

# A Method Involving Ice Nucleation for the Identification of Microorganisms Antagonistic to *Erwinia amylovora* on Pear Flowers

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## ABSTRACT

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A method was developed for the rapid selection of antagonists capable of suppressing growth of *Erwinia amylovora* on pear flowers. The population size of *E. amylovora* on flowers pretreated with potential antagonists was estimated by a flower-freezing assay. *E. amylovora* harboring the *iceC* gene from *Pseudomonas syringae* on the stable plasmid pVSP61 expressed high levels of ice nucleation activity. The threshold freezing temperature of flowers colonized by the ice nucleation-active (Ice<sup>+</sup>) strain of *E. amylovora* increased linearly with the logarithm of the population size of the pathogen. Bacterial and yeast strains were selected from the predominant microflora of pear flowers and tested for their ability to

lower the threshold freezing temperature of flowers subsequently inoculated with the Ice<sup>+</sup> *E. amylovora* strain. For each antagonist, the proportion of 20 treated flowers that had frozen was evaluated when approximately 95% of the control (*E. amylovora* only) had frozen. Among 257 strains tested, 45 (4 yeasts and 41 bacteria) were capable of reducing freezing of inoculated flowers by 70% or more. The reduction in the proportion of pretreated flowers that froze relative to the control for a number of antagonists was correlated ( $R^2 = 0.61$ ) with reduced *E. amylovora* population sizes. Over 50% of the antagonistic strains that reduced the proportion of frozen flowers by 50% or more during an initial screening conferred a similar reduction in the number of frozen flowers in a second test. Several strains were more antagonistic to *E. amylovora* in situ than *P. fluorescens* strain A506, which is used commercially for biological control of fire blight.

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 main site of infection by the pathogen, *Erwinia amylovora* (1,20,24). Establishment of large epiphytic populations on stigmatic surfaces precedes infection of flowers by *E. amylovora* (20,23,24). Streptomycin and oxytetracycline (Terramycin) as well as copper-containing compounds are the primary means of disease control (1,24); these bactericides have to be applied frequently during and after periods when weather favors the development of epiphytic populations of *E. amylovora*. 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 (1,7, 16,19,21,24). Other than cutting out infected parts of the tree, alternative controls are not available for this disease, and cultivars of pome fruits resistant to fire blight that have suitable horticultural characteristics have not been identified (1,24). The high costs of chemical control, failures in chemical control due to resistance development, and lack of other effective control measures, therefore, have generated considerable interest in the development of biological control of fire blight.

Because pear and apple blossoms are produced only once per year from dormant woody tissues, there is only a limited period of the year when antagonists can be tested on flowers for inhibition of *E. amylovora*. Because a biocontrol agent for fire blight will most likely function by reducing epiphytic populations of *E. amylovora* on the flower, initial testing of interactions between antagonists and *E. amylovora* on flowers would seem to be superior to tests done in other plant parts. Antagonists, thus, can be selected for their ability to reduce the size of epiphytic populations of *E.*

*amylovora* on stigma. However, this method can be quite labor-intensive because it requires techniques such as dilution plating of several flowers per antagonist, which limits the number of strains that can be tested. Because of the difficulty of reproducibly obtaining infections on detached branches, tests for control of flower infection have to be done on trees and, thus, must be limited to a small number of antagonists. For these reasons, the initial screening of antagonists of *E. amylovora* usually has been done by antibiosis assays on agar or the inoculation of other susceptible plant parts, such as immature fruits (4,12,26). Unfortunately, in most plant systems there is a very poor correlation between antibiosis in culture and biocontrol activity, even under greenhouse conditions (2). Although eliminating noninhibitory strains reduces the number of bacterial strains that must be tested on plants for biological control of fire blight, this approach may not be expedient, because many effective antagonists will be discarded and the antibiotic-producing strains that are retained may not be superior to a randomly selected group of strains in biological control.

In this study, we describe the development of a method by which an unbiased collection of bacterial and yeast strains can be rapidly and quantitatively assessed for their ability to reduce the epiphytic population size of *E. amylovora* on flowers. The utility of this method, based on the use of threshold freezing temperatures to estimate population sizes of a strain of *E. amylovora* that has been made ice nucleation active (Ice<sup>+</sup>), is illustrated by the analysis of the antagonistic properties of over 250 microbial strains on flowers.

## MATERIALS AND METHODS

**Strains used.** *E. amylovora* strain Ea8R, a spontaneous mutant of strain Ea8 isolated from an infected pear tree, is resistant to 100 µg of rifampicin per ml and has been described previously (28). *Pseudomonas fluorescens* strain A506, which inhibits the growth of *E. amylovora* on pear flowers, also has been described previously (28). A collection of potentially antagonistic bacteria was

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isolated from pear blossoms collected from three commercial Bartlett pear orchards near Ukiah and Hopland, CA. The trees had received sprays of a mixture of the antibiotics streptomycin and oxytetracycline at about 5-day intervals, as well as weekly applications of benomyl. Each blossom was suspended in 10.0 mM potassium phosphate containing 0.1% peptone and was sonicated for 7 min to dislodge bacteria. Appropriate 10-fold serial dilutions were plated on 10% strength King's medium B (KB). An isolated colony representative of the predominant colony type on a plate was selected. Strains that exhibited ice nucleation activity at  $-9^{\circ}\text{C}$  in a droplet-freezing assay were eliminated (15). All bacteria were stored in 15% glycerol at  $-80^{\circ}\text{C}$ . Yeasts were isolated by washing blossoms collected from an ornamental pear tree (*Pyrus communis* L.) in Berkeley and plating on malt extract agar. Isolates of *Cryptococcus laurentii* (172, 204, 231, 236, 239, and 255) and *Candida tenuis* (178, 193, and 194) from apple fruit were provided by C. L. Wilson (USDA/ARS, Kearneysville, WV).

**Production of pear flowers.** Dormant branches were cut from pear trees, cultivar Bartlett, on 10 February 1995 in a research orchard near Hopland, CA, and stored at  $-2^{\circ}\text{C}$ . Each branch was about 50 cm long and contained from 5 to 10 flower buds. The basal ends of the branches were cut off under water, and the branches were placed in containers of water to a depth of about 5 cm. The branches were forced to bloom by incubating them in a growth chamber with continuous fluorescent light at 26 to  $27^{\circ}\text{C}$ . Before use, branches with newly opened flowers (1 to 3 days old) were cut to 15 to 25 cm, and all unopened flowers were removed.

**Production of an Ice<sup>+</sup> strain of *E. amylovora*.** An Ice<sup>+</sup> strain of *E. amylovora* was produced by introducing pJEL1703 (a derivative of pVSP61 containing the *iceC* gene from *P. syringae* strain Cit7 [17]) into *E. amylovora* Ea8R by a three-parental mating. A 10-fold excess ( $10^9$  cells per ml) of recipient strain Ea8R was incubated overnight on Luria agar (LA) at  $28^{\circ}\text{C}$  with donor strains *Escherichia coli* DH5 $\alpha$  (pJEL1703) and (pRK2073). Cells were removed from the agar surface, and appropriate dilutions were plated onto LA containing 50  $\mu\text{g}$  of kanamycin (Km) and 100  $\mu\text{g}$  of rifampicin (Rif) per ml. A transconjugant strain of *E. amylovora* that grew on LA + Km + Rif, designated Ea8KI, was isolated and tested for ice nucleation activity. The stability of pJEL1703 in *E. amylovora* in the absence of antibiotic selection was verified by repeated sequential transfers in Luria broth followed by plating on LA + Km. The pathogenicity of Ea8KI was confirmed by stab inoculation of immature pear fruit incubated at  $28^{\circ}\text{C}$  (5). The ice nucleation activity of cell suspensions of *E. amylovora* Ea8KI was measured by a droplet-freezing assay as described previously (15).

**Measurement of *E. amylovora* populations on pear flowers.** Pear flowers (10 per treatment) were individually immersed in 30-ml tubes containing 10 ml of 10 mM potassium phosphate buffer (pH 7.0). The tubes were sonicated for 3 min, and appropriate dilutions of the flower washings were plated on LA supplemented with Rif, cycloheximide ( $100 \mu\text{g ml}^{-1}$ ), and benomyl ( $50 \mu\text{g ml}^{-1}$ ) with a spiral dilution dispenser and incubated at  $28^{\circ}\text{C}$ . Colonies were counted 2 days later.

**Screening of antagonists.** Bacterial strains to be screened as antagonists were grown on KB agar for 1 to 3 days at room temperature until abundant growth was present. Yeasts were grown on malt extract agar for 3 to 5 days at room temperature. Bacterial cells were removed from the agar surface with a cotton swab and suspended in 4.5 liters of 5 mM phosphate buffer, pH 7.0, to yield a suspension of  $\sim 10^7$  cells per ml. Suspensions of yeast cells were prepared in the same fashion, but their concentration was not estimated. Recently opened flowers were inoculated with candidate antagonists by briefly dipping branches in a 5-liter beaker containing the cell suspension. From 2 to 4 branches with a total number of 25 to 40 flowers were inoculated with a given antagonist. The branches inoculated with an antagonist were immediately enclosed in a plastic bag and incubated at room temperature ( $\sim 21^{\circ}\text{C}$ ) on a lab bench. About 20 antagonists were tested in an experiment. There

were two controls for each experiment: the first was composed of flowers dipped in buffer only; the second was composed of flowers dipped in a suspension of the antagonist *P. fluorescens* A506. After 48 h, the blossoms (including controls) were sprayed with a suspension of *E. amylovora* Ea8KI ( $10^4$  cells per ml) in 5 mM phosphate buffer. The branches again were enclosed in plastic bags and incubated for 48 h at room temperature. After which, 20 flowers were aseptically removed from branches inoculated with a given strain and placed individually in 10-ml tubes containing 3.5 ml of 10 mM phosphate buffer. The threshold freezing temperature (i.e., the warmest temperature that caused freezing) of each flower was determined by immersing the tubes in a circulating ethanol bath at  $-2.2^{\circ}\text{C}$  and lowering the temperature by an increment of  $0.1^{\circ}\text{C}$  every 25 min. The number of frozen tubes for each treatment was counted when 85 to 100% (usually 95%) of the control tubes (containing flowers inoculated only with *E. amylovora* Ea8KI) had frozen. The effectiveness of each strain was expressed as the relative freezing potential, which was calculated by dividing the number of flowers treated with an antagonist that had frozen by the number of flowers treated only with *E. amylovora* Ea8KI that had frozen.

## RESULTS

Plasmid pJEL1703 containing *iceC* was maintained in *E. amylovora* Ea8R over 60 generations without any detectable loss in the absence of antibiotic selection. This plasmid did not affect the pathogenicity of *E. amylovora* Ea8R when tested on immature pear fruit and compared to the parental strain lacking this plasmid. *E. amylovora* strain Ea8KI expressed high levels of ice nucleation activity. The number of ice nuclei expressed per cell increased nearly logarithmically with decreasing temperatures between  $-2$  and  $-4^{\circ}\text{C}$  (Fig. 1). Nearly half of the cells of strain Ea8KI contained ice nuclei that were active at temperatures warmer than about  $-5^{\circ}\text{C}$ .

The threshold freezing temperature of blossoms inoculated with strain Ea8KI was related to the population size of this strain (Fig. 2). Freezing of flowers was observed at temperatures between about  $-2.4$  and  $-2.7^{\circ}\text{C}$  only when bacterial populations were larger than  $10^3$  cells per flower. However, 100% of the flowers froze, even at temperatures as warm as  $-2.5^{\circ}\text{C}$ , when *E. amylovora* populations were larger than about  $10^{6.5}$  cells per flower. At a given temperature, the fraction of flowers that froze increased with increasing

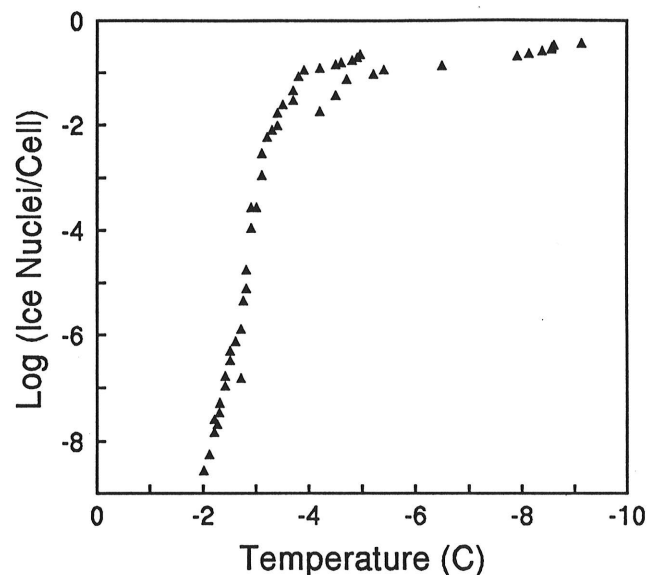


Fig. 1. Ice nucleation activity of cells of *Erwinia amylovora* Ea8KI assayed at progressively colder temperatures.

log-transformed population size (Fig. 2). A linear relationship was observed between logarithmic population size of *E. amylovora* strain Ea8KI on individual flowers and threshold freezing temperature (Fig. 3). Thus, the population size of strain Ea8KI on flowers could be estimated from samples with a given threshold freezing temperature.

A total of 229 bacterial and 28 yeast strains were tested for their ability to inhibit growth of *E. amylovora* on pear flowers by the flower-freezing assay. Over 69% of the bacterial strains caused less than a 50% reduction in the relative freezing potential (i.e., frozen treated flowers/frozen control flowers ratio) (Fig. 4). However, there were 41 bacterial isolates that greatly reduced the relative freezing potential of inoculated flowers (relative freezing potential of 0.2 or less) (Fig. 4). None of the yeasts isolated from pear flowers reduced the relative freezing potential of pear flowers to 0.5 or less when inoculated before strain Ea8KI (data not shown). However, two strains each of *Candida tenuis* (178 and 194) and *Cryptococcus laurentii* (231 and 239) isolated from apple fruit reduced the relative freezing potential of inoculated flowers to less than 0.3.

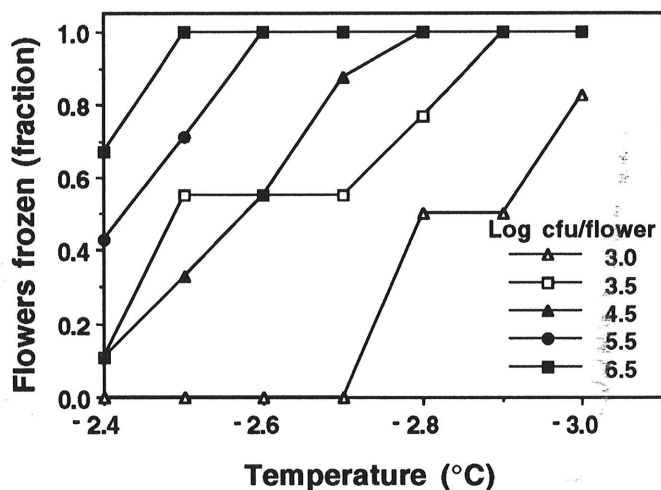


Fig. 2. Proportion of pear flowers inoculated with *Erwinia amylovora* Ea8KI that froze at different assay temperatures. There were approximately 10 flowers for each population size.

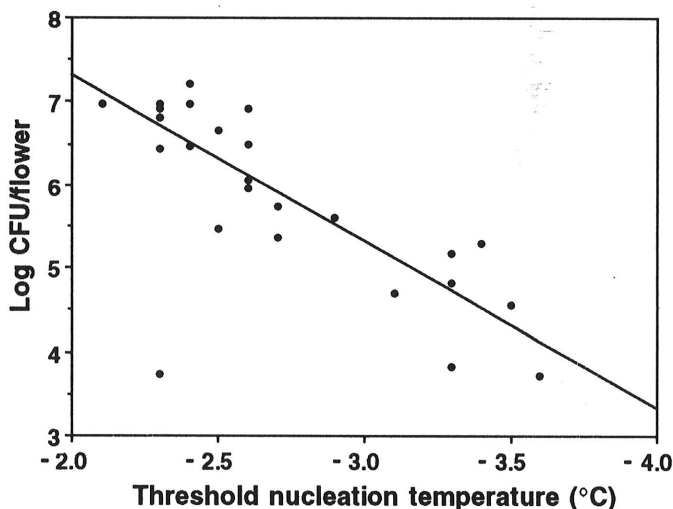


Fig. 3. Relationship between population size of *Erwinia amylovora* Ea8KI on pear flowers and the threshold freezing temperature (i.e., the warmest temperature that caused freezing) of 25 pear flowers suspended in buffer in test tubes. The line represents a linear regression  $y = 11.280 + 1.9856x$  ( $R^2 = 0.61$ ,  $P \leq 0.05$ ).

The population of *E. amylovora* Ea8KI was estimated by dilution plating of pear flowers pretreated with bacterial strains that had resulted in a range of flower-freezing potentials. There was a significant correlation between Ea8KI population sizes on flowers inoculated with a given strain and the relative freezing potential of the flowers (Fig. 5). Strains that lowered the relative freezing potential reduced the population size that *E. amylovora* Ea8KI had attained on the flowers. In contrast, strains that had not reduced the freezing potential of inoculated flowers allowed Ea8KI to increase to relatively large populations ( $>10^4$  cells per flower) (Fig. 5).

*P. fluorescens* A506 reduced the freezing potential of inoculated flowers to a range of 0.0 to 0.6 when tested on a number of samples on the same day (Fig. 6A). In most experiments conducted on different days, A506 also reduced the freezing potential of inoculated flowers greatly (0 to 0.4), but in a number of tests, A506 did not lower the relative freezing potential (Fig. 6B). The relative freezing potential of flowers inoculated with a given bacterial strain was usually, but not always, similar when measured in sequential experiments. All test strains that did not reduce the relative freezing potential of flowers inoculated with strain Ea8KI in an initial experiment remained ineffective when tested a second time (Fig. 7). Likewise, over 50% of the bacterial strains that reduced the freezing potential of inoculated flowers to less than 0.5 in an initial test conferred a similar reduction in flower-freezing potential in a second test (Fig. 7). However, ~30% of the strains that reduced flower-freezing potential to less than 0.5 in an initial test did not cause a similar reduction in a subsequent test (Fig. 7).

## DISCUSSION

Threshold freezing temperatures proved useful in estimating population sizes of an Ice<sup>+</sup> *E. amylovora* strain on pear blossoms (Figs. 3 and 5). Because ice nucleation activity at warm assay temperatures (such as  $-2^\circ\text{C}$ ) is relatively rare in cells of an Ice<sup>+</sup> bacterial strain population (Fig. 1), only plant samples containing a relatively large population of the Ice<sup>+</sup> strain will harbor even one ice nucleus. Thus, plant samples harboring different numbers of Ice<sup>+</sup> bacteria can be distinguished, because only those samples having a relatively large population of Ice<sup>+</sup> strains will freeze at a warm assay temperature (Fig. 2). This phenomenon has allowed the proportion of leaves with a large indigenous Ice<sup>+</sup> *P. syringae* population to be estimated (10). Because the proportion of cells in a population of an Ice<sup>+</sup> bacterium that are active as ice nuclei increases approximately logarithmically with decreasing temperature (14), a

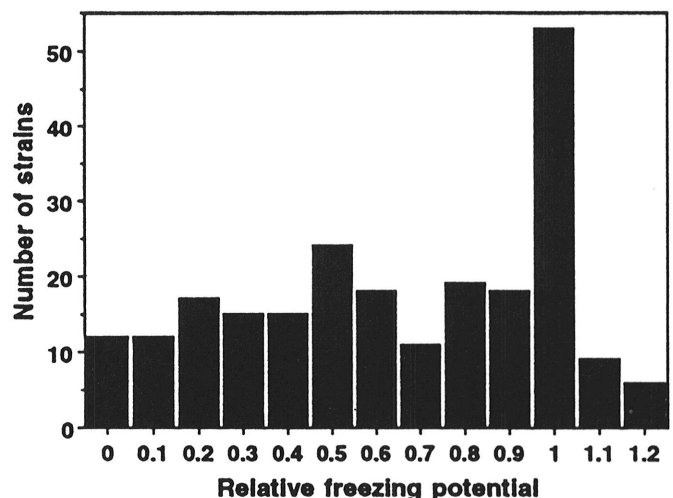


Fig. 4. Effectiveness of bacterial strains tested as antagonists of *Erwinia amylovora*. The effectiveness of antagonism was expressed as the relative freezing potential, i.e., frozen treated flowers/frozen control flowers ratio, when 85 to 100% of the control (*E. amylovora* only) flowers had frozen.

log-linear relationship is seen between threshold freezing temperature and bacterial population size (Fig. 3). The conversion of *E. amylovora* to an Ice<sup>+</sup> phenotype allows rapid estimation of its population size without labor-intensive methods, such as dilution plating. For this reason, a large number of microbial strains can be tested for antagonism toward *E. amylovora* in a quantitative fashion and in a relatively short time. In all cases, reduction of Ice<sup>+</sup> *E. amylovora* population sizes on flowers treated with an antagonist was related to the relative freezing potential of the inoculated flowers (Fig. 5).

Initial screening of antagonists of *E. amylovora* has traditionally been done on culture media and in immature pear fruit (4,12, 26). However, antagonists successful in the biocontrol of fire blight preemptively or competitively inhibit *E. amylovora* during its epiphytic phase on the pistil (9,27,28). Because flowers are the site of interaction between *E. amylovora* and bacterial antagonists and the chemical and physical attributes of culture media and the interior of the fruit are likely to be different from that of the surface of a flower, assays in blossoms appear to be more likely to reveal effective biocontrol agents than tests done in the other habitats. For example, *P. fluorescens* strain A506, an effective biocontrol agent of fire blight (28), did not antagonize *E. amylovora* on immature pear fruit and, thus, would not have been selected as an antagonist with this assay (18). Because strain A506 does not cause the formation of an inhibition zone on common culture media, it apparently does not produce antibiotics and instead inhibits the growth of *E. amylovora* on flowers by other means, possibly by competing for nutrients or causing changes in flower physiology. Antagonist strains that do not produce antibiotics would not be identified as an antagonist of *E. amylovora* in the immature pear fruit assay. Screening of bacterial strains on pear fruit tissue is likely to identify only antibiotic-producing strains, because this means of antagonism is related to the inhibition effectiveness of *E. herbicola* in this system (29). However, because antibiotic production is not the sole, nor probably the primary, means by which *E. amylovora* is antagonized on flowers, a system that allows the selection of all possible antagonists would be more useful. A further advantage of screening for antagonists on pear flowers is that strains that inhibit the growth of *E. amylovora* in this habitat are likely to be good colonizers of the pistil, the site of increase of *E. amylovora* populations. This further increases their likelihood of being effective in controlling fire blight in the field.

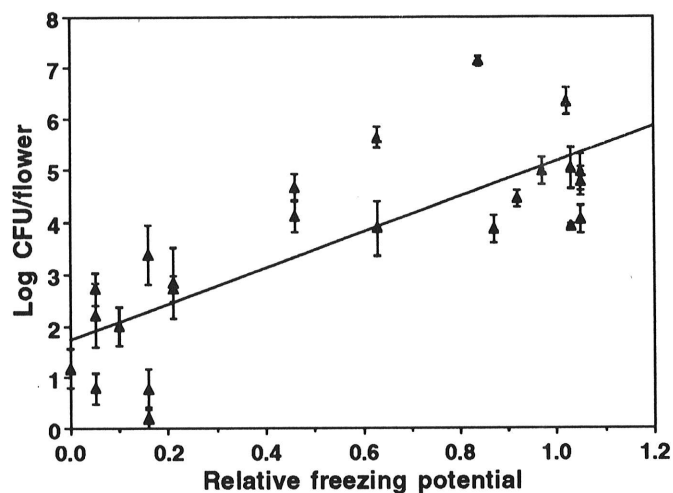


Fig. 5. Relationship between the extent of antagonism to *Erwinia amylovora* by various antagonists as evaluated by a flower-freezing assay and the population size of *E. amylovora* on pear flowers. For each antagonist, the relative freezing potential (frozen treated flowers/frozen control flowers ratio) of 20 flowers was measured; the population size of *E. amylovora* Ea8KI was estimated from 10 of the same flowers. The line represents the regression  $y = 1.7248 + 3.4191x$  ( $R^2 = 0.615$ ,  $P \leq 0.01$ ).

Several factors may influence estimates of apparent antagonism of Ice<sup>+</sup> *E. amylovora* strains measured by the flower-freezing assay. Although most bacterial strains tested yielded a similar flower-freezing potential in repeated tests, several strains that were retested did not perform as well in a second assay (Fig. 7). Although strain A506 usually caused a substantial reduction in the freezing potential of inoculated flowers, there were a few occasions when it did not cause a substantial change in the freezing potential of treated flowers (Fig. 6). Any screening procedure is likely to generate a number of false-positives, i.e., strains that apparently exhibit antagonism when they are not, in fact, antagonistic; such strains can be identified easily by their lack of efficacy in a second testing. A good screening procedure should allow the retention of a large proportion of all potential antagonists during the first test; although false-negative indications (strains that are antagonistic but are not identified as such) should be minimized (3). A screening procedure that is too selective is likely to generate too many false-negative results, and useful strains may be unnecessarily discarded. Although false-positive strains can be readily identified by subsequent retesting and discarded, false-negative strains would be prematurely discarded. A useful screening procedure is one that is sufficiently easy to perform so false-positive strains can be easily retested, but not so stringent that false-negative strains are prematurely discarded. It is possible that false-negative strains could have been identified if more ineffective antagonists had been retested.

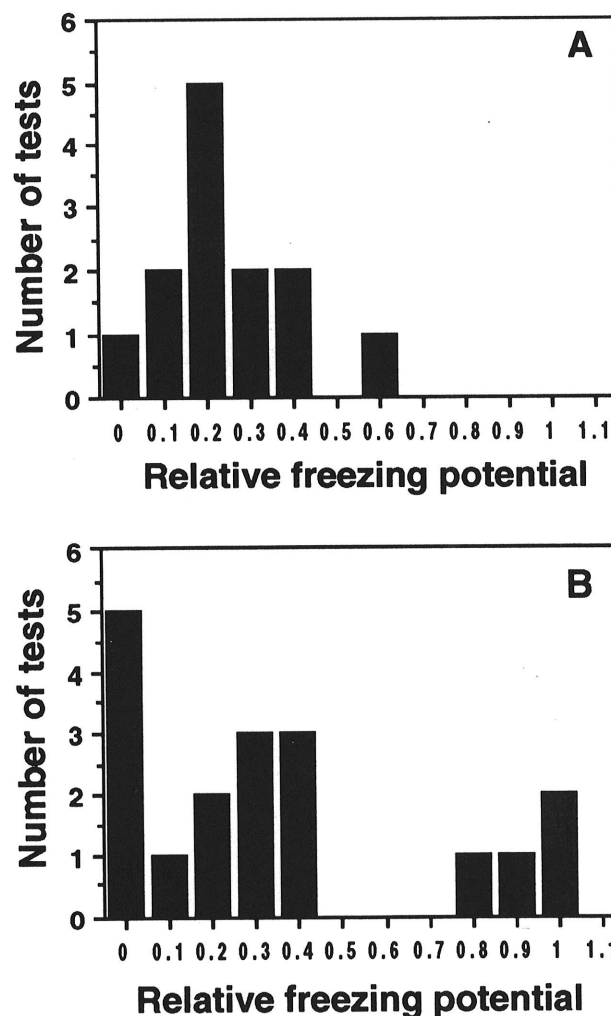


Fig. 6. Performance of *Pseudomonas fluorescens* A506 as an antagonist of *Erwinia amylovora* in different flower samples evaluated by the flower-freezing assay: A, flowers treated on the same day; B, flowers treated on different days. The effectiveness of antagonism was expressed as the relative freezing potential, i.e., frozen treated flowers/frozen control flowers ratio.

The antagonism of *E. amylovora* estimated by the flower-freezing assay was always closely related to results obtained by direct measurement of population sizes of *E. amylovora* (Fig. 5). Thus, the flower-freezing assay gave a reliable estimate of the relative population size of the Ice<sup>+</sup> *E. amylovora* in these studies. The false-positive strains identified in our tests are most likely attributed to variation in the flowers used in some assays. Although newly opened flowers usually had low (nondetectable) microbial populations, in some experiments they were heavily colonized by bacteria and fungi (data not shown). The flowers on small-diameter branches, especially branches that had been stored for several weeks, were often smaller and more prone to fungal colonization, which could have interfered with the antagonists. This is likely the reason why A506 performed poorly on a few occasions (Fig. 6B). It also is possible that some of the flowers used in this study were poor hosts for the multiplication of *E. amylovora*, causing an apparent inhibition of the pathogen that was attributed to the antagonist and resulting in its identification as a false-positive. Also, in certain experiments in which *E. amylovora* Ea8KI did not grow in control (untreated) flowers, antagonism from other strains, by definition, could not be observed. For these reasons, in any given test, normal freezing of flowers should be observed in the control (*E. amylovora* only) and a reduction of relative freezing potential in flowers treated with a known antagonist such as A506 should occur to be able to consider the results reliable. In our studies, experiments in which strain A506 did not exhibit antagonism were infrequent, and more careful selection of plant material used in assays should minimize the occurrence of false-negative and -positive results. Care in the selection and storage of branches used for forcing should be taken so only branches harboring flowers of normal size and a low level of microbial contaminants are used for such tests.

A relatively high proportion of bacterial strains from pear flowers was effective in inhibiting *E. amylovora* in pear flowers (Fig. 4). One possible reason is that such bacteria are likely to be good colonizers of this habitat, having been isolated from pear blossoms. In contrast, yeasts isolated from pear flowers were not effective antagonists of *E. amylovora*. However, because the number of strains tested was small (19 strains), it is possible that more extensive screening would identify some antagonistic yeast strains. Some of the yeast strains from apple fruits (*Cryptococcus laurentii* and *Candida tenuis*) were as antagonistic as bacterial strains against *E.*

*amylovora* on pear blossoms. Thus, it is possible that yeasts can inhibit *E. amylovora* and other bacterial pathogens on plant surfaces. Although yeasts have proven effective in biological control of some fungal diseases (6,25), they previously have not been antagonistic to bacterial plant pathogens. The yeast strains we tested were known to be antagonistic toward *Botrytis cinerea* and *Penicillium expansum* in wounds on apple fruit and to prevent rot caused by these pathogens (C. L. Wilson, *personal communication*). These strains, thus, may have a superior competitive ability, such as effectively utilizing nutritive resources on both fruit wounds and pear flowers. Five strains (one yeast and four bacteria) that reduced the population size of *E. amylovora* in flowers also were tested for control of fire blight in potted pear trees in the greenhouse and were compared to strain A506. All strains significantly reduced the percentage of flowers that became infected; three were as effective as strain A506 in controlling fire blight (J. Mercier and S. E. Lindow, *unpublished data*).

All gram-negative bacterial pathogens into which the *iceC* or *inaZ* gene have been inserted can be converted to an Ice<sup>+</sup> phenotype (8). *E. amylovora* strain Ea8R expresses ice nucleation activity (Fig. 1) even more highly than the *P. syringae* source strain for *iceC* (22). The high stability of plasmid pVSP61 containing *iceC* in all plant-pathogenic bacteria studied enables the convenient use of *iceC* in trans. It should be possible to rapidly estimate the population size of any plant-pathogenic bacterium by measuring threshold freezing temperature, as was first described for naturally occurring Ice<sup>+</sup> strains of *P. syringae* pv. *syringae* (10,14). The simplicity and speed of the flower-freezing assay demonstrated here also allows estimates of Ice<sup>+</sup> bacterial populations to be made on a large number of flowers. Because the population size of epiphytic bacteria varies greatly from leaf to leaf (11,13) and flower to flower (28), estimates of bacterial population size are best made with large numbers of individual plant parts (11,13). This approach should allow interactions between antagonistic bacteria and pathogens to be quantified on the surface of the plant part on which epiphytic populations increase prior to infection. Antagonists most capable of inhibiting the development of epiphytic populations of the pathogens should prove effective in biological control.

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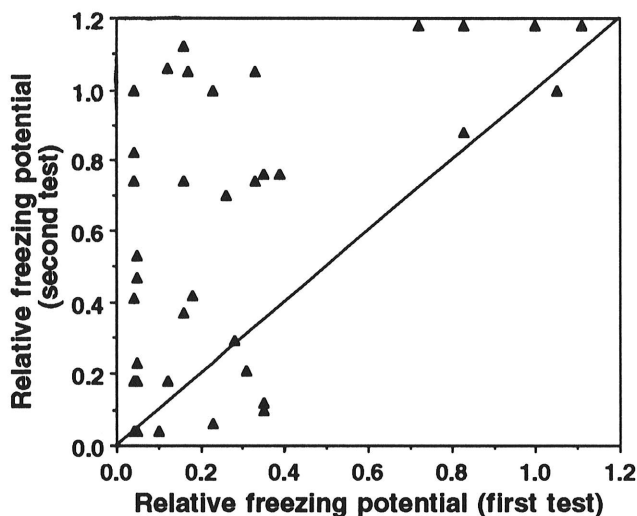


Fig. 7. Antagonism to *Erwinia amylovora* by various antagonists when evaluated by a flower-freezing assay in two successive tests. The relative freezing potential (i.e., frozen treated flowers/frozen control flowers ratio) of flowers treated with a given antagonist in an initial test (abscissa) and in a subsequent test (ordinate) is shown. The line indicates the expectation for equal antagonism in each test.

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