Interactions of Antibiotics with *Pseudomonas fluorescens* Strain A506 in the Control of Fire Blight and Frost Injury to Pear

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ABSTRACT

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Pseudomonas fluorescens strain A506 and the antibiotics streptomycin and oxytetracycline, to which this antagonist is resistant, were evaluated individually and in combination in field trials over a 16-year period to assess the interactions and relative efficacy of bactericides and antagonistic bacteria in the control of fire blight and frost injury to pear. Strain A506 maintained population sizes of greater than about 10^5 cells per fruiting spur for over 30 days on trees inoculated once with this strain. Over 90% of the flowers on inoculated trees harbored detectable populations of strain A506. The population sizes of strain A506 were as high on inoculated trees, to which streptomycin was applied, as on trees sprayed only with strain A506. The population sizes of ice nucleation active bacteria were about 100-fold lower on trees treated with strain A506 than on untreated control trees or trees sprayed only with antibiotics. The incidence of frost injury to pear fruit in natural frosts and of fire blight was reduced to about 40 and 50%, respectively, of that on untreated trees. The incidence of frost damage and fire blight was lower on trees treated both with strain A506 and antibiotics than with either agent alone. Strain A506 and antibiotics acted additively in the control of frost and disease.

Fire blight is one of the most serious diseases limiting the production of pear, apple, and other pome fruits in many parts of the world. Flowers are the most common site of infection by the pathogen *Erwinia amylovora* (1,25,34). Establishment of large epiphytic populations on stigmatic surfaces precedes infection of flowers by the pathogen (25,34). While relatively warm temperatures and the presence of free moisture facilitate the increase in population size of *E. amylovora* on flowers, epiphytic populations of the pathogen are common, even on flowers that subsequently do not become infected (25,33,34). Streptomycin and oxytetracycline (Terramycin), as well as copper-containing compounds, are the primary means of disease control (1,34); these bactericides are usually applied frequently during and after periods when weather favors the development of epiphytic populations. Antibiotic-resistant strains of *E. amylovora* have been found in many pear- and apple-growing regions, and lack of adequate disease control has been associated with the occurrence of resistant strains (1,6,22,24,27,34). Other than cutting out infected parts of the tree and reducing nitrogen fertilizer or water applications to reduce the rate of disease spread through the tree, other controls are not available for this disease; varieties of pome fruits resistant to fire blight disease and with suitable horticultural characteristics have not been identified (1,34). The high costs of chemical control, failures in chemical control due to resistance development, and lack of other effective control measures have, therefore, generated considerable interest in biological control of fire blight.

Flowers and young fruit of pome fruits have little or no tolerance of ice formation and, therefore, are subject to frost damage (5,7,30). These tissues can supercool and avoid damaging ice formation, however, at least to temperatures as cold as about –4°C (28,30). Ice nucleation active (Ice^+) bacteria occur in significant numbers on pear in many growing regions and apparently limit the supercooling ability of this species (9,14,15,16,17,21,28). Reductions of the size of epiphytic Ice^+ bacterial populations can reduce the incidence of frost damage at temperatures above about –5°C (8,16,17). While nonbacterial ice-nucleating agents (i.e., Ice^+ fungi) may also be present in or on the woody parts of stone fruit trees (3,4,10), Ice^+ bacteria are abundant on the herbaceous tissues of the trees and commonly limit the supercooling ability of flowers and young fruit (17,20,28). Reductions in the population size of Ice^+ bacteria by both chemical bactericides and non-Ice^+ bacterial strains have reduced the incidence of frost damage to pear (16,17,18,20).

While many reports of the biological control of diseases in the greenhouse and in limited field tests have appeared, reports of the efficacy of such agents in the field over many growing seasons and in many locations are lacking. Commonly, the biological control of diseases under field conditions is often substantially less than, and more variable than, that under controlled conditions. To be accepted by growers, biological control agents must be consistently efficacious in the field. In addition to variable efficacy in the field, most biological control agents usually control only a single pathogen; both of these factors have limited their utility.

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Because frost damage and fire blight both occur during the flowering period of pear, we evaluated whether the same biological control agent could reduce the incidence of both hazards.

Pseudomonas fluorescens strain A506 was found to inhibit the growth of Ice$^+$ *P. syringae* strains on corn leaves in an in vivo screening process (17). Since strain A506 was isolated from a healthy pear tree in a commercial orchard in California that was treated repeatedly with streptomycin and oxytetracycline, it was not unexpected to find that this strain was resistant to these antibiotics. Initial field studies revealed that application of strain A506 to pear trees at flowering for the control of frost damage reduced the incidence of fire blight (17). We, therefore, report the efficacy of this strain in reducing both frost injury and fire blight to pear over a 16-year period in California. Since the efficacy of this strain in biological control is consistently high, it has been the focus of commercialization efforts. The availability of an Experimental Use Permit allowed us to compare the efficacy of this strain to control frost damage and fire blight in large- and small-scale trials in commercial orchards to determine if biological control by an epiphytic bacterium is influenced by the spatial scale at which it is used. In addition, the compatibility of *P. fluorescens* strain A506 with antibiotics that may be applied to pear trees, as well as the interactions of these bactericides with this agent on the control of frost damage and fire blight, was evaluated.

MATERIALS AND METHODS

Bacterial strains and culture media. The source and characteristics of *P. fluorescens* strain A506 have been described previously (36). This strain is a spontaneous mutant resistant to rifampicin (100 μg/ml), selected as described in a previous study (29). Estimates of total bacteria were made on King's medium B (13) containing cycloheximide (100 μg/ml) (KB). Recovery of strain A506 was on KB containing rifampicin (100 μg/ml) (KBR). *E. amylovora* was quantified on modified Miller-Schroth medium (MS) (25). Inoculum of strain A506 sprayed onto trees in small-scale field tests was recovered from the surface of KBR plates after 2 days of growth at 24°C. The registered biological pesticide Blightban A506 (Plant Health Technologies, Boise, ID), which is a lyophilized preparation of cells (1.2×10^{11} cells per g) of strain A506 recovered from broth culture, was used in large-scale field trials.

Field plot design. All field studies were conducted in commercial orchards containing mature 'Bartlett' pear trees. The orchards used were in the major pear-growing regions of California located in Lake, Mendocino, Sacramento, Solano, and Yuba counties. The trees in most orchards were about 4 m apart within a row, and 7 m separated adjacent rows of trees. Most trees were about 4 to 5 m in height. Neither Ice$^+$ bacteria nor *E. amylovora* were applied to any tree in any plot. At least two trials were conducted in each of the 16 years of this study; a total of 37 small-scale and eight large-scale tests were conducted. Because of nonconducive weather conditions between sites and years, neither fire blight disease nor frost injury occurred in some of the plots. Results are presented for every trial in which any frost injury or fire blight was detected. Small-scale field tests were conducted on trees organized in a randomized complete block design with four replications in an orchard section in which antibiotics were not applied to the trees (except as in the experimental design). Each replication consisted of from one to four trees in a given row, depending on the experiment; a single tree separated treated trees within a block and different blocks were in adjacent rows of pear trees. While no bactericides were applied to the plot area, other fungicides such as benomyl and mancozeb were applied to control pear scab (*Venturia pyrina* Aderhold).

Bacterial cells were scraped from the surface of KBR plates and suspended in tap water. The cell concentration of the suspensions was determined from the turbidity of the suspensions, and the suspensions were diluted with tap water to a concentration of approximately 10^8 cells per ml. Bacteria were applied to wetness (about 3 liters/tree) to trees at about 20% bloom with a backpack mist blower as in other studies (2). Some of the trees treated with strain A506, as well as trees not treated with this strain, in the small-scale tests were sprayed at 7-day intervals with streptomycin sulfate (Agristrep D 21%) at a concentration of 100 μg/ml or oxytetracycline (Mycoshield 17%) at a concentration of 100 μg/ml. In a given trial, trees were sprayed with bactericides starting at the time that *P. fluorescens* strain A506 was applied to the trees; the antibiotics were applied to trees also treated with strain A506 within 1 h after the bacterium was inoculated onto the trees. The bactericides were applied either with a backpack mist blower or with a piston-pressurized handgun sprayer as described previously (2).

Large-scale trials also were conducted on trees organized in a randomized complete block design with four replications; each replication of each treatment consisted of groups of 25 to 200 trees, at least five rows wide and having at least five trees within a row. All of the trees in the large-scale tests were sprayed with solutions of streptomycin sulfate (100 μg/ml) or oxytetracycline (100 μg/ml) applied with commercial air-blast sprayers by cooperating growers. Bactericides were sprayed at intervals that the cooperators determined, generally at 3- to 7-day intervals, depending on prevailing weather conditions. Lyophilized preparations of *P. fluorescens* strain A506 were dispersed into tap water in either a commercial air-blast sprayer or a piston-pressurized handgun sprayer at a concentration of 10^8 cells per ml and sprayed onto trees within 1 h of preparation at a rate of about 3 liters/tree.

Enumeration of bacterial populations on plants. Epiphytic bacterial populations from 25 individual flowers or fruiting spurs were estimated for each treatment. Individual flowers or fruiting spurs were placed in small, sterile, sealable plastic bags immediately after collection in the field and transported on ice to the laboratory within about 4 h. Eight or 100 ml of sterile washing buffer (29) was added to the bags. The tissues were submerged in the buffer and the bags were sonicated for 7 min in an ultrasonic cleaning bath to dislodge bacteria from the leaves as in other studies (29). The bags were then manually agitated for 10 s to disperse the bacterial cells before enumeration. An appropriate dilution of the tissue washing was then plated onto KB, KBR, and MS using a spiral diluter/dispenser. Total bacteria were counted after 4 days of growth on KB at 24°C. Bacteria resembling *E. amylovora* on MS were enumerated after 6 days of growth at 24°C, while strain A506 was enumerated on KBR after 3 days at 24°C. Ice$^+$ bacteria were quantified after 6 days of growth on KB at 24°C by a "replica freezing" technique similar to that described elsewhere (21). Cells from colonies on dilution plates were transferred onto the surface of a paraffin-coated aluminum-foil sheet using a sterile velvet pad. The sheet was floated on the surface of a refrigerated bath maintained at –9°C and sprayed with a fine mist of sterile, distilled water. After about 30 s, Ice$^+$ bacterial colonies were readily apparent as "frosty" spots where the microdroplets of water had frozen.

Estimation of frost injury and fire blight disease. Approximately 200 fruit were harvested at maturity from each replicate of each treatment. The presence or absence of frost damage, evident as sunken necrotic spots or rings on the surface of the fruit (similar to that previously depicted [17]), were noted for each fruit; fruit either had obvious frost injury or injury was absent. Frost injury to flowers was apparent as browning of the petals, stamens, and pistil of flowers 12 h after a freezing event; newly opened flowers either had obvious frost damage or injury was absent. Fire blight infection was visually rated by viewing trees from ground level and counting the number of infected fruiting or vegetative shoots on each tree that exhibited wilting, discoloration, or both typical of fire blight disease. Most fire blight strikes were enumerated about 2 weeks after infections had become ap-

parent so that disease was obvious and not easily missed during observation.

Statistical methods. The goodness-of-fit of the normal distribution of log-transformed bacterial population sizes of individual tissue samples was tested by the Shapiro-Wilk W statistic and calculated using SAS (release 6.04, SAS Institute, Cary, NC). Since most distributions were adequately described by a log-normal distribution, all estimates of bacterial population sizes were log-transformed prior to analysis. All measurements of frost injury, expressed as the proportion of fruit injured, were subjected to inverse sine transformation before analysis. Samples in which population sizes of Ice$^+$ bacteria or *E. amylovora* were not detected were assigned the log-transformed value of 0. Analysis of variance of log-transformed epiphytic bacterial population sizes was computed using the general linear models procedure of SAS. Separation of treatment means was by Fisher's unprotected least significant difference test; this method controls the comparison-wise error rate.

RESULTS

Colonization of pear tissues. The mean population size of *P. fluorescens* strain A506 generally increased after spray inoculation and remained high for 30 or more days (Figs. 1 and 2). The results presented in these two figures are typical of the colonization observed in the many other trials that are not reported. Rainfall did not normally occur with a high frequency or abundance 30 days or more after first flowering, and decreases in the population size of strain A506 were associated with these conditions; this strain was usually undetectable at the time of harvest (data not shown). The population size of strain A506 on untreated control trees in small-scale trials (located an average of about 10 m from A506-treated trees) was frequently higher than 10^4 to 10^5 cells per g of fresh weight (Fig. 1A). Even in large-scale trials in which control trees were more than 50 m from A506-treated trees, more than 100 A506 cells per fruiting spur were often detected (Fig. 2A). The population sizes of strain A506 on treated trees were usually at least 10- to 100-fold higher than on untreated trees (Figs. 1 and 2).

The population sizes of Ice$^+$ bacteria were generally significantly lower on trees treated with A506, irrespective of whether antibiotics were also applied to the trees (Fig. 1). During the first 20 days after flowering, the population size of Ice$^+$ bacteria was 100-fold lower on trees treated with strain A506 than on untreated control trees (Fig. 1). While the population size of Ice$^+$ bacteria was 10-fold or more lower on trees treated with streptomycin than on control trees shortly after flowering (Fig. 1D), subsequent population sizes were usually similar to that of the controls (Fig. 1). During much of the early growing season, the population size of Ice$^+$ bacteria on trees treated with A506, and which subsequently received streptomycin sprays at 5-day intervals, was 1,000-fold lower than on untreated control trees, as well as on trees treated only with streptomycin (Fig. 1). More total bacteria were usually recovered from trees treated with strain A506 within the first 20 days after inoculation than from untreated control trees, especially in years when total epiphytic populations on control trees were low. For example, total bacterial populations on A506-treated trees were 30- to 50-fold higher during the first 20 days after inoculation compared with that on untreated control trees in 1994 (Fig. 2). Total bacterial populations were also about threefold higher on trees treated with both strain A506 and streptomycin than with streptomycin alone in 1993 (Fig. 1). In most studies, total bacterial populations on A506-treated and untreated trees were similar at samplings made 20 days or more after inoculation (Figs. 1 and 2; other data not shown).

Since the biological control of fire blight disease occurs at the scale of individual flowers, the distribution of population sizes of *P. fluorescens* strain A506 within a population of flowers on

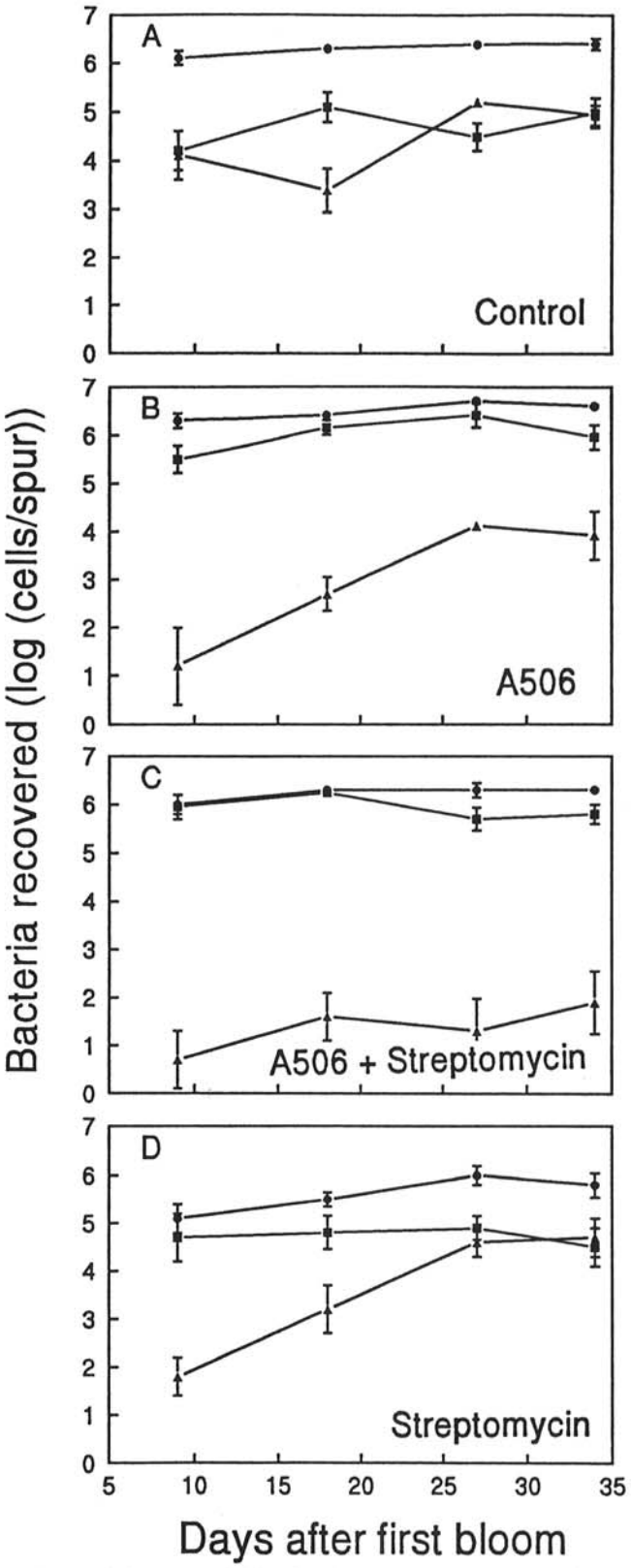

Fig. 1. Population sizes of all culturable bacteria (circles), *Pseudomonas fluorescens* strain A506 (squares), and ice nucleation active bacteria (triangles) recovered from individual fruiting spurs harvested at varying times after initial blossoms appeared on trees in small-scale tests in a commercial 'Bartlett' pear orchard located near Lakeport, CA. Trees were **A,** not sprayed; **B and C,** sprayed once with strain A506 at 4 days after first bloom; or **C and D,** sprayed weekly with streptomycin. The day of first bloom was 22 March 1993. The vertical bars represent the standard error of the determination of mean log bacterial population sizes.

A506-treated trees was determined. When measured 1 week after inoculation, over 95% of individual flowers on trees treated once with strain A506 had detectable populations of this strain and the mean population size was about 10^5 cells per flower (Fig. 3B). While the incidence of flower colonization by strain A506 was not substantially higher on trees treated twice with strain A506, the mean population size measured 1 week after the second inoculation was about 10^6 cells per flower (Fig. 3C). The population size of strain A506 on nearly all flowers treated twice with this strain was in excess of about 10^5 cells per flower. Detectable populations of strain A506 were found on untreated control trees located more than 20 m from the A506-treated trees (Fig. 3A). About 50% of the flowers from untreated control trees had population sizes of

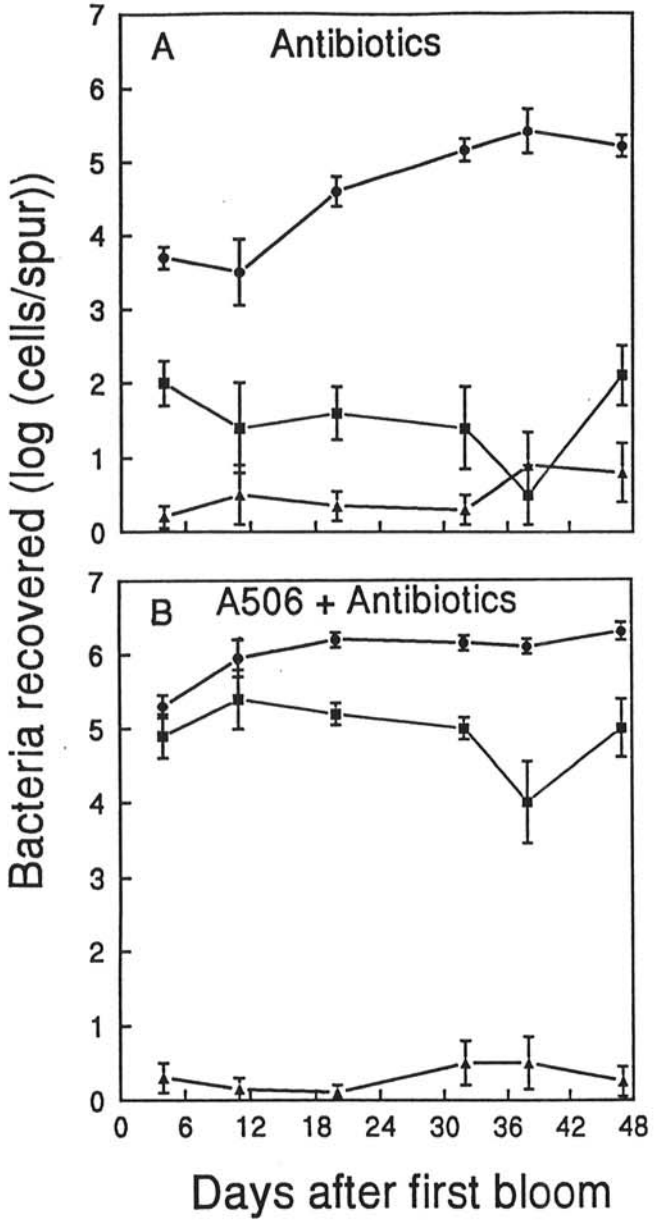

Fig. 2. Population sizes of all culturable bacteria (circles), *Pseudomonas fluorescens* strain A506 (squares), and ice nucleation active bacteria (triangles) recovered from individual fruiting spurs harvested at varying times after initial blossoms appeared on trees in a large-scale test in a commercial 'Bartlett' pear orchard located near Lakeport, CA. **A and B**, All trees were sprayed at 3-day intervals with a mixture of streptomycin and oxytetracycline. **B,** Trees were sprayed with strain A506 at 2 and 17 days after first bloom with a commercial air-blast sprayer. The day of first bloom was 25 March 1994. The vertical bars represent the standard error of the determination of mean log bacterial population sizes.

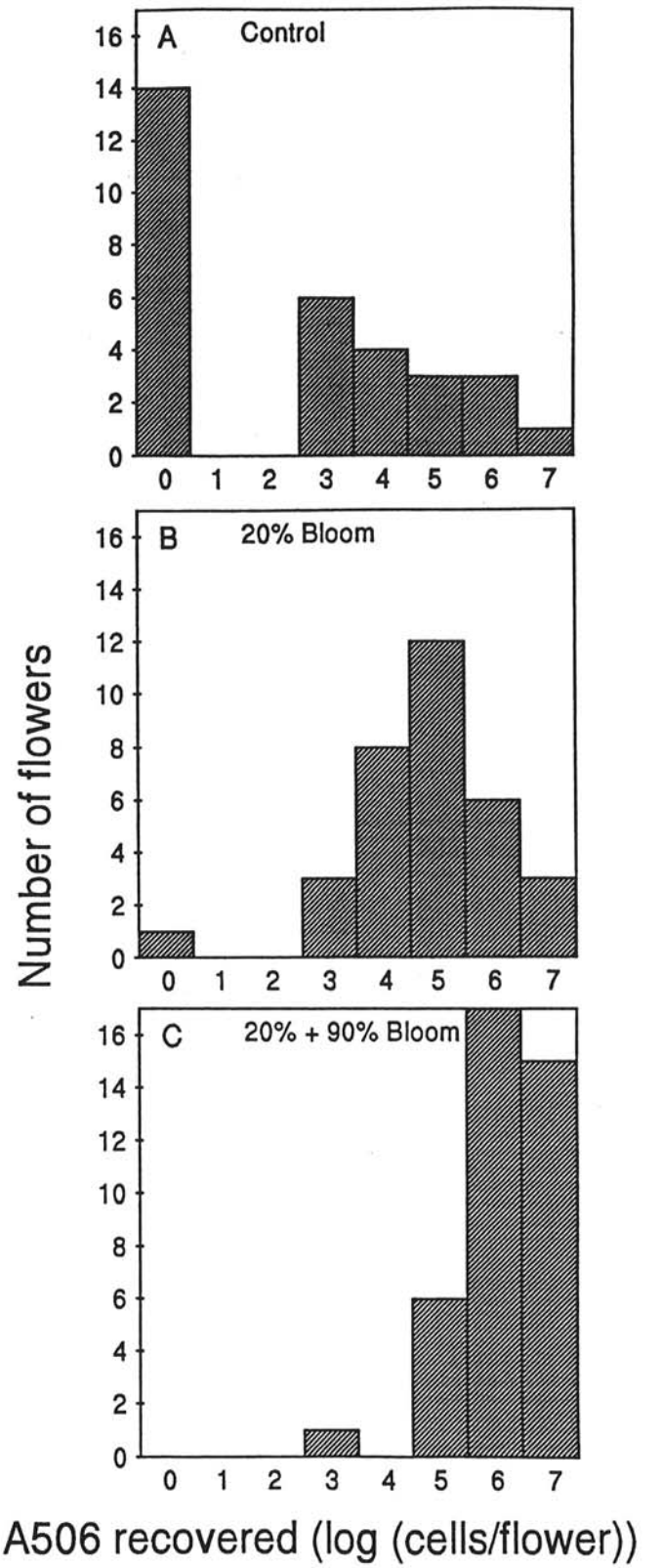

Fig. 3. Distribution of population sizes of *Pseudomonas fluorescens* strain A506 among individual flowers of 'Bartlett' pear and harvested from **A,** untreated control trees; and **B,** trees treated with strain A506 once (at about 20% bloom) or **C,** twice (at 20 and 90% bloom). The trees in a large-scale test in a commercial pear orchard near Hopland, CA, were spray-inoculated with strain A506 using a commercial air-blast orchard sprayer. All trees were also sprayed with streptomycin at 3-day intervals. Those flowers that had undetectable populations of strain A506 (<100 cells per flower) are depicted as having log populations of 0.

strain A506 greater than 10^3 cells per flower (Fig. 3A). However, few flowers from untreated trees had population sizes greater than about 10^5 cells per flower (Fig. 3A). Flowers of untreated trees that were closer to A506-treated trees often had higher numbers of this strain than trees further away (data not shown). Less than 5 CFU of strain A506 generally were deposited onto gravity sedimentation plates placed 20 m away from treated trees during, and shortly after, spray inoculation. The populations of strain A506 recovered in untreated control trees, therefore, apparently represented primarily growth of inoculum deposited during the treatment of nearby trees, as well as from subsequent immigration from inoculated trees.

The incidence of frost injury to pear fruit at harvest was reduced by strain A506 in all seven small-scale trials in which freezing temperatures were encountered. The incidence of frost damage to 'Bartlett' pear fruit on trees treated only with strain A506 was reduced to about 40% of that on untreated control trees (Table 1). In all cases, the reduction of frost damage on trees treated with strain A506 was at least as great as that conferred by antibiotic sprays alone; antibiotic sprays reduced the incidence of frost damage to about 57% of that on control trees (Table 1). The incidence of frost damage on trees treated with both strain A506 and either streptomycin or oxytetracycline was reduced to only about 23% of that on untreated trees (Table 1). This level of injury was similar to that expected if strain A506 and antibiotics had acted additively (e.g., 22% of the damage seen on untreated trees).

The incidence of frost injury to pear trees in all large-scale plots that were treated with strain A506 (as well as receiving twice weekly antibiotic sprays) that experienced freezing conditions was reduced to only about 31% that of trees treated only with antibiotics (Table 2). The frost event in both 1991 and 1994 was relatively mild (minimum air temperatures of about –2 to –4°C); thus, most frost-damaged flowers or young fruit did not drop from the trees and were available for rating at harvest. The incidence of frost-damaged flowers and fruit in 1991, therefore, were similar (Table 2). The frost-damaged fruit was generally sufficiently blemished that it was unsuitable for fresh market commerce. The reduction in the incidence of frost damage in trees treated both with strain A506 and antibiotics compared with that on trees treated with antibiotics alone in these large-scale trials was substantially greater than that observed in small-scale tests of these two treatments in other years (compare Table 2 with Table 1).

The incidence of fire blight strikes on trees treated with *P. fluorescens* strain A506 alone, antibiotics, or both was reduced in each of nine small-scale tests in which natural fire blight epidemics occurred (Table 3). Reductions in the incidence of fire blight by applications of strain A506 were not obviously related to disease pressure. While the incidence of fire blight infection was reduced by strain A506 by about 50% when applied alone, combination treatments including single applications of strain A506 and weekly applications of either streptomycin or oxytetracycline reduced disease incidence by about 70% compared with that on untreated trees (Table 3). In contrast, applications of either streptomycin or oxytetracycline on a weekly basis reduced the incidence of disease by only about 40% compared with that on untreated control trees (Table 3).

P. fluorescens strain A506 reduced the incidence of fire blight infection in each of six large-scale field trials conducted in commercial orchards in which natural fire blight epidemics occurred. All trees in these tests were sprayed with either streptomycin, oxytetracycline, or both at 3- to 7-day intervals as dictated by the prevailing weather conditions and the discretion of the cooperating growers. Comparisons of fire blight control conferred by two applications of strain A506 (once at about 20% bloom and again at about 95% bloom) in conjunction with antibiotic sprays with that of antibiotic sprays alone were conducted. The incidence of fire blight in trees treated with both strain A506 and antibiotics was reduced, on average, to 27% of that in trees treated with antibiotics alone (Table 4). The control of fire blight by strain A506 in large-scale trials was superior to that observed in many smaller

TABLE 1. Incidence of frost injury to pear fruit in trees treated with *Pseudomonas fluorescens* strain A506 and antibiotics in small-scale trials

	Injury (fraction of fruit)[w]						
Treatment	1980	1981	1985	1986	1991	1992	1995
Control	0.39 a	0.30 a	0.27 a	0.76 a	0.19 a	0.18 a	0.81 a
A506[x]	0.09 b	0.07 b	0.18 b	0.41 b	0.05 b	0.09 b	0.32 b
A506 + streptomycin[x,y]	0.10 b	0.04 b				0.06 b	0.34 b
A506 + oxytetracycline[x,z]					0.04 b		
Streptomycin[y]	0.24 ab	0.11 ab	0.19 b	0.56 ab		0.15 a	
Oxytetracycline[z]					0.10 ab		

[w] The fraction of fruit from pear trees exposed to indigenous populations of ice nucleation active bacteria that exhibited frost injury at harvest resulting from a natural frost in the field near the time of bloom is reported. Means in each column followed by the same letter do not differ significantly at $P = 0.05$ by Fisher's unprotected least significant difference test.

[x] Trees were treated a single time at about 20% bloom with a suspension of about 10^8 cells per ml of *P. fluorescens* strain A506.

[y] Trees were treated approximately weekly with a solution of 100 µg/ml of streptomycin sulfate starting at the time of application of *P. fluorescens* strain A506.

[z] Trees were treated approximately weekly with a solution of 100 µg/ml of oxytetracycline starting at the time of application of *P. fluorescens* strain A506.

TABLE 2. Incidence of frost injury to pear fruit and flowers on trees treated with *Pseudomonas fluorescens* strain A506 in large-scale field trials

	Injury (fraction of fruit or flowers)[x]					
	1991 - site 1		1991 - site 2	1991 - site 3	1994 - site 1	1994 - site 2
Treatment	Flowers	Fruit	Fruit	Fruit	Fruit	Fruit
Control[y]	0.52 a	0.26 a	0.33 a	0.016 a	0.20 a	0.26 a
A506[z]	0.16 b	0.09 b	0.14 b	0.003 b	0.07 b	0.08 b

[x] The fraction of fruit from pear trees exposed to indigenous ice nucleation active bacterial populations that exhibited frost injury at harvest resulting from a natural frost in the field near the time of bloom. The fraction of flowers that exhibited browning because of freezing injury about 12 h after a natural radiative frost in the field is also reported. Means in each column followed by the same letter do not differ significantly at $P = 0.05$ by Fisher's unprotected least significant difference test.

[y] All trees in the trial were treated by cooperating growers with 100 µg/ml of streptomycin sulfate, 100 µg/ml of oxytetracycline, or both at a frequency of from every 3 to 7 days starting about 5 days after the application of *P. fluorescens* strain A506.

[z] *P. fluorescens* strain A506 was applied at a concentration of about 10^8 cells per ml using commercial air-blast orchard sprayers at about 20% bloom and again at about 90% bloom.

scale trials; in small-scale trials, application of both strain A506 and antibiotics reduced disease incidence to only 41% of that in trees treated with antibiotics alone (Table 3).

The population size of *E. amylovora* was generally reduced from 10- to 100-fold on individual flowers of trees in large-scale plots treated with both strain A506 and antibiotics compared with that on trees sprayed only with antibiotics. On 3 May 1992, for example, the mean population size of *E. amylovora* on flowers of trees in a commercial orchard near Sacramento, CA, treated with strain A506 at both 20 and 100% bloom was $10^{1.9}$ cells per flower, while flowers from trees treated only with antibiotics contained $10^{3.7}$ cells of *E. amylovora*. These differences in population size were significantly different ($P < 0.001$). Similar reductions of *E. amylovora* population sizes were observed in both large- and small-scale trials during the course of this study (data not shown).

DISCUSSION

Evaluations of bacteria for biological control of fire blight will better reflect their efficacy when used in commercial orchards in which disease is incited by indigenous populations of *E. amylovora*. The incidence of flower infections in these trials was usually much less than that in other studies in which flowers were inoculated. For example, if we assume that an average mature 'Bartlett' pear tree has approximately 20,000 flowers, then the average of about 10 infections per tree that occurred because of indigenous inoculum in these studies indicates that only about 0.05% of the flowers became infected. In contrast, 50% or more of the flowers were infected in some studies in which *E. amylovora* was inoculated onto flowers (35). Johnson et al. (12) reported that relative disease control was much greater in trees treated with *P. fluorescens* strain A506 when *E. amylovora* was applied at low doses using inoculated honeybees than when the pathogen was sprayed onto trees. Thus, while spray inoculation of newly opened flowers with 10^5 to 10^8 cells per ml of *E. amylovora* will result in a high incidence of flower infection, thereby facilitating measurements of disease, such high levels of infection are seldom seen under field conditions. A high incidence of infection will allow disease to be estimated on small trees or on single treated branches, but such procedures may minimize the apparent efficacy of a biological control agent.

Reductions in the population size of *E. amylovora* in pear flowers treated with *P. fluorescens* strain A506 is apparently due to competition (36). While application of strain A506 to flowers 1 day or more before inoculation with *E. amylovora* greatly reduces the final pathogen population sizes compared with those on control flowers, pathogen populations are not reduced substantially when strain A506 is coinoculated with the pathogen (36). Biological control agents such as strain A506, which do not kill the pathogen because of antibiotic production, apparently must sequester resources before the arrival of *E. amylovora* on flowers. Because strain A506 primarily reduces the potential increase in the population size of *E. amylovora* that might otherwise occur on flowers, application of a large number of *E. amylovora* cells overcomes the need for its growth and, therefore, negates its sensitivity to control. It seems likely that the number of bacterial strains capable of reducing fire blight is higher than that estimated from previous screening studies using flowers that were challenge-inoculated with large populations of *E. amylovora* (35). Screening procedures that incorporate knowledge of the normal population dynamics of the pathogen in flowers should maximize the likelihood of identifying antagonistic microorganisms.

P. fluorescens strain A506 and antibiotics appear to act additively to prevent infection of pear flowers by *E. amylovora* and in reducing frost damage. While, on average, only 49 and 63% as many infections occurred on trees treated with strain A506 or antibiotics alone as compared with those on untreated control trees, the incidence of infection in trees treated with both agents was, on average, only 30% that of untreated trees and equaled the product of these two relative effects ($0.49 \times 0.63 = 0.30$) (Table 3). Likewise, on average, there were only 41% as many infections on trees receiving both A506 and antibiotics treatments as compared with those on trees receiving antibiotic treatments alone, which is close to the 49% predicted if strain A506 acted independently from antibiotics (Tables 3 and 4). These results indicate that application of strain A506 to trees, especially in many California orchards in which streptomycin resistance is common (27), can yield similar disease control to that of the application of

TABLE 3. Incidence of fire blight strikes on pear trees in small-scale trials that were treated at flowering with *Pseudomonas fluorescens* strain A506 and antibiotics

	Disease incidence (no. strikes per tree)[w]								
Treatment	1980	1981	1984	1985 - site 1	1985 - site 2	1987	1988	1989	1990
Control	20.0 a	480 a	15.8 a	6.2 a	3.5 a	13.6 a	6.3 a	9.0 a	4.2 a
A506[x]	8.1 b	450 a	21.0 a	1.2 b	1.0 a	6.1 b	3.3 b	1.2 b	3.2 ab
A506 + streptomycin[x,y]	7.2 b	330 b	5.0 b	0.5 b					
A506 + oxytetracycline[x,z]						6.4 b	3.1 b	0.0 b	0.4 b
Streptomycin[y]		470 a	19.8 a	1.5 b	1.5 a	1.4 b			
Oxytetracycline[z]						6.6 b	5.0 a	0.1 b	2.8 b

[w] The incidence of fire blight strikes that resulted from indigenous populations of *Erwinia amylovora* in trees in commercial pear orchards. Means in each column followed by the same letter do not differ significantly at $P = 0.05$ by Fisher's unprotected least significant difference test.

[x] Trees were treated a single time at about 20% bloom with a suspension of about 10^8 cells per ml of *P. fluorescens* strain A506.

[y] Trees were treated approximately weekly with a solution of 100 µg/ml of streptomycin sulfate starting at the time of application of *P. fluorescens* strain A506.

[z] Trees were treated approximately weekly with a solution of 100 µg/ml of oxytetracycline starting at the time of application of *P. fluorescens* strain A506.

TABLE 4. Incidence of fire blight strikes on pear trees treated with *Pseudomonas fluorescens* strain A506 in large-scale field trials

	Disease incidence (no. strikes per tree)[x]					
	1990	1992 - site 1	1992 - site 2	1993	1994 - site 1	1994 - site 2
Control[y]	9.46 a	2.29 a	3.96 a	3.29 a	0.10 a	0.31 a
A506[z]	3.53 b	0.69 b	0.79 b	0.86 b	0.02 b	0.10 b

[x] The incidence of fire blight strikes that resulted from indigenous populations of *Erwinia amylovora* in trees in commercial pear orchards. Means in each column followed by the same letter do not differ significantly at $P = 0.05$ by Fisher's unprotected least significant difference test.

[y] All trees in the trial were treated by cooperating growers with 100 µg/ml of streptomycin sulfate, 100 µg/ml of oxytetracycline, or both at a frequency of from every 3 to 7 days starting about 5 days after the application of *P. fluorescens* strain A506.

[z] *P. fluorescens* strain A506 was applied at a concentration of about 10^8 cells per ml using commercial air-blast orchard sprayers at about 20% bloom and again at about 90% bloom.

streptomycin alone or mixtures of streptomycin and oxytetracycline. In addition, treating trees once or twice with strain A506 in addition to normal antibiotic sprays can improve control of fire blight (Tables 3 and 4). Since the incidence of fire blight was consistently less in trees treated with both strain A506 and antibiotics than with either treatment alone, it appears that the presence of strain A506 may reduce the growth of *E. amylovora* on flowers after partial eradication with antibiotic sprays. Since streptomycin and oxytetracycline are readily degraded under field conditions (1), it seems likely that residues drop to levels that are not sufficient to prevent the growth of *E. amylovora* within the 3- to 7-day interval between antibiotic sprays. Strain A506 may limit the growth of *E. amylovora* during periods when antibiotic residues are too low to prevent growth of the pathogen.

Not only did strain A506 and antibiotics act additively to control fire blight and frost injury, but antibiotic sprays generally did not largely affect the population size of strain A506. For example, the population size of strain A506 on trees treated with both streptomycin and strain A506 were indistinguishable from those observed on trees treated with strain A506 alone (Fig. 1). In no instance did we observe a reduction in the population size of strain A506 on streptomycin-treated trees (data not shown). While the population size of strain A506 was generally high on trees to which oxytetracycline was subsequently applied, its population size was often even higher (twofold to fivefold) on trees on which this antibiotic was not also applied (data not shown). Thus, *P. fluorescens* strain A506 appears to be completely compatible with subsequent applications of streptomycin, and its population size is not greatly reduced in the presence of oxytetracycline. Stockwell et al. (32) also report that the population size of strain A506 is not decreased by oxytetracycline or by streptomycin applied throughout the bloom period.

Strain A506 achieved and maintained relatively large population sizes on a high proportion of the flowers, even when applied with standard spraying equipment to trees in commercial orchards that were also sprayed with other pesticides for the control of insects and fungal pathogens (Fig. 3). The population sizes of *E. amylovora* vary greatly between different flowers; the colonization of individual flowers probably occurs independently from one another. Therefore, the interactions between A506 and *E. amylovora* that reduce the population size of the pathogen also occur independently in the many individual flowers on a tree. If we assume that the efficacy of biological control is dependent on the presence of a substantial population size of an antagonist, disease control will be greater in an orchard having a high proportion of flowers that have a moderately high population size of the biological control agent than in an orchard in which the average population size of the antagonist is high but the antagonist is restricted to a few flowers. Thus, the high population sizes of strain A506 on most individual flowers or spurs (Fig. 3) contributed to the effectiveness of this strain in controlling frost damage and fire blight.

The efficacy of biological control of fire blight generally increased with increasing size of the area treated with *P. fluorescens* strain A506. In large-scale trials, the incidence of fire blight on trees treated with both strain A506 and antibiotics was reduced, on average, to only about 28% that of trees treated with antibiotics alone, while in small-scale trials this reduction was only to about 41% (Tables 3 and 4). Antagonists such as strain A506 may commonly move between treated and untreated plants, as observed here (Figs. 1, 2, and 3). The application of strain A506 to large continuous blocks of trees may not only reduce the growth of *E. amylovora* in each treated tree but, as a result, it would also reduce sources of local inoculum. For example, substantial numbers of cells of *E. amylovora* may immigrate to a tree from adjacent untreated trees. Hence, a single tree treated with strain A506 might receive substantially more inoculum from adjacent untreated trees than would a similarly treated tree in a larger contiguous block treated with this strain. The greater control of fire blight and frost injury observed in large-scale compared with small-scale trials observed here suggest that some apparent failures of biological control under field conditions may be attributable to the design of the experiment. For example, uninoculated plants might commonly harbor substantial numbers of the antagonist because of immigration and subsequent growth of such strains from adjacent treated plants. In such cases, plants free of the antagonist are not available for control purposes. The prevalence of such cross-contamination of foliar bacteria needs to be further evaluated and should be considered when designing experiments.

Even though strain A506 was selected as an antagonist of Ice$^+$ *P. syringae* strains on corn leaves (17), it also apparently competes well with other bacteria, apparently by preemptive resource acquisition (37). The reductions in population size of Ice$^+$ *P. syringae* strains and *E. amylovora* strains on pear trees were similar in magnitude in these studies (Figs. 1 and 2). Strain A506 apparently can catabolize many of the nutrients that are available to bacteria on leaf surfaces including those catabolized by Ice$^+$ *P. syringae* strains (37) and presumably also by indigenous *E. amylovora* strains. If the primary mechanism of antagonism of *E. amylovora* and *P. syringae* on leaves is the sequestration of nutrient resources, selection for strains of *P. syringae* or *E. amylovora* that could avoid antagonism by strain A506 would appear unlikely. The biological control of fire blight and frost injury with a strain with broad resource acquisition capabilities such as strain A506 might be considered more "durable" than that conferred by a strain producing a specific antibiotic.

P. fluorescens strain A506 consistently reduced the incidence of frost damage to pear flowers and fruit more than did antibiotic sprays. Surveys of Ice$^+$ *P. syringae* strains on pear in California revealed that from 1 to nearly 100% of the strains were resistant to streptomycin or oxytetracycline (S. Lindow, *unpublished data*). Resistant strains have also been found in Oregon and Washington (31). It is unlikely that such strains would be controlled by streptomycin or oxytetracycline sprays, but infrequent applications of either of these antibiotics may allow the establishment of antibiotic-sensitive Ice$^+$ strains on leaves in the intervals between applications. Although antibiotic-sensitive strains would be killed by subsequent antibiotic sprays, the nonviable cells may retain some ice nucleation activity (20,23). If used alone or in combination with antibiotics, antagonistic bacteria such as strain A506 may reduce the total number of bacterial ice nuclei on a plant by preventing these transient populations from developing.

The reductions in population size of Ice$^+$ bacteria on pear by strain A506 was sufficient to account for the reductions in frost injury observed. For example, the population size of Ice$^+$ bacteria was frequently reduced as much as 100-fold on trees treated with strain A506 compared with that on untreated control trees or trees treated with antibiotics alone (Fig. 1). The temperature to which a plant part will supercool before freezing is directly related to the logarithm of the population size of Ice$^+$ bacteria on those plants (11,19). A 100-fold reduction in the population size of Ice$^+$ *P. syringae* strains should reduce the temperature at which ice is initiated in plants by about 1°C, assuming that most of the ice nuclei on the plant are bacterial in origin (19). Since most of the freezing events encountered in this study were of very short duration (air temperatures less than 0°C occurred for less than 3 h) and minimum air temperatures were normally warmer than about –3°C, the reduced Ice$^+$ bacterial populations on A506-treated trees should have substantially increased the fraction of plant parts which could supercool to at least the minimum air temperatures encountered here.

Many factors apparently influence the process of initial ice formation in woody plants. Ice nucleation not associated with viable bacteria has been reported in several woody plant species (3,4,10). While able to catalyze ice formation at relatively warm temperatures, these agents are of apparently low abundance on the foliage,

flowers, and fruit (4,14). Exposed vegetative tissues such as flowers and immature fruit are also often colder than woody tissues during radiative frosts such as those in these studies (26). The duration of freezing temperatures, the minimum temperature to which plants were cooled, the mean population size of indigenous Ice^+ bacteria, and the plant phenological stage at which freezing occurred all can affect the process of ice nucleation and propagation (20). Differences in these parameters in studies conducted in various locations likely account for the reduced levels of frost control to fruit trees obtained in other studies (8).

P. fluorescens strain A506 has several attributes which would be desirable in a biological control agent. This strain appears to readily colonize pear tissues, even in commercial orchards in which antibiotics and other pesticides are repeatedly applied. Since biological control conferred by this strain is additive to chemical control, growers have the option of integrating chemical and biological control to manage frost injury and fire blight; growers unwilling to completely replace bactericides with a biological control agent may be willing to substitute the biological control agent for at least some applications of the bactericide. Most importantly, the ability of strain A506 to reduce the colonization of two important pests of pear would maximize the likelihood that growers would realize the benefit from the early-season applications of this strain to their orchards. For example, neither frost damage nor fire blight occur every year in a given pear orchard, while both may occur in some years or in some orchards. Since the efficacy of strain A506 is increased when it is applied to plant tissues before arrival of the target organism (36), growers must apply this strain before either Ice^+ bacteria or *E. amylovora* are detected. The likelihood that growers will realize an economic benefit from the protective application of strain A506 is increased if more than one problem can be managed.

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