater long-term affect in frost control.

Freezing experiments by Lindow (43, S. E. Lindow, unpublished data) indicated no significant difference in survival between INA and non-INA *P. syringae* strains, and preliminary work in our lab showed no effect of freezing for one hour at temperatures as low as -12°C on the survival of either strain on oat plants. However, the damage inflicted upon colonized oat plants after freezing at -5°C was very different for the two strains. Therefore, in a second set of experiments, bacterial populations were also measured 24 hours after freeze damage to oat plants. These experiments showed a significant difference between the two strains. The INA strain, Cit7, which caused severe freeze damage to oats at -5°C, also showed a 10-fold or higher population increase 24 hours after inflicting freeze damage. The non-INA strain, Cit7dell1b, did not cause freeze damage to oats at -5°C and did not change significantly in population size. Plants colonized by strain Cit7dell1b required cooling to -12°C before damage comparable to that caused by strain Cit7 at -5°C occurred, and at this temperature both strains demonstrated similar increases in population size after 24 hours.

These results indicate a competitive advantage of INA strains over non-INA strains in mild freezing environments. For example, plants colonized by INA strains suffer freeze damage, followed by a subsequent population increase in INA bacteria, at a temperature where plants colonized by non-INA bacteria are not damaged and bacterial populations do not change significantly. Thus, for a particular population of plants in a mild freezing environment, INA strains may
increase in relative proportion to non-INA strains and have a larger pool to serve in recruitment to other plants.

Of course, in nature mixed populations of INA and non-INA strains coexist on leaf surfaces. Further freezing experiments with mixed populations on plants are needed to clarify the adaptive significance of the INA phenotype. It has been observed in mixed population studies that the proportion of INA and non-INA bacteria on plant species remains approximately constant throughout the growing season, with the relative proportion probably dictated by the relative size of the initial inoculum (S. E. Lindow, unpublished data). If the relative proportion of INA and non-INA strains also remains constant during growth on damaged plants, INA populations will tend to increase in relative proportion to non-INA strains, since only leaves dominated by INA strains will suffer frost damage, and any increases in non-INA populations will be matched by proportionate increases in INA strains.

The unprecedented release of genetically engineered organisms into the environment and concern over the potential ecological impact of field application of recombinant non-INA strains for use in frost control has resulted in governmental regulations which required extensive laboratory testing prior to approval for field testing of these strains. No evidence indicates an adaptive advantage of the non-INA phenotype. The occurrence of naturally-arising non-INA deletion mutants in only very low numbers within INA strains supports this view (24, 50, 62, S. E. Lindow, unpublished data). The information presented here suggests a competitive advantage of INA Pseudomonas syringae Cit7 over the genetically engineered non-INA strain Cit7del1b in mild freezing environments.
This helps to explain the ubiquity of INA bacteria and raises questions regarding the appropriate application of non-INA strains to insure their long-term effectiveness as biological frost control agents on crops. Considering the apparent competitive advantage of INA strains in freezing environments and their constant immigration from the environment onto plant leaf surfaces, perhaps several spray applications of the genetically engineered strain will be required seasonally to prevent the establishment of INA bacterial populations which cause frost damage to crops.
BIBLIOGRAPHY


