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Competitive Exclusion of Epiphytic Bacteria by Ice⁻ *Pseudomonas syringae* Mutants

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The growth of ice nucleation-active and near-isogenic ice nucleation-deficient (Ice⁻) *Pseudomonas syringae* strains coexisting on leaf surfaces was examined to determine whether competition was sufficient to account for antagonism of phylloplane bacteria. The ice nucleation frequency spectra of 46 Ice⁻ *P. syringae* mutants, obtained after mutagenesis with ethyl methanesulfonate, differed both quantitatively and qualitatively, but the mutants could be grouped into four distinct phenotypic classes. The numbers of ice nucleation-active bacteria and ice nuclei active at −5°C were reduced on plants colonized with Ice⁻ *P. syringae* mutant strains before challenge inoculations with an Ice⁺ *P. syringae* wild-type strain. Frost injury to plants pretreated with Ice⁻ *P. syringae* strains was also reduced significantly compared with that to control plants and was correlated with the population size of the Ice⁺ *P. syringae* strain and with the numbers of ice nuclei active at −5°C. An Ice⁻ *P. syringae* strain colonized leaves, flowers, and young fruit of pears in field experiments and significantly reduced the colonization of these tissues by Ice⁺ *P. syringae* strains and *Erwinia amylovora* as compared with untreated trees.

Bacteria, including several plant pathogens, live epiphytically on healthy host and nonhost plant species (4, 18) and can develop large epiphytic populations (6, 15, 18, 20, 22, 27, 35). The simple presence of many bacterial plant pathogens on plant surfaces is not directly correlated with incidence of disease on these plants. However, the incidence of brown spot disease of bean caused by *Pseudomonas syringae* pv. *syringae* increases with increasing epiphytic populations of this pathogen above an apparent threshold population size (21). Biological, chemical, and physical features of the leaf surface which determine the epiphytic population size of a phytopathogenic bacterial species will determine the likelihood of disease on those plants (18).

Frost injury is a serious abiotic disease of plants that causes losses in agricultural production in the United States of over one billion dollars yearly (38). Frost-sensitive plants are distinguished from frost-resistant plants by their relative inability to tolerate ice formation within their tissues (8, 10, 11). Frost injury to sensitive plants results from ice formation at −1.5 to −5°C caused by ice nucleation-active pathovars (14) of *P. syringae* (2, 15–17, 23, 32), strains of *Erwinia herbicola*, or three other bacterial species (26, 40) on leaf surfaces (22, 23, 25, 38). Ice nucleation-active bacteria are commonly found on nearly all plants in nature, with strains of *P. syringae* being the most abundant ice-nucleating bacteria on most plant species examined (15, 16, 27, 30). The incidence of frost injury to plants is related directly to the logarithm of the population size of ice nucleation-active bacteria and to the logarithm of the numbers of bacterial ice nuclei on plants at the time of freezing (22, 30). Treatments that reduce the numbers or the ice nucleation activity (or both) of bacteria on plants reduce the incidence of plant frost injury (1, 22, 23, 25, 28–30).

Certain bacterial strains that are not ice nucleation active, including *Pseudomonas fluorescens*, *Pseudomonas putida*, and *E. herbicola*, when established on plant surfaces reduce the subsequent colonization of these plants by ice nucleation-active bacteria under both controlled (28) and natural (22, 24, 25, 29, 30) environmental conditions. The population size of ice nucleation-active bacteria on plants is negatively correlated with the population size of epiphytic antagonistic bacteria colonizing these plants (28, 30).

Little is known of the importance of antibiosis or the role of other factors such as nutrient competition in the interaction of microorganisms on leaves. While many plant pathogens are inhibited in vitro as well as on plants by certain antagonistic bacteria used as biological control agents (3, 24, 36), not all strains effective in biological control are inhibitory to target organisms in vitro (6, 36, 39).

Some bacteria that are antagonistic to *P. syringae* on plants are antagonistic in vitro (24; S. E. Lindow, Phytopathology 72:986, 1982). Population sizes of ice nucleation-active bacteria on plants are not correlated with the presence or size of antibiosis zones produced by antagonistic bacteria in vitro. Antibiosis-deficient mutants of antibiotic-producing strains do not differ from the appropriate parental strain in reducing epiphytic population sizes of *P. syringae* (24). Antibiosis does not appear to be necessary for the exclusion of *P. syringae* from leaf surfaces by antagonistic bacteria. It is unknown whether other density-dependent mechanisms, such as modification of the chemical environment by nutrient utilization (4, 5) or acid production (4, 12), can account for the antagonism observed on plant surfaces. This paper addresses the role of competition in antagonism among bacteria on plant surfaces. Since antibiosis has been shown not to be necessary for interspecific antagonism of bacteria on leaf surfaces (24), this study was undertaken to determine whether competition alone could account for antagonism on leaf surfaces. Near-isogenic strains of *P. syringae* deficient in ice-nucleating ability (Ice⁻) were constructed, and competition between Ice⁺ and Ice⁻ strains on plant surfaces was studied.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The sources and biochemical characteristics of *P. syringae* 31 and *E. herbicola* M232ASR11 have been reported previously (2, 28). *P. syringae* Cit13 was isolated from the surface of a healthy

navel orange (*Citrus sinensis* L) leaf that was grown near Visalia, Calif. Spontaneous rifampin-resistant mutants (Cit13R and 31R1) of strains Cit13 and 31 were obtained by spreading approximately 10^9 cells of these parental strains on plates of King medium B (KB) (19) containing 100 μg of cycloheximide ml^{-1} (KBC) and 100 μg of rifampicin ml^{-1} (KBCR). A spontaneous chloramphenicol-resistant mutant (31R13C) of strain 31R1 was obtained by spreading 3×10^9 cells of strain 31R1 per plate of KBC containing 20 μg of chloramphenicol ml^{-1} (KBCC). Single mutant colonies arising after 3 days of incubation at 30°C were restreaked onto KBCR or KBCC as appropriate. Strains Cit13R, 31R1, and 31R13C were selected as isolates with in vitro growth rates and ice nucleation activities similar to those of the parental strains. All *P. syringae* strains were routinely cultured on KBCR at 24°C. *E. herbicola* M232ASR11 was routinely cultured on KBC containing 100 μg of streptomycin ml^{-1} (KBCS) at 24°C. All bacterial strains were stored at 5°C on slants of nutrient agar containing 2.5% glycerol. Neither strain Cit13R nor strain 31R1 was pathogenic to any plant species tested, including corn (*Zea mays*), bean (*Phaseolus vulgaris*), pear (*Pyrus cummunis*), almond (*Prunus dulcis*), navel orange (*C. sinensis*), wheat (*Triticum aestivum*), potato (*Solanum tuberosum*), tomato (*Lycopersicon esculentum*), tobacco (*Nicotiana tabacum*), oats (*Avena sativa*), and alfalfa (*Medicago sativa*).

Mutagenesis and selection of Ice⁻ strains. Bacterial strains were grown at 30°C with vigorous agitation in KB broth to the mid-logarithmic growth stage (3×10^8 cells ml^{-1}). Ethyl methanesulfonate (50 mg ml^{-1}) was added, and the cultures were shaken vigorously to ensure dissolution of the added ethyl methanesulfonate and then incubated with agitation for 35 min at 30°C. Cells were washed three times in KB broth, grown for 4 h at 30°C, dilution plated (about 40 colonies per plate) on KBCR, and incubated for 48 h at 30°C. Colonies containing ice nuclei were determined by a replica freezing technique described previously (27). Bacterial colonies were transferred to velvet pads which were subsequently printed onto sheets of aluminum foil coated with paraffin. The foil sheets were placed on a circulating alcohol bath maintained at −5°C and were sprayed with a fine mist of sterile water. Microdroplets containing Ice⁺ colonies freeze rapidly, and the colonies obtain a frosty appearance. Cells from colonies that did not freeze were sampled directly from the aluminum surface and subcultured on KBCR.

Evaluation of bacterial antagonism on plants in greenhouse trials. Approximately 120 three-leaf-stage corn (*Z. mays* L) plants per treatment were sprayed with suspensions of Ice⁻ derivatives of *P. syringae* 31R1 (ca. 10^6 cells ml^{-1} in distilled water; ca. 1.0 ml $plant^{-1}$) or with water alone and placed in a mist chamber in a greenhouse at 24°C for 2 days. The plants were then sprayed with an aqueous suspension of *P. syringae* 31R13C (10^5 cells ml^{-1}; ca. 0.1 ml $plant^{-1}$) or with water alone, incubated for an additional 2 days in a mist chamber at 24°C, allowed to dry, and then cooled to −5°C for 30 min. All three leaves of every corn plant were rated for frost injury and scored as damaged if any injury was apparent. Darkened flaccid areas, usually encompassing the entire leaf, were deemed evidence of frost injury. Damage was expressed as the fraction of leaves that showed frost injury in each treatment.

Measurement of bacterial populations and ice nuclei on leaves. Bacterial population sizes were determined as reported previously (27). Each sample of greenhouse-grown corn plants consisted of five three-leaf-stage plants grown in sterile vermiculite (sample size, about 9 g). Samples of pear tissue (sample size, about 20 g) from field trials consisted of 8 to 15 entire fruiting spurs, containing leaves, flowers, or young fruit, and a 1-cm segment of the fruit spur itself. Each sample was placed in 100 ml of washing buffer (0.1 M potassium phosphate buffer, 0.1% Bacto-Peptone [Difco Laboratories, Inc.] [pH 7.0]), and the flasks were sonicated in an ultrasonic cleaning bath (Bransonic 52; Branson Sonic Power Co.) for 7 min. Serial dilutions of leaf washings were plated on KBC and KBCR or KBCC as appropriate. The population size of Ice⁻ *P. syringae* strains was determined on KBCR after 3 days of incubation at 24°C. In most samples of greenhouse plants, the population sizes of strain 31R13C were less than 1% of those of Ice⁻ *P. syringae* strains. However, when population sizes of strain 31R13C determined on KBCC were 1% or more of those of Ice⁻ *P. syringae* strains in a sample, the numbers of 31R13C colonies on KBCR were determined by the replica freezing technique described above and substracted from the total numbers of colonies enumerated on KBCR to estimate the population sizes of Ice⁻ *P. syringae* strains. Total numbers of bacteria and numbers of *Erwinia amylovora* on pear fruiting spurs were enumerated after 4 days of incubation on KBC and Miller-Schroth medium (33), respectively, at 21°C. The population size of ice nucleation-active bacteria was estimated from KBC plates by the replica freezing technique.

Leaf surface ice nuclei active at −5°C were quantified by using a droplet-freezing procedure similar to that reported previously (26). At least 40 10-μl droplets of appropriate dilutions of leaf washings were placed on the surface of paraffin-coated aluminum foil held at −5°C on an alcohol bath and scored for ice formation. When ice nuclei were not detected in undiluted leaf washings, particulates in the leaf washings were concentrated 40-fold by centrifugation as described previously (30). The cumulative ice nucleus concentration at −5°C was calculated by the method of Vali (37) from the fraction of droplets unfrozen at −5°C.

Measurement of ice nucleus concentrations active at different temperatures in vitro. The cumulative concentration of ice nuclei active at temperatures in the range of 0 to −12°C for bacterial strains was measured and calculated as reported previously (26). Forty 10-μl droplets of appropriate serial dilutions of suspensions of bacterial cultures (grown for 3 days at 21°C on nutrient agar containing 2.5% glycerol) in distilled water were placed on an ice nucleus spectrometer (26). The cooling rate of the aluminum surface, measured with a thermistor, was 0.15°C min^{-1}. The temperature at which individual droplets froze was recorded as the temperature was decreased. The cumulative number of ice nuclei was calculated by the method of Vali (37). The number of CFU $milliliter^{-1}$ was estimated from the turbidity of undiluted suspensions by using a standard curve relating optical density at 600 nm and CFU $milliliter^{-1}$ for strain 31R1. The ice nucleus concentration at each temperature was divided by the cell concentration in each droplet observed, to obtain the ice nucleation frequency [$\log_{10}$(ice nuclei $cell^{-1}$)] at each temperature for the cell population.

Field trial conditions. An experimental plot was established in a 15-year-old commercial pear orchard near Pope Valley, Calif., in 1983. The experimental plot consisted of a randomized complete block design with four replications. Each replication consisted of four adjacent trees in two rows. At least one untreated tree separated each treatment group within a replication. Bacteria applied to pear trees were harvested by being washed from KBC plates after 4 days of growth at 24°C, suspended in tap water, diluted to a

concentration of ca. 10^7 cells ml^{-1}, and applied (ca. 2.5 liters $tree^{-1}$) to trees at approximately 5% bloom on 28 March 1983. The application was done within 30 min of harvest with a backpack mist blower similar to that used in a previous study (30).

RESULTS

Phenotypes of Ice$^-$ *P. syringae* mutants. Mutants of *P. syringae* 31R1 that were deficient in expression of ice nucleation activity were isolated readily after ethyl methanesulfonate mutagenesis. Mutants of *P. syringae* 31R1 that had reduced ice nucleation activity were isolated at a frequency of approximately 8.0×10^{-4} $cell^{-1}$ by the replica freezing technique when cells were grown at 30°C. A lower frequency of ice nucleation-deficient mutants (approximately 10^{-5} $cell^{-1}$) was detected when cells were grown at 21°C, the optimum temperature for expression of ice nucleation by the parental strain. A total of 46 different mutants of strain 31R1 that were deficient in expression of ice nucleation when grown at 21 or 30°C were obtained in this study.

Mutants of *P. syringae* 31R1 could be grouped into four broad phenotypic classes on the basis of ice nucleation spectra (Fig. 1). Class 1 Ice$^-$ mutants, exemplified by mutants 31R1-36, 31R1-4, and 31R1-23 (Fig. 1), expressed very low frequencies of ice nucleation at temperatures of −5°C or higher (usually lower by a factor of at least 10^3 as compared with the parental strain). However, the nucleation frequency of class 1 mutants approached that of the parental strain at temperatures of −9°C or lower. Class 2 Ice$^-$ mutants, exemplified by mutants 31R1-8, 31R1-11, and 31R1-16 (Fig. 1), expressed very low frequencies of or no ice nucleation activity at temperatures of −5°C or higher (generally less than 10^{-8} ice nuclei $cell^{-1}$) and also very low nucleation frequencies at temperatures of −9°C or lower (less than 10^{-6} ice nuclei $cell^{-1}$). Like class 1 mutants, however, class 2 mutants generally expressed a higher proportion of their total ice nucleation activity at temperatures lower than −9°C than did the parental strain (nucleation frequency at −9°C was higher than that at −5°C by a factor of greater than 100 and less than 10, respectively). Class 3 mutants, exemplified by mutants 31R1-13, 31R1-33, and 31R1-29 (Fig. 1), exhibited significantly lower ice nucleation frequencies at −5 and −9°C than did the parental strain. Unlike class 1 and class 2 mutants, however, the nucleation frequency of class 3 mutants at −9°C was less than 10-fold higher than that at −5°C. Class 4 mutants expressed no detectable ice nucleation activity at temperatures above −12°C. Of the 46 Ice$^-$ mutants of *P. syringae* 31R1 isolated, 5, 13, 22, and 6 fell into classes 1, 2, 3, and 4, respectively.

The *P. syringae* 31R1 *ice* gene has recently been cloned and expressed in *Escherichia coli* (34). The cloned gene complements completely, both quantitatively and qualitatively, all of the ice nucleation-deficient mutants used in the present study that were tested (34). Mutations in a rather small contiguous genetic region responsible for ice nucleation, and not pleiotropic effects of mutations in other genes, probably accounted for the Ice$^-$ phenotype of these 46 mutants. The reversion frequency of Ice$^-$ for all 46 *P. syringae* mutants used in this study was less than 10^{-8} $cell^{-1}$ (data not shown). The in vitro growth rates of all mutant strains did not differ significantly from that of the parental strain (data not shown).

Competition of Ice$^-$ and wild-type *P. syringae* strains on greenhouse plants. Frost injury to corn plants that were challenge inoculated with the ice nucleation-active parental strain *P. syringae* 31R13C was only about 25% of that of controls when Ice$^-$ mutants of this strain or a naturally occurring ice nucleation-inactive *E. herbicola* strain were

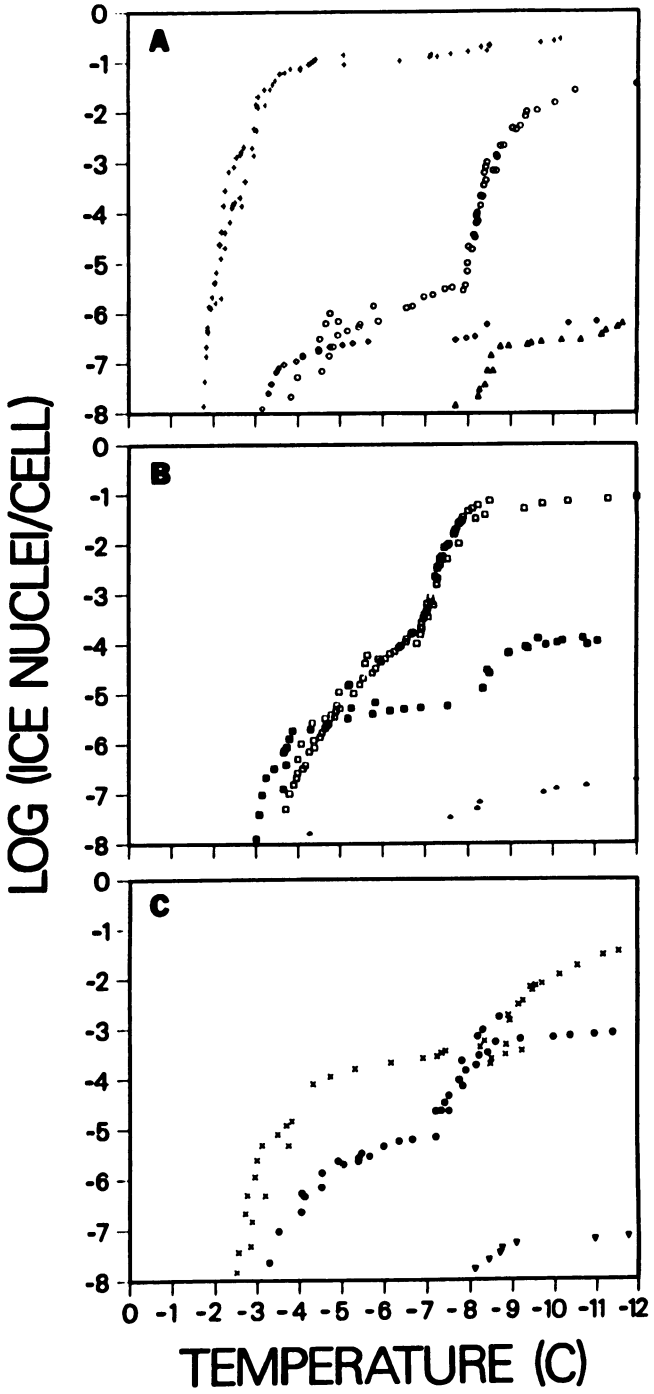

FIG. 1. Logarithm of the cumulative fraction of bacterial cells exhibiting ice nucleation activity at or above a given temperature. (A) Parental strain *P. syringae* 31R1 (+) and ice nucleation-deficient mutants 31R1-36 (○), 31R1-13 (◇), and 31R1-8 (△). (B) Mutants 31R1-4 (□), 31R1-33 (■), and 31R1-11 (▲). (C) Mutants 31R1-23 (×), 31R1-29 (●), and 31R1-16 (▽).

TABLE 1. Reduction of frost injury to corn colonized with ice nucleation-deficient *P. syringae* mutants before challenge inoculations with the parental strain

Pretreatment[a]	Ice nucleation frequency[b] (log ice nuclei cell^{-1})	*P. syringae* 31R13C treatment[c]	Bacterial population {log(CFU g [fresh weight]$^{-1}$)}		Frost damage[d] (fraction of leaves)
			Total[e]	Antagonist[f]	
Water[g]		+	5.81	ND	0.89a
Dry[h]		+	5.63	ND	0.90a
31R1-31	−7.2	+	5.50	5.06	0.38b
M232ASR11	−10.0	+	5.88	5.43	0.36bc
31R1-28	−7.9	+	5.24	4.59	0.35bcd
31R1-26	−7.8	+	5.97	4.88	0.27cde
31R1-16	−8.1	+	5.31	4.87	0.26cde
31R1-1	−8.1	+	5.74	4.56	0.25e
31R1-12	−8.4	+	5.52	4.60	0.25e
31R1-13	−6.4	+	5.56	4.76	0.23e
31R1-11	−8.0	+	5.70	4.75	0.21e
Water[g]		−	4.09	ND	0.02f
31R1-28	−7.9	−	4.78	4.93	0.01f
F (treatment)			4.19[i]	2.19	85.87[i]
LSD 5%			0.81		0.08

[a] Plants were treated with Ice⁻ mutants of *P. syringae* 31R1 or given other treatments 4 days before enumeration of bacterial populations and freezing of plants. LSD, Least significant difference.
[b] Nucleation frequency of Ice⁻ *P. syringae* mutants at −5°C.
[c] Plants were treated with Ice⁺ *P. syringae* 31R13C (+) or with water (−) 2 days before being frozen.
[d] Means followed by the same letter do not differ significantly ($P = 0.05$) according to Duncan's multiple-range test.
[e] Total bacterial populations enumerated on KBC.
[f] Populations of Ice⁻ mutants of *P. syringae* 31R1 enumerated on KBCR or of *E. herbicola* M232ASR1 enumerated on KBCS. ND, Antibiotic-resistant colonies not detected.
[g] Plants were sprayed with water alone and placed in a mist tent 4 days before being frozen.
[h] Plants were left dry on a greenhouse bench until *P. syringae* 31R13C was applied, 2 days before plants were frozen.
[i] *F* test significant ($P = 0.01$).

previously established on the plants (Table 1). Five of the eight Ice⁻ *P. syringae* strains tested as in vivo antagonists of the parental strain reduced frost damage to corn up to almost twofold more than did *E. herbicola* M232ASR11. Significant differences in protection against frost injury to corn were observed among the Ice⁻ *P. syringae* mutants tested in this study (Table 1).

The total bacterial population size determined on KBC on plants not treated with any bacteria (Table 1, water pretreatment) differed significantly from that observed with all other treatments. No other significant differences in bacterial populations were observed among treatments. The total bacterial population size after 4 days of incubation on plants pretreated with any Ice⁻ mutant strain did not differ from that on plants treated only with Ice⁺ bacteria 2 days later.

Ice⁻ *P. syringae* mutants generally comprised over 10% of the total bacteria isolated on KBC from plants pretreated with these strains (Table 1). Ice⁻ mutants were not detected on plants not pretreated with these strains. However, no significant differences in the population sizes of the Ice⁻ *P. syringae* mutants or *E. herbicola* M232ASR11 were observed (Table 1).

Ice⁻ *P. syringae* strains and *E. herbicola* M232ASR11 applied as antagonists reduced the population size of ice nucleation active-bacteria and numbers of ice nuclei active at −5°C on plants. Frost injury to corn was correlated significantly with both the logarithm of numbers of *P. syringae* 31R13C (Fig. 2) and the logarithm of numbers of ice nuclei active at −5°C (Fig. 3) on plants at the time of freezing. However, the regressions of the logarithm of numbers of Ice⁻ bacteria with frost injury and of the logarithm of numbers of ice nuclei active at −5°C with frost injury had different slopes ($m = 0.31$ and 0.20, respectively). A significant linear relation between the logarithm of *P. syringae* 31R13C population sizes and the logarithm of numbers of ice nuclei active at −5°C on plants was also observed (Fig. 4).

Exclusion of bacteria from pear tissue with Ice⁻ *P. syringae* mutants. The population size of ice nucleation-active strains of *P. syringae* and other bacteria exhibited large seasonal variations on untreated pear trees in the field. Total bacterial population sizes determined on KBC were relatively low (less than about 10^5 cells g [fresh weight]$^{-1}$) at budbreak in late March but increased to about 10^7 cells g (fresh weight)$^{-1}$

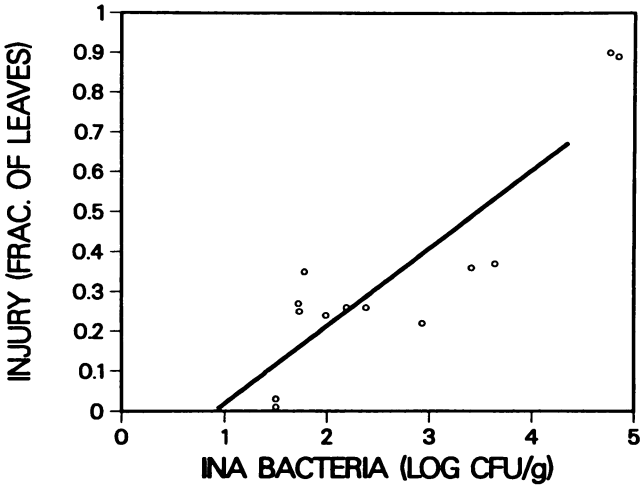

FIG. 2. Relationship between leaf surface population size of *P. syringae* 31R13C and frost injury for the experiment described in Table 1. The line represents the regression of the fraction of leaves injured against the number of Ice⁺ bacteria recovered before freezing [log(CFU g [fresh weight] of leaves^{-1})] ($r = 0.889$; $P < 0.01$). INA, Ice nucleation active.

by mid-April (Fig. 5). A decrease in the total numbers of bacteria detected on pear leaves and young fruit in late April and May was associated with warm, dry weather. Population sizes of ice nucleation-active bacteria (exclusively *P. syringae* strains) were quite low at budbreak (ca. 200 cells g [fresh weight]$^{-1}$) and increased to over 10^5 cells g (fresh weight)$^{-1}$ by mid-April before decreasing during periods of warm, dry weather. The numbers of ice nuclei active at −5°C on plants increased with increasing populations of ice nucleation-active bacteria (Fig. 5). *E. amylovora*, a bacterial pathogen of pears, was not detected on pear flowers and leaves until mid-April, when warm temperatures conducive to insect vector movement and bacterial multiplication became favorable for its colonization of asymptomatic pear fruiting spurs (Fig. 5).

The total population size of bacteria on trees treated at budbreak with Ice$^-$ *P. syringae* Cit13R-12 exhibited large seasonal variations, as did that of bacteria on control trees, but the population sizes of several epiphytic species were reduced compared with those on untreated trees. Strain Cit13R-12 comprised over 95% of the total bacteria recovered on KBC from plant leaves and flowers for over 30 days from trees treated once at budbreak with this strain (Fig. 6). However, the population size of total bacteria on trees colonized by strain Cit13R-12 differed significantly from that of bacteria on untreated trees only on 6 April. The population size of strain Cit13R-12 increased on pear tissue for 3 weeks after its application on 28 March. The population sizes of ice nucleation-active bacteria were not decreased immediately upon inoculation of pear buds; both untreated control trees and trees treated with strain Cit13R-12 had populations of less than about 1,000 cells (fresh weight)$^{-1}$ for 1 week after treatment. However, no subsequent increases in the population size of ice nucleation-active *P. syringae* strains occurred on trees colonized by strain Cit13R-12, in contrast to that on untreated trees (Fig. 6). Concentrations of ice nuclei on trees colonized by strain Cit13R-12 also were reduced about 10-fold compared with those in untreated trees at most sampling dates (Fig. 6). The population size of

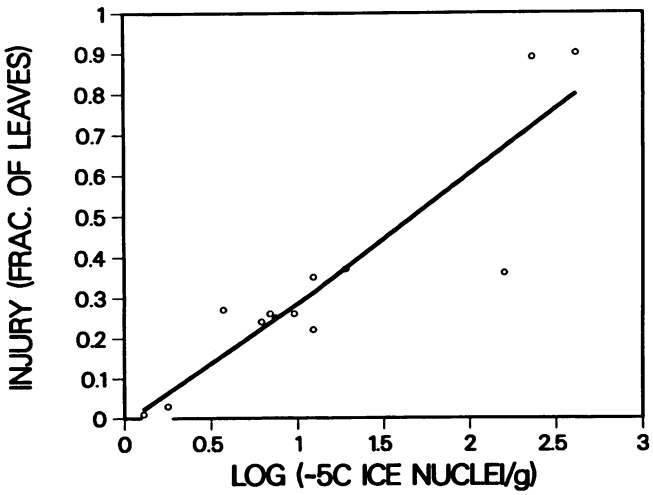

FIG. 3. Relationship between frost injury to corn plants and the numbers of ice nuclei active at −5°C on leaves at the time of freezing for the experiment described in Table 1. The line represents the regression of the fraction of leaves injured against the number of ice nuclei active at −5°C [log(ice nuclei g [fresh weight]$^{-1}$)] recovered before freezing (r = 0.904; $P < 0.01$).

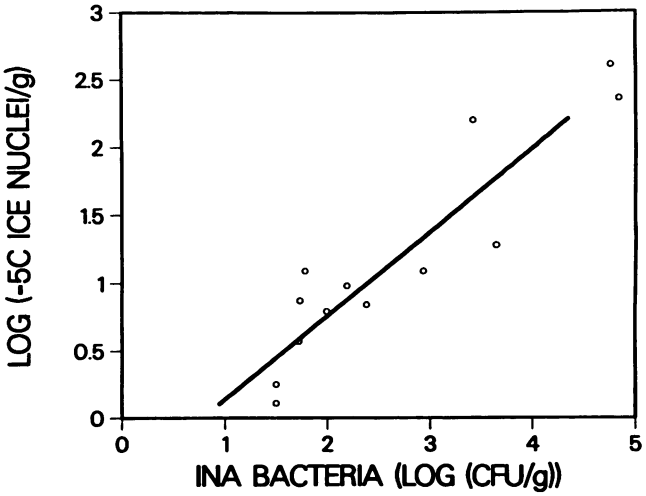

FIG. 4. Relationship between numbers of ice nuclei active at −5°C on corn plants treated with Ice$^-$ mutants of *P. syringae* and leaf surface populations of *P. syringae* 31R13C for the experiment described in Table 1. The line represents the regression of log(−5°C ice nuclei g [fresh weight]$^{-1}$) against log(cells g [fresh weight]$^{-1}$). (r = 0.913; $P < 0.01$). INA, Ice nucleation active.

E. amylovora was at least 100-fold lower on trees colonized by strain Cit13R-12 than on untreated trees after April 14 (Fig. 6).

DISCUSSION

Competition for sites of colonization or for particular nutrients has been suggested as mechanisms of mutual microbial antagonism on the phylloplane (4–6). The apparent carrying capacity of leaf surfaces for bacteria (approximately 10^7 cells g [fresh weight]$^{-1}$ or 10^6 cells cm^{-2}) (19, 27) (Fig. 5 and 6) is lower than that of other habitats, such as root surfaces (3, 7). Sites on leaves protected from extremes of environmental fluctuations (9), with sufficient nutrients, or suitable for bacterial adhesion to leaf surfaces may therefore limit the population size of epiphytic bacteria. Bacteria competing successfully for such limiting resources may exhibit considerable antagonism against strains requiring similar resources. While two different strains of bacteria may occupy the same leaf surface, antagonism between those two strains may occur only if they utilize the same phylloplane resources. Antagonism due to competition of one strain with another would increase proportionally to the overlap of their ecological niches. Isogenic bacterial strains should exhibit considerable antagonism toward each other on leaf surfaces if competition is sufficient to explain antagonism on plants.

Class 2 and 3 mutants of *P. syringae* strains, obtained after ethyl methanesulfonate mutagenesis, exhibited sufficiently low ice nucleation activity at temperatures above −5°C (Ice$^-$) to be used in studies of competition of near-isogenic Ice$^+$ bacterial strains. It is noteworthy that only 14% of the Ice$^-$ mutants of *P. syringae* 31R1 were devoid of all ice nucleation activity and that only 41% of the mutant strains lacked ice nucleation activity at temperatures above −5°C. Because our assays for ice nucleation activity are capable of quantifying reductions of the Ice phenotype of as much as 10^8-fold, many mutations leading to large but incomplete changes in the expression of this phenotype were selectable. Such mutant strains may be useful in studies of structure-function relationships for the determination of ice nucle-

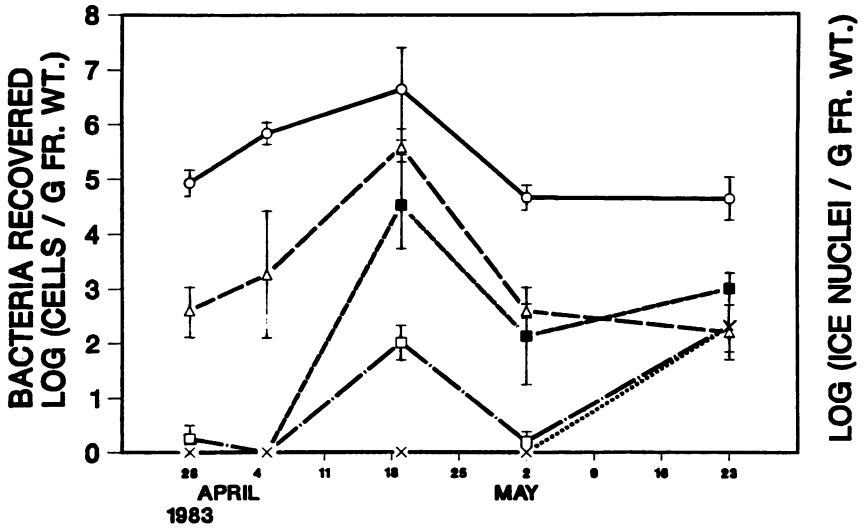

FIG. 5. Population sizes of total bacteria (○) and ice nucleation-active bacteria (△) enumerated on KBC, *E. amylovora* (■) enumerated on Miller-Schroth medium, and rifampin-resistant bacteria enumerated on KBCR (×) and numbers of ice nuclei active at −5°C (□) on leaves and flowers on untreated Bartlett pear trees during the 1983 growing season in Pope Valley, Calif. The vertical bars represent the standard error of the mean population size (logarithmic transformation). FR. WT., Fresh weight.

ation, as has recently been attempted for this phenotype in *Pseudomonas fluorescens* (13).

A large reduction in the potential population size of Ice⁺ *P. syringae* strains could be attributed to the presence of a large population of Ice⁻ *P. syringae* strains on corn and pear leaves. The total bacterial population size on both corn and pear leaves was generally unaffected by inoculation with Ice⁻ *P. syringae* strains (Table 1; Fig. 5 and 6). *P. syringae* strains comprised only 1% of the total bacteria supported by pear tissue on untreated plants (Fig. 5) but comprised 10 to 95% of the bacteria on treated pear plants (Fig. 6). Some interspecific as well as intraspecific bacterial interactions may have occurred on the plants in this study. The potential carrying capacity of the leaves for *P. syringae* and for total bacteria may be rather constant, and the reduction of Ice⁺ *P. syringae* strains on plants previously treated with Ice⁻ strains may represent a competitive exclusion by Ice⁻ strains previously established on the phylloplane. Interspecific antagonism of ice nucleation-active bacteria on corn leaves by an ice nucleation-inactive bacterial strain not exhibiting in vitro antagonism to such strains requires the prior establishment of the antagonist on leaves for successful exclusion of ice nucleation-active bacteria (28). Ice nucleation-active bacteria established on corn leaves are not displaced by subsequent inoculations of ice nucleation-inactive bacteria (28). Both intraspecific and interspecific

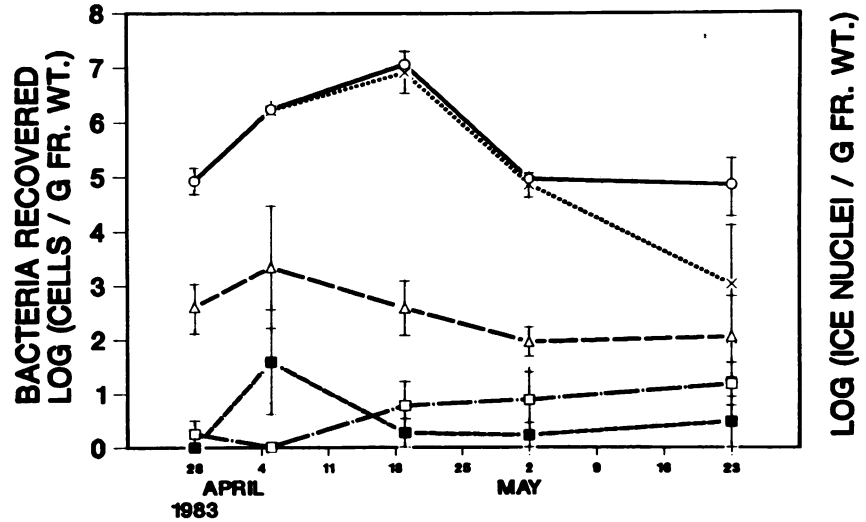

FIG. 6. Population sizes of total bacteria (○) and ice nucleation-active bacteria (△) enumerated on KBC and *E. amylovora* (■) enumerated on Miller-Schroth medium and numbers of ice nuclei active at −5°C (□) on leaves and flowers on Bartlett pear trees treated on 28 March with Ice⁻ *P. syringae* Cit13R-12 (×), enumerated on KBCR during the 1983 growing season in Pope Valley, Calif. The vertical bars represent the standard error of the determination of the mean of log populations. FR. WT., Fresh weight.

antagonism of bacteria on leaves probably represent competitive exclusion of newcomers to an occupied niche rather than competitive displacement from such a niche.

Ice$^-$ mutants of *P. syringae* reduced plant frost injury by reducing the number of ice nucleation-active bacteria on plants (Fig. 2). Although the number of ice nuclei on plants colonized by Ice$^-$ strains was also reduced compared with that on control plants and was log-linearly related to frost injury (Fig. 3), the number of ice nuclei on plants was not directly related to the population size of Ice$^+$ *P. syringae* 31R13C (Fig. 4). The apparent nucleation frequency of strain 31R13C increased with decreasing population size (from about 10^{-2} ice nuclei cell^{-1} at a population size of 10^4 cell [fresh weight]$^{-1}$ to about 10^{-1} ice nuclei cell^{-1} at a population size of 10^2 cells g [fresh weight]$^{-1}$). This apparent population size-dependent variation in nucleation frequency may be explained by (i) in vivo ice nucleation frequencies of Ice$^-$ *P. syringae* mutants which are much greater than those quantified in vitro or (ii) nonviable *P. syringae* cells serving as cryptic sources of ice nuclei on these treated plants. Unless the in vivo ice nucleation frequencies of the Ice$^-$ *P. syringae* strains used in this study were at least 10^{-5} cell^{-1} (higher than in vitro frequencies by a factor of more than 10^3), ice nuclei on plants heavily colonized with Ice$^-$ *P. syringae* strains (10^5 cells g^{-1}) (Table 1) could not be attributable to this source. This possibility appears unlikely since the Ice$^-$ strain 31R1-28 did not produce appreciable numbers of ice nuclei or contribute to frost injury to corn plants (Table 1). It is more likely that a high proportion of the ice nuclei detected on plants with high population sizes of Ice$^-$ *P. syringae* strains and thus low numbers of the Ice$^+$ strain 31R13C are attributable to nonviable cells of strain 31R13C remaining from the original inoculum. If only 10% of the inoculum of strain 31R13C was captured by the corn plants, as observed in other studies (28, 31) and verified in this study (data not shown), then approximately 10^3 cells of strain 31R13C were initially deposited per g of plant. Since the final population sizes of strain 31R13C on plants colonized by Ice$^-$ strains were generally in the range of 10^2 to 10^3 cells g (fresh weight)$^{-1}$, no net growth occurred and many cells of this strain apparently died before the final viable bacterial population size and numbers of ice nuclei were determined. Since even nonviable cells can express ice nucleation activity (1, 31, 32), approximately 10 ice nuclei g (fresh weight)$^{-1}$ are likely to have been deposited during inoculation with the Ice$^+$ strain (the in vitro nucleation frequency of the inoculum was ca. 10^{-2} ice nuclei cell^{-1}). Thus, ice nuclei contributed by inoculated cells of strain 31R13C that succumbed due to unsuccessful competition and survival in the presence of established Ice$^-$ strains probably caused the apparent nucleation frequency to increase when normalized only for viable cells of this strain. The ice nucleation frequency of strain 31R13C on plants having high populations of this strain (a lower proportion of dead to viable cells) was about 10^{-2} ice nuclei cell^{-1}, similar to that observed in a previous study (31).

The specificity of competition among leaf surface bacteria is probably not stringent. Strains that are nearly isogenic to *P. syringae* 31R1 are more competitive with each other than is another effective bacterial antagonist (28, 29) on leaf surfaces (Table 1). However, the Ice$^-$ strain Cit13R-12 greatly reduced the population sizes of Ice$^+$ *P. syringae* strains and *E. amylovora* colonizing pears under field conditions (Fig. 5 and 6). The natural epiphytic populations of *P. syringae* on pears in this study probably represented a diversity of ecotypes of this species, as described in other reports (16). Since no isogenic competitor of *E. amylovora* was available for this study, it is unknown whether such a strain would have reduced *E. amylovora* populations more than strain Cit13R-12 did. Conversely, *P. fluorescens*, *P. putida*, and *E. herbicola* strains, when applied to plants before *P. syringae*, decreased subsequent colonization of leaf surfaces by *P. syringae* (22, 24, 28–30). While a high degree of biochemical, genetic, and thus ecological similarity may be required to maximize competitive exclusion of phylloplane bacteria, many bacteria may normally compete for similar limiting resources. Biological control to achieve disease or frost control by competitive exclusion of epiphytic bacteria appears to be an attractive possibility. The results of this study suggest that competitive exclusion is sufficient to account for antagonism of ice nucleation-active *P. syringae* on the phylloplane of corn and pear.

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