Control of Fire Blight by *Pseudomonas fluorescens* A506 and *Pantoea vagans* C9-1 Applied as Single Strains and Mixed Inocula

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ABSTRACT

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The biological control agents *Pseudomonas fluorescens* A506 and *Pantoea vagans* C9-1 were evaluated individually and in combination for the suppression of fire blight of pear or apple in 10 field trials inoculated with the pathogen *Erwinia amylovora*. The formulation of pathogen inoculum applied to blossoms influenced establishment of the pathogen and the efficacy of biological control. *Pantoea vagans* C9-1 suppressed fire blight in all five trials in which the pathogen was applied as lyophilized cells but in none of the trials in which the pathogen was applied as freshly harvested cells. In contrast, *Pseudomonas fluorescens* A506 reduced disease significantly in only one trial. A mixture of the two

strains also suppressed fire blight, but the magnitude of disease suppression over all field trials (averaging 32%) was less than that attained by C9-1 alone (42%). The two biological control agents did not antagonize one another on blossom surfaces, and application of the mixture of A506 and C9-1 to blossoms resulted in a greater proportion of flowers having detectable populations of at least one bacterial antagonist than the application of individual strains. Therefore, the mixture of A506 and C9-1 provided less disease control than expected based upon the epiphytic population sizes of the antagonists on blossom surfaces. We speculate that the biocontrol mixture was less effective than anticipated due to incompatibility between the mechanisms by which A506 and C9-1 suppress disease.

Additional keywords: Biolog Phenotype Microarray, *Erwinia herbicola*, *Malus*, *Pantoea agglomerans*, pome fruit, *Pyrus*, stigma.

Severe epidemics of fire blight occur when periods of warm (>15°C), wet weather are coincident with open flowers on pear or apple trees (35). Under these conditions, the pathogen *Erwinia amylovora* colonizes flower stigmas and establishes large population sizes on stigmatic surfaces (35). Free moisture (heavy dew or rain) facilitates migration of the pathogen from the stigmas to the nectary (35). The pathogen gains entry into the plant tissues through the nectarthodes (35). After infection, the pathogen can spread internally throughout the tree (35). There is no cure for fire blight after infection and diseased tissues must be removed by pruning.

Pear and apple growers apply antibiotics during bloom to suppress epiphytic growth and subsequent infection by the pathogen. Streptomycin has been the most effective antibiotic used for control of fire blight (15) but streptomycin-resistant strains of *E. amylovora* are now prevalent in pear-production regions of the United States (15,20). Streptomycin resistance in indigenous populations of *E. amylovora* has increased the risk of fire blight epidemics, thereby increasing the need for alternative control measures.

The pathogen *E. amylovora* can be recovered from pear or apple flowers when trees are in full to late bloom (35) but generally not during earlier phases of bloom. This delay in the emergence of populations of the pathogen in orchards provides a period of time during early bloom for introduction and establish-

ment of bacterial antagonists on flowers (12–14,17,25,35,36). Preemptive colonization of stigmas by antagonists can restrict the epiphytic growth phase of *E. amylovora* and reduce the incidence of blossom blight (12). One commercially available antagonist for management of fire blight is *Pseudomonas fluorescens* strain A506 (A506), the active ingredient in BlightBan A506 (NuFarm Americas, Burr Ridge, IL) (32). A506 was isolated from pear in California and was demonstrated to decrease the severity of frost injury and russetting of pear fruit, and to reduce the incidence of fire blight in orchards in California by 50 to 80% (17–19). A506 is resistant to streptomycin and tolerant of oxytetracycline and can be combined with antibiotic sprays in orchards to increase the efficacy of control of fire blight (17–19). In growth-chamber studies, A506 suppressed growth of *E. amylovora* when applied prior to the pathogen; when applied in combination with *E. amylovora*, the growth of the pathogen was unaffected (41). In this study, Wilson and Lindow introduced the concept that the sequence of introduction of two bacterial strains has an important influence on their interactions on floral surfaces. They also proposed that A506 suppresses fire blight by preemptive exclusion, limiting access of the pathogen to sites and nutrients required for its growth on stigmatic surfaces. More recently, Temple et al. (34) found that A506 produces an antibiotic inhibitory to *E. amylovora* in iron-amended media but the bioavailable iron concentrations on pome fruit flowers were too low to induce antibiosis. Therefore, preemptive exclusion of the pathogen is still considered to be the primary mechanism for fire blight suppression by *Pseudomonas fluorescens* A506.

Strains of *Pantoea* spp. (formerly *E. herbicola*) represent another genus of effective antagonists for control of fire blight (7,16,21,23,25,27,30,33,36–38,40,44–46). Two strains, *Pantoea vagans* C9-1 (formerly *Pantoea agglomerans*) (28) and *Pantoea*

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*The e-Xtra logo stands for “electronic extra” and indicates that the online version contains a supplemental table.

agglomerans E325, are registered with the United States Environmental Protection Agency as microbial pesticides for fire blight management. Each of these strains produces antibiotics in culture that inhibit the growth of *E. amylovora* (7,27). Strain C9-1 produces at least two antibiotics, herbicolin O and herbicolin I, that contribute to the suppression of fire blight by C9-1 based on immature pear fruit assays (3,7). The structure and role of herbicolin I in suppression of fire blight on flowers have not been determined. Herbicolin O is synonymous with pantocin A and mceEh252, small peptide antibiotics whose inhibition is reversible with L-histidine (9,10,38,39). In orchard trials, *Pantoea agglomerans* strain Eh252, which produces mceEh252, reduced the incidence of fire blight by 55% and an antibiotic-deficient mutant of Eh252 reduced the incidence by 30%, indicating that antibiosis by *Pantoea* spp. contributes to control of fire blight (30). The small though significant reduction in the incidence of fire blight by the antibiotic-deficient mutant of Eh252 compared with water treatment was attributed to preemptive exclusion of the pathogen from sites and nutrients (30).

A 1:1 mixture of A506 and C9-1R, a spontaneous rifampicin-resistant mutant of C9-1, was tested by Johnson et al. (13) for suppression of fire blight in pear orchard trials inoculated with honey-bee-vectored lyophilized cells of *E. amylovora* strain Ea153N (13). In that study, the two antagonists co-colonized flowers and reduced the incidence of fire blight by $\approx 50\%$ (13). A similar level of disease control was obtained by spraying A506 alone (50 to 80%) in California orchards with natural populations of the pathogen (17–19) or *Pantoea agglomerans* Eh252 alone (55%) in orchards challenged with an aqueous suspension of lyophilized cells of Ea153N (30). It is unclear whether control efficacy by the mixture of A506 and C9-1R is different than the level of control exerted by individual strains because the orchard trials were conducted in different areas and with different pathogen-inoculation methods.

A primary objective of this study was to directly compare the antagonists A506 and C9-1R applied alone and in combination for the biological control of fire blight. Our hypotheses were that (i) the complementary mechanisms of biocontrol by C9-1R and A506 would enhance efficacy and decrease variation in control and (ii) establishment of C9-1R and A506 jointly on flowers would collectively result in utilization of a greater range of nutrients required by the pathogen. To examine the compatibility of the antagonists on flowers, we did replacement-series experiments, which have been used successfully in the past to evaluate bacterial interactions on leaves and flowers (11,42,43). In a replacement-series experiment, strains that compete equally for the same limiting nutrients and niches on plant tissues establish the same total population size when applied in combination or as single-strain inoculants representing a low level of coexistence (43). A negative deviation from linearity indicates suppression of growth by at least one of the co-inoculants via competition for niches or antibiosis. A positive deviation from linearity (greater total populations of bacteria when applied as mixtures compared with single strains) indicates a high level of coexistence via utilization of different nutrient resources or by niche differentiation (43). To assess co-utilization of nutrients by the antagonists and the pathogen, we determined their catabolic profiles in culture to estimate the niche overlap index (NOI). The NOI is a measure of co-utilization of nutrients, generally carbon sources, by bacterial strains in vitro (8,11), and has been used to predict the level of coexistence of bacterial isolates on leaves. For example, Ji and Wilson (8) demonstrated a significant positive correlation between similarity in carbon source utilization (high NOI) (i.e., NOI approaching 1 or all tested carbon sources were utilized by each isolate) and efficacy of various strains of pseudomonads in suppression of bacterial speck caused by *Pseudomonas syringae* pv. *tomato*. Thus, combining bacterial antagonists to increase the NOI of the antagonist population with

the pathogen may more effectively exhaust nutrients required by the pathogen, leading to better suppression of the pathogen via preemptive exclusion or competition.

Here, we report that A506 and C9-1R co-colonize flowers and establish larger total populations than single-strain inoculants in 10 replicated field trials done in pear and apple orchards over a 7-year period. Despite the larger population sizes on flowers achieved by the combined antagonists, the efficacy of control was not significantly increased compared with single-strain inoculants. Finally, our results demonstrate that *Pantoea vagans* C9-1 is an extremely effective antagonist that provided consistent suppression of fire blight.

MATERIALS AND METHODS

Bacterial strains. *Pseudomonas fluorescens* strain A506 was provided by S. E. Lindow, University of California–Berkeley. A506 is resistant to rifampicin and streptomycin (both at 100 $\mu\text{g/ml}$) (17–19). *Pantoea vagans* strain C9-1 (formerly *Pantoea agglomerans* C9-1 and *E. herbicola* C9-1) (7,28) was kindly provided by C. A. Ishimaru, University of Minnesota. For field trials, strain C9-1R, a spontaneous rifampicin-resistant mutant of C9-1, was used. Like the parental strain, C9-1R produces two antibiotics called herbicolin I and O (7) and has a similar growth rate in culture (13).

E. amylovora strain Ea153 was isolated from a fire-blight canker on ‘Gala’ apple in Milton-Freewater, OR (13). For field trials, Ea153N, a spontaneous nalidixic acid-resistant (100 $\mu\text{g/ml}$) mutant of Ea153, was used. Ea153N is similar to the parental strain in growth rate and colony morphology and is sensitive to streptomycin and oxytetracycline. Ea153N has been used in numerous field trials (11,13,22–25,27,29–31). Strains were stored in nutrient broth with 15% glycerol at -80°C .

Nutrient utilization by strains. The capacity of wild-type strains C9-1, A506, and Ea153 to utilize nutrients was assessed with phenotype microarray (PM) assays (2) at 22°C by the Biolog PMServices (Hayward, CA), Biolog GN plates, and growth rate in broth. The Biolog PMServices provided average peak heights for each well in the test plates. Values from internal negative control wells were subtracted from each treatment well, resulting in an average peak height for each compound. Duplicate plates were run in each PM assay and the assays were repeated. Duplicate Biolog GN plate assays were conducted at 22°C according to the recommendations by the manufacturer and optical density (OD) at 590 nm was measured at 24 and 48 h. The OD of the internal negative control well was subtracted from each well to generate relative OD values for each compound tested. Growth rate of bacterial strains in broth culture was measured at 20°C in 5 ml of Davis broth (Difco Laboratories, Detroit) amended with 0.1 mM FeCl_3 , 0.1 mM nicotinic acid, 0.1 mM thiamine, and a 1% (wt/vol) final concentration of the following sugars or amino acids (Sigma-Aldrich, St. Louis) representing some of the major chemical constituents of apple and pear stigma exudates: α -D-glucose, D-fructose, D-sorbitol, sucrose, L-asparagine, L-glutamic acid, L-glutamine, or L-proline (26). Washed cells from overnight cultures of A506, C9-1, and Ea153 in modified Davis medium containing α -D-glucose were placed in the test media, incubated with agitation (200 rpm), and the OD at 600 nm was measured every 30 to 60 min during the exponential growth phase.

Co-utilization of carbon sources between strains was calculated with the NOI described by Wilson and Lindow (41). The NOI also was calculated for nitrogen sources. The NOI was calculated as the number of carbon or nitrogen sources catabolized in common by two strains divided by the number of nutrients catabolized by only one strain.

Experimental design of orchard trials. Field experiments were conducted in 0.5-ha orchards of mature pear (*Pyrus communis* L. cvs. Bartlett and Bosc) and apple (*Malus $\times$ domesti-*

ca Borkh., cv. Golden Delicious) trees at the Oregon State University, Botany and Plant Pathology Field Laboratory near Corvallis, OR; and at the Southern Oregon Research and Extension Center near Medford, OR on Bartlett pear. Treatments were assigned to individual trees in field trials in a randomized complete block design. Each treatment was applied to single trees in 4 to 10 replicate blocks (Table 1).

Treatments. A506 and C9-1R were cultured separately for 4 days at 25°C on nutrient agar (Difco Laboratories) containing 1% glycerol. Bacteria were scraped from the agar surface and suspended in sterile 20 mM phosphate buffer at a cell concentration of $\approx 1 \times 10^{10}$ CFU/ml based on spectrophotometric readings (Bausch and Lomb Spectronic 20), and the concentrated stock solution was transported to the field in an ice chest. In the field plots, bacterial suspensions were diluted with local groundwater to 5×10^7 or 1×10^8 CFU/ml prior to spraying. The bacterial cell concentration of the applied suspension was confirmed after treatment by dilution plating. Treatment dates and environmental conditions are provided in Table 1.

Trees were treated at 20% bloom and again at 70% bloom at sunrise with an aqueous suspension of A506 (1×10^8 CFU/ml), C9-1R (1×10^8 CFU/ml), or a 1:1 mixture of each strain at 5×10^7 CFU/ml (total cell concentration of 1×10^8 CFU/ml). Individual trees were sprayed with 2 to 4 liters of a bacterial suspension until near run-off using 12-liter backpack sprayers fitted with adjustable brass nozzles attached to a handheld spray wand (Table 1). Additional trees in each plot were treated with water or streptomycin sulfate (Agriprep 17, 100 µg/ml a.i.; Syngenta, Greensboro, NC) as controls for disease suppression. Oxytetracycline (Mycoshield, 200 µg/ml a.i.; Syngenta) was included as a disease control treatment in three experiments. Antibiotic and water treatments were repeated within 3 days after inoculation with Ea153N.

At full bloom, trees were inoculated after sundown under calm conditions with Ea153N by one of three methods. In 1992 and 1995, flowers were inoculated with a backpack sprayer by misting trees with a suspension of cells (termed fresh cells) from 4-day-old cultures scraped from the surface of nutrient agar amended with 1% glycerol. The suspension of fresh cells of Ea153N was adjusted to 1×10^6 CFU/ml with a spectrophotometer. In other trials or blocks within trials, lyophilized cells of Ea153N were used to inoculate flowers. In 1995 and 1998, a lyophilized cell preparation ($\approx 1 \times 10^{11}$ CFU/g) of Ea153N (13) was suspended in water to a concentration of 1×10^6 CFU/ml and misted onto flowers with a backpack sprayer. The final inoculation method,

used in 1992, 1993, and 1995, consisted of dusting a lyophilized formulation of Ea153N onto flowers. The formulation consisted of the lyophilized cell preparation (1×10^{11} CFU/g) of the pathogen diluted to 1×10^9 CFU/g with powdered skim milk ground to a fine powder. The formulated pathogen was dusted onto trees in full bloom at night with a handheld Chapin duster (Chapin Manufacturing, Batavia, NY); ≈ 10 to 20 g of material was applied to each tree.

Recovery of bacteria from flowers. Open flowers with dehiscid anthers were harvested after the treatment at 70% bloom and periodically thereafter until symptoms of fire blight were visible. In total, 36 to 40 flowers per treatment were collected among the replicate trees in each trial. Flowers were placed in individual wells of 12-well sterile plastic plates (Corning Inc., Corning, NY) and transported to the laboratory. The pistil and hypanthium of each pear flower or the pistil of each apple flower was removed and placed in 1 ml of sterile 10 mM potassium phosphate buffer, pH 7.1, and sonicated for 3 min. After sonication and vortexing, 10 µl of the sample buffer and two 100-fold dilutions were spread onto *Pseudomonas* agar F (Difco Laboratories) with rifampicin at 100 µg/ml and cycloheximide at 50 µg/ml for enumeration of C9-1R and A506, and CCT medium (6) containing nalidixic acid at 100 µg/ml for recovery of the pathogen. C9-1R and A506 were distinguished by colony morphology. The detection limit was 1×10^2 CFU/flower.

Disease assessment. Flower clusters with symptoms of fire blight (necrosis, wilting, or bacterial ooze) were counted and removed immediately from trees over a 2-week period starting at the first visible symptoms. The sum of fire blight infections (strikes) per tree was calculated.

Data analyses. Mean population size and standard error of bacterial strains on individual flowers were calculated by averaging the logarithm (base 10) of population values obtained on each sample date. The incidence of detection of bacterial strains was recorded for each sampled tree at each time and the data were arcsine square root transformed before mean separation by Fisher's protected least significant difference (LSD) test at $P = 0.05$ using the analysis of variance (ANOVA) procedure of SAS (Statistical Analysis Systems, Cary, NC).

To evaluate growth of A506 and C9-1R on flowers, the relative area under the population curve (RAUPC) was calculated for each tree in each trial. The area under the population curve (AUPC) was calculated for each trial with the formula (30)

$$AUPC = \sum_{i=1}^n [(y_i + y_{i-1})/2] \cdot (t_i - t_{i-1})$$

TABLE 1. Cultivar, location, replications, treatment dates, and environmental conditions for orchard trials

Year	Cultivar	Location	Reps ^a	Applications ^t	Inoculation ^u	Environmental conditions ^v			
						Mean (°C) ^w	Max (°C) ^x	Incidence ^y	Rain (mm) ^z
1992	Bartlett	Corvallis	8	20 and 23 March	26 March	11.1	17.5	0.50	18
	Bartlett	Medford	10	21 and 24 March	27 March	12.0	19.4	0.25	8
1993	Bartlett	Corvallis	5	4 and 7 April	9 April	10.1	14.7	1.00	100
	Bartlett	Medford	5	2 and 7 April	10 April	9.2	14.1	0.64	53
1994	Bosc	Corvallis	8	8 and 12 April	...	12.0	18.1	0.50	23
	Bartlett	Medford	9	31 March and 4 April	...	11.4	18.4	0.28	47
1995	Golden Delicious	Corvallis	4	10 and 14 April	16 April	9.2	14.4	0.60	46
1998	Bartlett	Corvallis	5	4 and 8 April	10 April	9.1	14.5	0.57	41

^a Replications = number of blocks in orchard experiment. Individual trees were assigned to treatments in a complete randomized block design.

^t Bacterial antagonists applications. All treatments were applied to trees in 20 and 70% bloom. Water and antibiotic treatments were repeated within 3 days after pathogen inoculation.

^u Date of pathogen inoculation. Fire blight pathogen, *Erwinia amylovora* strain Ea153N, was inoculated during full bloom.

^v Summary of environmental conditions during bloom recorded by Agrimet (Northwest Cooperative Agricultural Weather Network, Bonneville Power Administration, and the U.S. Bureau of Reclamation, Boise, ID) weather stations located near the experimental field laboratories near Corvallis and Medford. The weather data can be accessed from the Agrimet website.

^w Mean daily temperature.

^x Mean daily maximum temperature.

^y Incidence of days with rain.

^z Total rainfall.

where y is the mean population size of a bacterial strain on the i th sample date and t is the corresponding sample time. The AUPC was converted to the RAUPC by dividing the AUPC by the elapsed number of days during the sampling period (30). The LSD at $P = 0.05$ procedure was used to separate mean RAUPC values obtained for each bacterial treatment of each trial.

Intergeneric competition of bacterial antagonists was evaluated graphically with replacement series analysis and by ANOVA with the methods described by Wilson and Lindow (42,43) and Johnson et al. (11). For ANOVA, the enumerated populations of each antagonist near petal fall (≈ 8 to 14 days after pathogen inoculation) were adjusted to the proportion of the bacterium in the initial inoculum by the equation $\log_{10}(\text{population size}) - \log_{10}(\text{inoculum proportion})$. In most cases, the mixtures were in a 1:1 mixture; therefore, the proportion of a strain in the inoculum was 0.5. The mean adjusted populations were compared with mean populations achieved by single-strain inoculants by Fisher's protected LSD test at $P = 0.05$.

The number of diseased flower clusters per treated tree was converted to a relative disease incidence by dividing the total number of fire blight strikes on treated trees by the number of strikes on the water-treated tree within each block. The relative disease incidence data were arcsine square root transformed before analysis. For analysis of individual experiments, Fisher's protected LSD test at $P = 0.05$ was used to separate treatment means of the transformed, relative disease incidence values. Rank sum analysis was used to compare relative efficacy of water, biological control treatments, and streptomycin for disease control among trials; oxytetracycline was excluded from the analysis because it was not included in every trial. The relative incidence of disease for five treatments within a trial was sorted from high to low and the disease incidence was replaced with a value for its rank from 1 for the highest disease incidence through 5, the lowest incidence in a trial. Fisher's protected LSD test at $P = 0.05$ was used to separate rank sum means.

RESULTS

Nutrient utilization by strains. The metabolic profiles of Ea153, C9-1, and A506 were evaluated with Biolog PM assay using plates PM1, PM2A, PM3B, and PM4A. Of 190 compounds tested as sole carbon sources, 112 were used by at least one bacterium (Supplementary Table 1). Ea153 catabolized 100 compounds as sole carbon sources, C9-1 utilized 96 compounds, and A506 utilized 80. The NOI of C9-1 compared with Ea153 was 0.96, or 96% of the sole carbon sources utilized by Ea153 also were metabolized by C9-1. The NOI of carbon sources utilized by Ea153 and A506 was 0.71. Similarly, 68 of the carbon sources metabolized by C9-1 were co-utilized by A506 (NOI = 0.71). Quinic acid and gentiobiose were utilized as sole carbon sources only by Ea153, not C9-1 or A506, in the PM assay. Of the 112 carbon sources catabolized by at least one bacterium, 110 were utilized by C9-1 or A506. The NOI of the combined antagonists compared with Ea153 was 0.98.

Several of the carbon sources evaluated with the PM also were tested with Biolog GN plates. Generally, there was a positive correlation between the assays; high OD readings (>1.00) obtained with Biolog GN plates corresponded with compounds metabolized in the PM assay. Noted exceptions were D-arabitol and D-galactonic acid- γ -lactone, which were utilized by Ea153 and C9-1 in the Biolog GN assay (OD > 0.5) but not in the PM assay. Glycerol was not utilized by A506 in the PM assay but was in the Biolog GN assay. Ea153, C9-1, and A506 had similar OD readings (>0.5) in Biolog GN plates for quinic acid but, in PM assays, only Ea153 utilized quinic acid as a sole carbon source.

Of 95 compounds tested as sole nitrogen sources in PM assays, 70 were used by at least one bacterium. Compounds utilized as

sole nitrogen sources in the PM assay also were catabolized in Biolog GN plate assays. Ea153 used 48 compounds as sole nitrogen sources, C9-1 used 56 compounds, and A506 used 65 of the compounds. The NOI of nitrogen sources was 1.00 comparing C9-1 to Ea153 and 0.98 comparing A506 to Ea153. Of the 56 compounds catabolized by C9-1 as sole nitrogen sources, 51 also were catabolized by A506 (NOI = 0.91). Of the 70 nitrogen sources utilized by at least one bacterium, all were utilized by C9-1 or A506.

Overall, the metabolic profiles of C9-1 and Ea153 were similar and had a high level of overlap, with NOI values of 0.94 to 1.00 for sole carbon and nitrogen sources. Both C9-1 and Ea153 metabolized 53 to 59% of the total number of compounds in the PM test as sole carbon and nitrogen sources. The metabolic profile of A506 differed from the enteric bacteria C9-1 and Ea153. A506 utilized a greater range of nitrogen sources (68%) compared with carbon sources (42%) in the PM assay. The predicted NOI of combined antagonists C9-1 and A506 to Ea153 is 0.99 for sole carbon sources and 1.00 for nitrogen sources.

Carbohydrates are major constituents of secretions in stigmas and the nectary of pear and apple (26). Ea153, C9-1, and A506 metabolized each of the major sugars detected on pistil tissues in the Biolog assays (Table 2). Of these three strains, A506 had lower average peak heights for the carbohydrates than C9-1 and Ea153. L-Asparagine, L-glutamic acid, L-glutamine, L-proline, and L-serine are the major amino acids of the 21 reported in stigmatic secretions of pear and apple flowers (26), and each were metabolized by Ea153, C9-1, and A506 as sole carbon and nitrogen sources in the Biolog assays (Table 2). None of the strains utilized L-lysine, a minor amino acid in stigmatic secretions, as a carbon or nitrogen source. Ea153 metabolized 16 of the amino acids detected in stigma secretions. C9-1 utilized 17 of the amino acids but did not metabolize L-leucine which could be metabolized by Ea153 as a sole carbon source. A506 metabolized all of the amino acids in stigma secretions that are utilized by C9-1 or Ea153 (Table 2).

Generation times in broth media supplemented with compounds found on stigmas. Generation times of A506 and Ea153 were not significantly different in Davis medium amended with L-asparagine, L-glutamic acid, L-glutamine, or L-proline (Table 3). Generation times of C9-1 also were similar to A506 and Ea153 for the amino acids tested, except L-proline, which supported a slower growth rate for C9-1 compared with the other two bacteria.

C9-1 had a significantly faster growth rate in broth amended with glucose, fructose, sorbitol, or sucrose compared with Ea153 (Table 3). The faster growth rate of C9-1 compared with Ea153 was not anticipated in light of the PM analyses, where peak heights of Ea153 and C9-1 were similar and indicated a similar rate of catabolism of carbohydrates (Table 2). A506 had a faster growth rate than Ea153 in glucose and sorbitol compared with Ea153 but the growth rate of A506 in broth was similar to Ea153 in fructose and sucrose (Table 3).

Populations of A506 and C9-1R as single-strain inoculants on flowers. A506 established mean populations in excess of 10^5 CFU/flower by petal fall in six of seven orchard trials when applied as a single-strain inoculant (Table 4). A506 was detected on 31 to 66% of the flowers sampled on treated trees. Similarly, C9-1R established mean populations in excess of 10^5 CFU/flower by petal fall in five of seven trials when applied as a single-strain inoculant (Table 4). C9-1R was detected on 46 to 90% of the flowers sampled on treated trees among trials. Subsequent inoculation of trees with *E. amylovora* applied by different methods did not significantly affect the population sizes or incidence of establishment of A506 or C9-1R on flowers. Mean populations of A506 or C9-1R on flowers recovered from trees inoculated with fresh cells of *E. amylovora* did not differ significantly from mean populations on trees inoculated by dusting the pathogen (data not shown).

Populations of A506 and C9-1R applied as mixed inoculants on flowers. When C9-1R and A506 were applied as a 1:1 mixture of the two strains, the mean established populations of the sum of the bacteria were in excess of 10^5 CFU/flower by petal fall in every trial; and in four of seven trials, the mean populations were $>10^6$ CFU/flower (Table 4). The incidence of detection of at least one antagonist on flowers treated with the mixture of bacteria was higher than single-strain inoculants at 56 to 98%. Of the flowers colonized by at least one of the antagonists, 96 to 100% of colonized flowers had detectable populations of both antagonists, except in 1995, when 86% of the colonized flowers had populations of both antagonists. Similar to single-strain inoculants, the method of inoculation of *E. amylovora* did not significantly affect the populations of the antagonists on flowers (data not shown).

The mean population size of A506 on flowers with detectable populations of C9-1R was significantly greater than the population size of the antagonist on flowers treated with A506 alone in five of seven trials (Fig. 1). The mean population size of C9-1R on flowers co-colonized by A506 was significantly greater than the population size of the C9-1R on flowers treated with that strain alone in three of seven trials (Fig. 1). In two of these trials, the population size of A506 also was significantly greater than expected compared with populations of A506 applied as a single-strain inoculant. In two trials, A506 and C9-1R were applied at 1:1, 9:1, and 1:9 proportions. In these trials, C9-1R established populations similar to C9-1R applied as a single-strain inoculant (Fig. 1E and F). In contrast, A506 established significantly greater mean population sizes when C9-1R was present on the flower, regardless of proportion in the inoculum. In one trial but not in

TABLE 2. Catabolism of compounds reported in pome fruit stigma secretions by *Pseudomonas fluorescens* A506, *Pantoea vagans* C91, and *Erwinia amylovora* Ea153

Compound ^x	Relative abundance ^y	Average peak height by 48 h at 22°C ^z					
		A506	Source	C9-1	Source	Ea153	Source
Carbohydrates							
α- D-Glucose	M	20 ± 4	...	57 ± 4	...	73 ± 12	...
D-Fructose	M	17 ± 2	...	50 ± 2	...	62 ± 13	...
D-Galactose	M	20 ± 2	...	96 ± 1	...	93 ± 19	...
D-Sorbitol	...	15 ± 3	...	78 ± 5	...	73 ± 9	...
L-Arabinose	M	28 ± 4	...	105 ± 1	...	87 ± 13	...
m-Inositol	...	13 ± 1	...	120 ± 1	...	96 ± 23	...
Sucrose	M	11 ± 1	...	56 ± 3	...	101 ± 16	...
Amino acids							
Hydroxy- L-proline	...	20 ± 2	C	0		0	...
L-Alanine	...	62 ± 10	CN	115 ± 21	CN	67 ± 4	CN
L-Arginine	...	60 ± 11	N	100 ± 4	N	66 ± 8	N
L-Asparagine	M	58 ± 6	CN	134 ± 24	CN	109 ± 6	CN
L-Aaspartic Acid	...	57 ± 6	CN	125 ± 15	CN	103 ± 8	CN
L-Cysteine	...	44 ± 1	S	98 ± 18	NS	77 ± 55	NS
L-Glutamic Acid	M	82 ± 4	CN	124 ± 14	CN	102 ± 8	CN
L-Glutamine	M	82 ± 6	CN	144 ± 14	CN	119 ± 14	CN
L-Glycine	...	39 ± 8	N	63 ± 14	N	32 ± 11	N
L-Histidine	...	67 ± 11	CN	97 ± 24	CN	39 ± 15	CN
L-Isoleucine	...	84 ± 4	N	0	...	0	...
L-Leucine	...	51 ± 11	CN	0	...	29 ± 13	C
L-Lysine	...	0	...	0	...	0	...
L-Methionine	...	47 ± 21	CS	107 ± 17	CS	109 ± 5	CS
L-Phenylalanine	...	10 ± 5	N	73 ± 12	N	39 ± 17	N
L-Proline	M	87 ± 19	CN	108 ± 10	CN	90 ± 9	CN
L-Serine	M	53 ± 16	CN	110 ± 29	CN	71 ± 10	CN
L-Threonine	...	31 ± 13	N	0	...	0	...
L-Tryptophan	...	13 ± 2	N	76 ± 11	N	51 ± 7	N
L-Tyrosine	...	60 ± 6	N	71 ± 10	N	35 ± 25	N
L-Valine	...	56 ± 13	N	10 ± 1	N	0	...

^x Carbohydrates were tested for their metabolism as sole carbon sources. Most amino acids were tested as sole carbon and nitrogen sources in separate assays. Hydroxy- L-proline was evaluated only as a sole carbon source. L-Cysteine was tested as a sole nitrogen and sulfur source. L-Methionine was tested as a sole carbon, nitrogen, and sulfur source. L-Threonine, L-tryptophan, L-tyrosine, and L-valine were tested only as sole nitrogen sources.

^y "M" indicates that the compound was reported as a major constituent of stigmatic secretions on pear or apple flowers (26). All other compounds were minor constituents, meaning that they were not consistently detected or were present in low concentrations.

^z Values were determined with Phenotype Microarray analyses by Biolog PM services. Positive peak height values were obtained for an amino acid evaluated as sole carbon (C), nitrogen (N), or sulfur (S) sources. Values for metabolism of amino acids are presented from evaluation as a sole nitrogen source, unless the amino acid only was utilized as a sole carbon source. Values for metabolism of L-cysteine and L-methionine are from evaluation of these amino acids as sole sulfur sources. Average peak height $\pm$ one standard error of duplicate readings after subtracting the peak heights of negative control wells. Value of zero indicates that the compound was not catabolized.

TABLE 3. Average generation time (minutes) of *Pseudomonas fluorescens* A506, *Pantoea vagans* C9-1, and *Erwinia amylovora* Ea153 at 20°C cultured in Davis minimal medium broth amended with compounds at a concentration of 1% (wt/vol)^z

Strain	Carbohydrates				Amino acids			
	α -D-Glucose	D-Fructose	D-Sorbitol	Sucrose	L-Asparagine	L-Glutamic acid	L-Glutamine	L-Proline
A506	93 $\pm$ 5 A	155 $\pm$ 32 AB	111 $\pm$ 9 A	130 $\pm$ 16 AB	96 $\pm$ 12 A	131 $\pm$ 2 A	130 $\pm$ 9 A	102 $\pm$ 8 A
C9-1	121 $\pm$ 11 A	143 $\pm$ 12 A	126 $\pm$ 4 A	119 $\pm$ 8 A	116 $\pm$ 9 A	136 $\pm$ 6 A	145 $\pm$ 2 A	208 $\pm$ 37 B
Ea153	191 $\pm$ 24 B	225 $\pm$ 26 B	205 $\pm$ 34 B	175 $\pm$ 20 B	89 $\pm$ 15 A	117 $\pm$ 5 A	126 $\pm$ 1 A	117 $\pm$ 2 A

^z Different letters within a column indicate a significant difference in the average generation time for a compound among strains by Fisher's protected least significance difference at $P = 0.05$.

the other, A506 mean populations were greater when applied with C9-1R in a 1:9 proportion (Fig. 1E and F).

The total population size of antagonists on co-colonized flowers was greater than the population size of single-strain inoculants. Overall, applying these bacteria as a mixture of genera resulted in a greater proportion of flowers colonized by at least one strain and a higher total population size of the antagonists. There was no evidence of antagonism between the two biological control agents.

Populations of *E. amylovora* Ea153N applied as fresh cells.

When applied as fresh cells, the incidence of Ea153N on water-treated flowers at petal fall varied among the three trials, with 5 to 50% of the flowers sampled (Table 5). Within trials, the proportion of flowers harboring detectable populations of Ea153N was greatest on trees treated with water, A506, and the mixture of the antagonists (Table 5). The incidence of detection of the pathogen on trees treated with A506 or the mixed inoculum did not differ significantly compared with water treatment in any of the three trials. Incidence of detection of the pathogen on trees treated with C9-1R was significantly lower than that observed on trees treated with water or A506 in two of three trials. In two trials, populations of Ea153N were monitored on trees treated with streptomycin; the response to treatment with streptomycin was similar to treatment with C9-1R (Table 5).

Populations of *E. amylovora* Ea153N applied as lyophilized cells. In five trials, populations of *E. amylovora* Ea153N applied as dusted lyophilized cells were monitored on trees treated with water, the mixture of biological control agents, or streptomycin. The incidence of detection of Ea153N by petal fall was 35 to 80% on flowers of trees treated with water (Table 5). The incidence of Ea153N detected on trees treated with the mixture of biological control agents was 32 to 88% and did not differ significantly from the incidence on water-treated trees in four of five trials. The mean detectable population size of *E. amylovora* on inoculated water-treated controls or trees treated with the mixed inocula was $\log_{10}$ 3.2 to 6.5 and did not differ significantly by treatment, except in the 1995 trial, when significantly greater populations of Ea153N were recovered from trees treated with A506 and C9-1R compared with water-treated trees (Table 5). Treatment with streptomycin consistently resulted in the lowest incidence of detection of the pathogen in all four trials where populations of the pathogen on treated trees were monitored. The detection incidence of Ea153N on streptomycin-treated trees was similar to

that on C9-1R-treated trees in three of four trials. The mean detected population size of the pathogen did not differ significantly between the water-treated control, biological treatments, or streptomycin in four of five trials (Table 5). In the one trial where significant differences in mean detected population sizes were observed, the lowest values were obtained on trees treated with streptomycin or C9-1R.

Suppression of fire blight by antagonists or antibiotics.

Trees were inoculated with streptomycin-sensitive Ea153N by three methods: spraying with an aqueous suspension of fresh cells scraped from solidified media, spraying with an aqueous suspension of lyophilized cells, or dusting flowers with lyophilized cells. Inoculation of flowers with aqueous suspensions of lyophilized cells resulted in the greatest average number of strikes per water-treated tree (Table 6). Spraying with aqueous suspensions of fresh cells resulted in the lowest number of strikes on water-treated trees (Table 6). Dusting trees with lyophilized cells resulted in an intermediate level of disease (Table 6) compared with the other two methods.

In all but two trials, streptomycin significantly reduced the average number of strikes per tree by 73% compared with the level of disease on water-treated trees (Table 6). Oxytetracycline did not significantly reduce the relative incidence of fire blight compared with the water treatment in the three trials where that product was evaluated.

The performance of A506 for disease control varied greatly among trials. In one trial (Corvallis 1992, dusted inoculum), treatment with A506 reduced the relative disease incidence by 80% compared with water-treated trees (Table 6). In most trials, however, A506 did not significantly reduce the incidence of disease compared with water-treated trees. In two trials, the average number of strikes per tree was greater on A506-treated trees compared with water-treated trees (Corvallis 1992, fresh inoculum; Medford 1993, dusted inoculum) (Table 6).

C9-1R provided better control of fire blight than A506. The relative incidence of fire blight was reduced between 7 and 44% on C9-1R-treated trees inoculated with fresh cells of the pathogen but the reduction was not significant compared with water-treated controls (Table 6). Treatment with C9-1R significantly reduced the relative disease incidence in each trial inoculated with lyophilized cells of the pathogen (Table 6). In these trials, C9-1R-treated trees had 17 to 88% fewer strikes per tree compared with inoculated water-treated controls (Table 6). The level of disease

TABLE 4. Relative area under the population curve and incidence of detection of *Pseudomonas fluorescens* A506 and *Pantoea vagans* C9-1R applied as single strains or in combination^a

Treatment ^b	Year and host ^c							
	1992		1993		1994		1995	
	Bartlett	Medford	Bartlett	Medford	Bosc	Medford	Apple	
A506	5.58 ± 0.43 (0.31 c)	4.61 ± 0.33 (0.43 b)	5.41 ± 0.26 (0.62 a)	5.59 ± 0.23 (0.65 a)	5.37 ± 0.37 (0.65 b)	5.48 ± 0.37 (0.66 b)	5.09 ± 0.41 (0.47 b)	
C9-1R	4.89 ± 0.25 (0.52 b)	5.84 ± 0.17 (0.90 a)	5.14 ± 0.43 (0.65 a)	5.46 ± 0.23 (0.63 a)	5.26 ± 0.39 (0.48 c)	4.81 ± 0.37 (0.46 c)	5.14 ± 0.23 (0.62 a)	
A506 and C9-1R (1:1)	6.19 ± 0.32 (0.82 a)	6.23 ± 0.11 (0.98 a)	6.38 ± 0.19 (0.68 a)	5.98 ± 0.15 (0.70 a)	6.40 ± 0.16 (0.82 a)	5.65 ± 0.28 (0.87 a)	5.67 ± 0.26 (0.56 ab)	
A506 and C9-1 R (9:1)	...	...	...	...	6.04 ± 0.46 (0.85 a)	5.85 ± 0.22 (0.76 b)	...	
A506 and C9-1 R (1:9)	...	...	...	...	6.21 ± 0.15 (0.90 a)	5.64 ± 0.30 (0.68 b)	...	

^a Data indicate average relative area under the population curve ± one standard error from application to petal fall or ≈8 to 14 days after inoculation of trees with the pathogen. Values for mixtures of antagonists represent the sum of the antagonist population enumerated from flower samples. Numbers in parentheses = average incidence of detection of an antagonist at 8 to 14 days after pathogen inoculation. Incidence data were transformed (square root of the arcsine) prior to mean separation with Fisher's least significant difference. Different letters after incidence data represent significant difference within columns at $P = 0.05$.

^b *Pseudomonas fluorescens* A506 and *Pantoea vagans* C9-1R were applied as single strain inoculants at 1×10^8 CFU/ml to trees in complete randomized block design at 20 and 70% bloom. Experimental orchards were located in Corvallis Oregon, except for trials conducted in Medford Oregon, as indicated in the Table. The strains were applied as a 1:1 mixture with each strain at 5×10^7 CFU/ml or a total of 1×10^8 CFU/ml. In 1994, combinations of one part antagonist to 9 parts of the other antagonist were included.

^c Hosts: Bartlett = Bartlett pear at Corvallis, OR; Medford = Bartlett pear at Medford, OR; Bosc = Bosc pear; and Apple = Golden Delicious apple.

control by C9-1R was similar to treatment with streptomycin in six of eight trials.

Deploying C9-1R and A506 as a mixture provided levels of disease control intermediate to the strains applied as individual inoculants. The 1:1 mixture of C9-1R and A506 significantly reduced the incidence of fire blight compared with water-treated controls in 4 of 10 trials (Table 6). On trees inoculated with fresh cells of the pathogen, the mixture of antagonists did not significantly reduce the mean number of strikes per tree. In two of three of these trials, the mean number of strikes per tree on trees treated with C9-1R and A506 was greater than the average number of strikes on water-treated controls. The combination of C9-1R and A506 reduced disease by 54 to 63% on trees inoculated with an aqueous suspension of lyophilized cells, and control was significant compared with water-treated controls in one trial (Table 6). In three of five trials, the combination of antagonists significantly reduced the incidence of fire blight on trees dusted with lyophilized cells of the pathogen compared with water-

treated controls (Table 6). The level of control with a C9-1R and A506 mixture was not better than single inoculants on trees dusted with the pathogen. In one trial (Corvallis 1992, dusted inoculum), disease control by C9-1R combined with A506 was significantly less than that obtained with C9-1R or A506 applied as single strains.

Rank sum analysis was used to compare disease control efficacy by treatments among the trials regardless of inoculation method. As expected, water-treated controls had the highest level of disease and streptomycin provided the best control of the streptomycin-sensitive strain of the pathogen (Table 6). A506 did not significantly reduce fire blight compared with water-treated controls. C9-1R provided the best control among antagonist treatments but suppression of fire blight by C9-1R was significantly less than streptomycin against the streptomycin-sensitive pathogen. The combination of C9-1R and A506 provided significantly less control of fire blight compared with C9-1R alone; however, a significant reduction in the incidence of fire blight was obtained by the mixture compared with water treatment.

DISCUSSION

The biological control agent *Pantoea vagans* C9-1R reduced the incidence of fire blight by an average of 42% compared with water treatments in eight orchard trials. C9-1R did a better job than A506 of suppressing fire blight and the establishment of *E. amylovora*, assessed as the incidence of flowers with detectable populations of the pathogen. The superior control by C9-1R is probably due, at least in part, to its production of herbicolin O, an antibiotic synonymous to mceEh252, which was shown to contribute to biological control fire blight by strain Eh252 in orchards (7,30,39). In previous efficacy trials, Eh252 reduced the incidence of colonization of flowers by the pathogen and reduced the incidence of fire blight by 55% (30). In this study, the level of disease control by C9-1R was similar to streptomycin in six of eight trials. When all eight trials were analyzed together, the level of disease control of the streptomycin-sensitive pathogen strain by streptomycin was superior to C9-1R alone. These results highlight the promise of *Pantoea vagans* as an effective biological control agent for fire blight, especially in the many pome-fruit-growing regions where streptomycin-resistant populations of the pathogen are now prevalent (15).

Pseudomonas fluorescens A506 did not significantly reduce the incidence of detection of *E. amylovora* on flowers or consistently reduce the incidence of fire blight in this study. In only one trial, A506 provided significant control of fire blight and the level of control was similar to that obtained with streptomycin. The lack of consistent disease control by A506 in pathogen-inoculated orchard trials has been reported by others (33) and contrasts with reports that A506 reduced the incidence of fire blight by 50 to 80% in many trials in California (17–19). The trials in California relied on natural sources of the pathogen and the average number of strikes per tree in those trials was low (1 to 5 per tree). It is possible that A506 provides better control efficacy for fire blight under low disease pressure compared with disease pressure imposed by artificial inoculation with the pathogen. Additionally, the evaluation trials of A506 in California were conducted in growers' orchards that were treated with routine antibiotic applications (17–19). The excellent control of fire blight reported in growers' orchards may indicate that A506 performs well in an integrated disease control program in large orchards and is less effective when evaluated as a stand-alone treatment on artificially inoculated trees.

Inoculation of pear and apple trees with *E. amylovora* can be a challenge. Application of too high a dose can overwhelm the system, such that even potent chemicals like streptomycin are ineffective. Too low an inoculum dose may result in disease levels insufficient for discrimination of treatment effects. Formulation of

Fig. 1. Mean detectable population sizes (CFU × 100,000 per flower) of *Pseudomonas fluorescens* strain A506 (open triangles) and *Pantoea vagans* strain C9-1R (open diamonds) on flowers sampled at petal fall or 8 to 14 days after full bloom in **A**, Bartlett pear, Corvallis 1992; **B**, Bartlett pear, Medford 1992; **C**, Bartlett pear, Corvallis 1993; **D**, Bartlett pear, Medford 1993; **E**, Bosc pear, Corvallis 1994; **F**, Bartlett pear, Medford 1994; and **G**, Golden Delicious apple, Corvallis 1995. Filled squares represent the sum of antagonist populations applied as single-strain inoculants or in combination. Dashed lines represent the expected population size of each strain and the sum of two strains if interstrain competition is equivalent to intrastain competition. Significantly greater population size of a single strain applied in 1:1 mixed inoculum compared with the strain applied as a single strain is indicated by the “+” sign after the strain name; a “0” after the strain name indicates no significant difference between the strain applied in combination and as a single-strain inoculant; P values are included.

the inoculum also can influence establishment of the pathogen on flowers (29). In this study, *E. amylovora* was introduced to flowers with three different methods: spraying trees with aqueous suspensions of fresh cells harvested from agar surfaces or lyophilized cells, or dusting flowers with lyophilized cells. The dusted formulation of the pathogen was similar to honey-bee-vectored inocula used by Johnson et al. (13) to evaluate fire blight control in experimental orchard trials. Inoculation with lyophilized cells resulted in detectable populations of the pathogen on a greater proportion of flowers than inoculation with fresh cells, which agrees with previous research concluding that lyophilized cells of the pathogen establish better on flowers than fresh cells (29). Correspondingly, the average number of blossom cluster strikes was greater on trees inoculated with lyophilized cells compared with fresh cells of the pathogen. The dose and

formulation of the pathogen applied with the three methods was adequate to detect treatment effects. Among trials, streptomycin provided the best disease control, which was expected because the inoculated strain of the pathogen is sensitive to streptomycin. Although the pathogen also is sensitive to oxytetracycline in vitro, oxytetracycline treatment did not significantly reduce the incidence of fire blight compared with water treatment. On trees inoculated with fresh cells, only treatment with streptomycin significantly reduced the incidence of fire blight (Table 6). C9-1R significantly reduced the incidence of fire blight in each trial inoculated with lyophilized cells of the pathogen but it did not significantly reduce disease incidence on trees inoculated with fresh cells of the pathogen. The inability to detect significant disease control with C9-1R on trees inoculated with fresh cells of the pathogen is noteworthy, given that fresh cell inocula of the

TABLE 5. Average detectable population size and incidence of detection of *Erwinia amylovora* Ea153N on flowers of trees treated with water, biological control agents, or antibiotics

Inoculum, treatment ^y	Year and host ^z				
	1992		1993		1995
	Bartlett pear	Bartlett pear (Medford)	Bartlett pear	Bartlett pear (Medford)	Golden Delicious apple
Fresh cells					
Water	4.19 A (0.20 bc)	2.64 A (0.05 b)	NT	NT	5.01 B (0.50 abc)
A506	5.09 A (0.10 c)	2.00 A (0.05 b)	NT	NT	5.07 B (0.44 abc)
C9-1R	3.45 A (0.02 d)	ND (0.00 c)	NT	NT	4.09 B (0.28 c)
A506 and C9-1R	4.30 A (0.08 c)	4.12 A (0.10 b)	NT	NT	6.05 A (0.62 ab)
Streptomycin	2.00 A (0.02 d)	ND (0.00 c)	NT	NT	NS
Dusted lyophilized cells					
Water	5.25 A (0.50 a)	4.87 A (0.35 a)	4.06 A (0.62 ab)	3.79 AB (0.80 a)	4.17 B (0.41 abc)
A506	NS	NS	3.76 A (0.77 a)	4.43 A (0.82 a)	NT
C9-1R	NS	NS	2.89 B (0.43 b)	3.23 B (0.70 a)	NT
A506 and C9-1R	4.71 A (0.32 b)	3.23 A (0.38 a)	3.78 A (0.73 a)	4.24 A (0.88 a)	6.53 A (0.47 abc)
Streptomycin	ND (0.00 d)	4.59 A (0.05 b)	2.52 B (0.15 c)	3.22 B (0.12 b)	NS

^y Each inoculum formulation was applied to trees in full bloom. Fresh cells consisted of suspensions of bacterial lawns scraped from solidified nutrient agar plus 1% glycerol and adjusted to 1×10^6 CFU/ml and sprayed onto trees. Dusted lyophilized cells consisted of a lyophilized formulation of the pathogen added to powdered milk, as a carrier, to a population of 1×10^9 CFU/g and dusted onto trees with a handheld Chapin duster. *Pseudomonas fluorescens* A506 and *Pantoea vagans* C9-1R were applied as single strain inoculants and as a 1:1 mixture with each strain at 5×10^7 CFU/ml or a total of 1×10^8 CFU/ml to trees in 20 and 70% bloom.

^z Data indicated average detectable population $\log_{10}$ (CFU/flower) of *E. amylovora* Ea153N 8 to 14 days after pathogen inoculation. Enumerated populations were $\log_{10}$ transformed prior to mean separation with Fisher's least significant difference. Experimental orchards were located in Corvallis OR, except for trials conducted in Medford, OR, as indicated. Different uppercase letters after the populations represent significant difference within columns at $P = 0.05$. Numbers in parentheses = average incidence of detection of *E. amylovora* Ea153N at 8 to 14 days after pathogen inoculation. Incidence data were transformed (square root of the arcsine) prior to mean separation with Fisher's least significant difference. Different lowercase letters after incidence data represent significant difference within columns at $P = 0.05$. NT = not tested, ND = not detected (detection limit was 100 CFU/flower), and NS = not sampled.

TABLE 6. Relative incidence of fire blight on blossom clusters on trees treated with water, biological control agents, and antibiotics

Treatment ^v	Pathogen formulation, host, and year ^w										
	Fresh inoculum ^x			Lyophilized cells dusted ^y					Suspension of cells sprayed ^z		
	Bartlett 1992	Medford 1992	Apple 1995	Bartlett 1992	Medford 1992	Bartlett 1993	Medford 1993	Apple 1995	Apple 1995	Bartlett 1998	Mean rank sum
Water	100 AB (5.6 ± 1.1)	100 A (2.7 ± 0.8)	100 AB (24.2 ± 7.5)	100 A (121.4 ± 41.3)	100 A (12.8 ± 1.9)	100 A (13.1 ± 3.3)	100 A (16.2 ± 1.9)	100 A (48.8 ± 17.0)	100 A (124.2 ± 53.5)	100 AB (188.0 ± 22.8)	1.6 A
A506	129 A	74 AB	98 AB	19 C	70 ABC	76 AB	159 A	NT	74 AB	NT	2.0 AB
A506 and C9-1R	122 A	48 AB	124 A	42 B	63 AB	55 BC	94 B	52 AB	37 B	46 B	2.6 B
C9-1R	93 AB	67 AB	56 AB	12 C	52 BC	53 BC	83 B	NT	44 B	NT	3.8 C
Oxytetracycline	NT	NT	72 AB	NT	NT	NT	NT	NT	77 AB	155 A	...
Streptomycin	16 C	26 B	45 B	11 C	30 C	19 C	24 C	35 B	28 B	40 B	5.0 D

^v *Pseudomonas fluorescens* A506 and *Pantoea vagans* C9-1R were applied as single strain inoculants and as a 1:1 mixture with each strain at 5×10^7 CFU/ml or a total of 1×10^8 CFU/ml to trees in 20 and 70% bloom. Water, streptomycin, and oxytetracycline were applied to trees at 20 and 70% bloom and repeated within 3 days after inoculation with the pathogen at full bloom.

^w Each pathogen inoculum formulation was applied to trees in full bloom. Experiments were conducted in experimental pear (cv. Bartlett) and apple (cv. Golden Delicious) orchards located near Corvallis, OR, except for Bartlett pear trials that were located in Medford, OR. Different letters within a column following the incidence of fire blight relative to water-treated trees indicate a significant difference in the arcsine square root transformed disease incidence data by Fisher's protected least significance difference at $P = 0.05$. Numbers in parentheses are the average number of fire blight strikes on water-treated trees ± standard error. NT = not tested.

^x Fresh cells consisted of suspensions of bacterial lawns scraped from solidified nutrient agar plus 1% glycerol and adjusted to 1×10^6 CFU/ml and sprayed onto trees.

^y Dusted lyophilized cells consisted of a lyophilized formulation of the pathogen added to powdered milk, as a carrier, to a population of 1×10^9 CFU/g and dusted onto trees with a hand-held Chapin Duster.

^z Resuspended lyophilized cells consisted of lyophilized cells of the pathogen suspended in water to a concentration of 1×10^6 CFU/ml and misted onto trees.

pathogen are used routinely in field trials evaluating antagonists for fire blight control (5,21,33). Subsequent fire blight trials in Oregon have relied on spraying suspensions of lyophilized cells of the pathogen, which permits consistent establishment of low populations of the pathogen in an attempt to mimic the population dynamics of natural inocula (13,29).

Successful biological control of fire blight depends on the establishment of suppressive populations of an antagonist on stigmas, the site of epiphytic growth of the pathogen. Populations of antagonists in excess of 10^5 CFU/flower are required to suppress colonization and growth of *E. amylovora* on flowers (12–14,22,41). When applied together, A506 and C9-1R colonized flowers and achieved suppressive population sizes ($>10^6$ CFU/flower). This observation is in agreement with many other studies demonstrating that these bacteria colonize floral tissues as single-strain inoculants (4,14,19,23,25,29,30,32,36,40,41,43) or when applied in combination (11,13,22). In this study, the average population size of the sum of the antagonists and the incidence of colonization was significantly greater with the mixture than when strains were applied as single-strain inoculants. The replacement series experiments also indicated that the antagonists A506 and C9-1R have a high level of coexistence on flowers in orchards, either through occupation of distinct microsites on stigmas or utilization of different nutrients available on flowers. Nonetheless, an additive or synergistic effect was not observed for control of fire blight with the mixed inocula.

The metabolic versatility of *Pseudomonas* spp., *Pantoea* spp., and *Erwinia* spp. is widely recognized and was confirmed by the phenotypic arrays employed in this study. Both antagonists and the pathogen used a large proportion of the 190 carbon and 95 nitrogen substrates tested. Given that versatility, it is not surprising that a high overlap in NOIs was observed between each strain pair. Considering the high NOIs between A506 and C9-1, one might expect that the antagonists would compete for similar resources on floral tissues, as proposed by others evaluating bacterial interactions on leaf surfaces (8,11). In contrast to this expectation, the antagonists were highly compatible on flowers. There are many possible explanations for the observed compatibility of strains having high NOIs. First, NOIs determined from the phenotype arrays may not be predictive of those on flowers. However, when we based the NOI calculations only on those nutrients detected in floral tissues (24,26), the NOI between A506 and C9-1 was absolute. Second, the nutritional preferences of *Pseudomonas* spp. and *Pantoea* spp. are different. Although both strains of antagonists could grow on similar substrates in the phenotypic array experiments, their distinct preferences may dictate their catabolism on floral surfaces. Third, nutrients may not be limiting to bacterial growth on stigmas throughout bloom. Stigmas of flowers are nutrient-rich tissues, supporting populations in excess of 10^6 CFU/tissue or 10^{10} CFU/g (12). Pusey et al. (26) estimated that the concentration of monosaccharides alone on pome fruit flowers was ≈ 3 μ g and adequate to support large populations of bacteria. Additionally, flowers are not “static” tissues but undergo many changes rapidly during bloom. During anthesis, the cuticle covering the stigmatic surfaces ruptures (12), which releases more nutrients (26). These secretions may provide additional nutritional niches that could be used by bacteria as flowers age. If nutrients are not limiting to bacterial growth on floral surfaces, the NOI is not likely to be useful in predicting the level of co-existence of bacterial isolates on flowers, as it has been done on leaf surfaces (8,11).

We had anticipated increased disease control by combining A506 with C9-1R compared with single-strain inoculants. One of our hypotheses was that the two antagonists collectively would use a large proportion of the nutrients required by the pathogen for growth on floral surfaces. In actuality, each of the individual antagonists had very high NOI with the pathogen, which was increased only slightly by combining the antagonists. The greater

population size of the sum of the antagonists on flowers was expected to result in diminished establishment of the pathogen on flowers. However, the mixture of the two strains did not perform as well as C9-1R alone for disease control. The mixture did not reduce the incidence of detection of the pathogen on flowers compared with water treatment. The mixture significantly reduced the incidence of fire blight compared with water treatment in 4 of 10 trials but the performance varied among trials (Table 6). This type of variation in control efficacy is one of the factors that discourages growers from relying solely on biologically based methods for disease control.

The lack of synergy in disease control by C9-1R and A506 is not unique to this specific mixture of bacterial antagonists for fire-blight management. Vanneste and Yu (37) found that combining Eh252 with A506 did not improve control of fire blight compared with single-strain inoculants in a field trial on apple cv. Granny Smith or Asian pear cv. Kosui. Disease control by mixtures of the commercial formulations BlightBan A506 + BlightBan C9-1S in orchard trials in the eastern United States was not better than control by BlightBan C9-1S alone (33). There are several possible reasons why the level of disease control by the mixture of A506 with *Pantoea* spp. was less than expected. One possibility is that *E. amylovora*, in the face of competition from two antagonists and reduced nutritional resources, may induce the expression of genes of the *hrp* cluster, releasing effectors that kill plant cells and creating new physical niches and associated nutrients. Johnson et al. (11) demonstrated that the gene *dspE*, which encodes for a pathogenesis-related protein, is transcribed in a portion of the population of *E. amylovora* residing on stigmatic tissues. They suggested that the pathogen may cause microlesions which expand epiphytic niches and, thus, escape interactions with bacterial antagonists. A second possibility is that combining A506 with C9-1R may interfere with mechanisms of antagonism. In addition to competition, antibiosis contributes to disease control by strains of *Pantoea* spp. C9-1R produces herbicolin O, which is synonymous to pantocin A and mceEh252, peptide antibiotics inhibitory to *E. amylovora* (7,9,38,39). Anderson et al. (1) discovered that A506 is insensitive to herbicolin O and produces an extracellular metalloprotease that inactivates herbicolin O. The extracellular protease of A506 also inactivates mceEh252 (1), which is produced by *Pantoea agglomerans* Eh252 and has been shown to contribute to suppression of fire blight (30,38). We speculate that A506 and C9-1R occupy sites in close proximity on stigmas. The extracellular protease secreted by A506 may inactivate herbicolin O secreted by C9-1R, such that the mechanism of antibiosis of C9-1R is quelled by A506. We speculated that combining C9-1 with a derivative of A506 that did not produce the extracellular protease may lead to better control of fire blight. The management of fire blight with an extracellular protease-deficient mutant of A506 combined with strains of *Pantoea* spp. is addressed in a companion article (31).

Consistent and highly effective biologically based management of fire blight has been an elusive goal. By understanding the interactions among biological control agents, we may improve the efficacy of control and reduce variation, factors cited as a deterrent to widespread adoption of biological-based methods for disease control by growers. Unfortunately, using intergeneric bacterial mixtures did not result in improved control of fire blight in this study. In the design of mixtures, cohabitation and niche-filling are factors that should be considered for disease suppression by preemptive exclusion but the impact of intergeneric interactions on other mechanisms of control also should be considered.

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