Antibiosis Contributes to Biological Control of Fire Blight by *Pantoea agglomerans* Strain Eh252 in Orchards

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ABSTRACT

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Fire blight, caused by *Erwinia amylovora*, is the most serious bacterial disease of pear and apple trees. Biological control with strains of *Pantoea agglomerans* (syn. *Erwinia herbicola*) may provide an effective disease management strategy for fire blight. Most strains of *P. agglomerans* evaluated for suppression of fire blight produce compounds that inhibit the growth of *E. amylovora* in culture. The role of these inhibitory compounds in fire blight suppression in orchard environments has not been studied. In seven field trials in Oregon, we compared the population dynamics and disease suppression with *P. agglomerans* Eh252, a strain that produces a single antibiotic, with its near-isogenic antibiotic-deficient derivative, strain 10:12. Water or suspensions of Eh252 or 10:12 (1×10^8 CFU/ml) were applied at 30 and 70% bloom to pear or apple trees. Aqueous suspensions of freeze-dried cells of *E. amylovora* (3×10^5 CFU/ml) were applied at full bloom. Additional trees were treated with streptomycin or oxytetracycline at 30 and 70% bloom and in some experiments, 1 day after application of the pathogen. Population sizes of Eh252 or 10:12 on pear blossoms were estimated by spreading dilutions of blossom washes on culture media. Average population sizes of Eh252

and 10:12 on blossoms ranged from 10^5 to 10^7 CFU, and in five of six trials, the relative area under the population curve of Eh252 was not significantly different than that of its derivative 10:12. Both Eh252 and 10:12 reduced the growth of the pathogen on blossoms compared with inoculated water-treated controls. Eh252 significantly decreased the incidence of fire blight in six of seven field trials compared with the incidence on water-treated trees, and 10:12 similarly reduced the incidence of fire blight in four of seven trials. In three of seven field trials, trees treated with Eh252 had a significantly lower incidence of fire blight compared with trees treated with 10:12. Overall, Eh252 reduced the incidence of fire blight by $55 \pm 8\%$, 10:12 by $30 \pm 6\%$, streptomycin by $75 \pm 4\%$, and oxytetracycline by $16 \pm 14\%$. The effectiveness of strain 10:12 compared with water treatment indicates that other mechanisms (e.g., competitive exclusion or habitat modification) also contribute to disease suppression by *P. agglomerans*. The increased suppression of fire blight by the parental strain Eh252 compared with the antibiotic-deficient mutant 10:12 indicates that antibiosis is an important mechanism of biological control of fire blight.

Additional keywords: herbicolin, *Malus*, microcin, pantocin, pome fruit, *Pyrus*.

Strategies for control of bacterial diseases of plants often focus on host resistance and cultural methods, which can be difficult to implement with perennial tree fruits. Moreover, chemical control measures are limited, due in part to the dearth of registered, effective, nonphytotoxic bactericides for agricultural use. Two antibiotics, streptomycin and oxytetracycline, are registered in the United States for control of fire blight, a serious disease of pear and apple caused by the bacterium *Erwinia amylovora*. Streptomycin was an effective chemical for the management of fire blight until pathogenic strains resistant to the antibiotic emerged in several pome fruit growing regions (17–19,28). Oxytetracycline is registered for use only on pear and is considered less effective than streptomycin for suppression of antibiotic-sensitive populations of *E. amylovora* (18).

As pathogen resistance has compromised the effectiveness of streptomycin for fire blight management, interest in biological control of fire blight has increased. Biological control focuses primarily on suppression of the epiphytic growth phase of the pathogen on blossoms prior to infection and endophytic growth

(10). The earliest studies on biological suppression of fire blight with a bacterium were conducted in the 1930s with a “yellow pigmented” bacterium, which has since been classified as *Pantoea agglomerans* (syn. *Erwinia herbicola*) (1). Over a period of several decades, many strains of *P. agglomerans* have been evaluated as potential biocontrol agents for fire blight, in part, due to their production of antibiotics inhibitory to *E. amylovora* in synthetic culture media (41). Production of antibiotics is a common phenotype of *P. agglomerans*, as 42% of 346 field-collected strains assayed inhibited the growth of *E. amylovora* in culture (40). The antibiotics, referred to as herbicolins (8), pantocins (2,43), or microcins (36), are characterized principally by the reversal of inhibition by addition of exogenous amino acids (e.g., histidine, leucine, and arginine) to culture media (40), sensitivity to heat, pH extremes, and by inactivation by proteases or penicillinases (14). Some strains of *P. agglomerans* produce a single antibiotic (e.g., strains Eh252, Eh112Y, and Eh1087) (1,14,35), whereas others produce multiple compounds inhibitory to *E. amylovora* (e.g., strains Eh318 and C9-1) (8,42,43). The chemical structure of one arginine-reversible, tripeptide pantocin B produced by *E. herbicola* Eh318 has been published (2) and other antibiotics produced by strains of *P. agglomerans* are also likely to be low molecular weight compounds with a peptide component (43). The genes encoding for antibiotic biosynthesis in *P. agglomerans* have been localized to the chromosome in several strains (3,32,33,35,42,43) and on plasmids in others (4,15,16).

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Several lines of evidence indicate that antibiotics produced by *P. agglomerans* contribute to fire blight suppression. First, nearly every isolate of *E. amylovora* tested has been sensitive to the antibiotics in culture plate assays (2,4,8,14–16,21,30,32,33,35–43). *P. agglomerans* or semipurified preparations of antibiotics applied to slices of immature pear fruit inoculated with *E. amylovora* reduced necrosis of the host tissue and delayed the production of bacterial ooze (8,14–16,21,30,33,35–37,39,41–43). In some experiments, non-antibiotic producing mutants of *P. agglomerans* exhibit reduced capacity to inhibit symptom development of inoculated immature pear fruit slices compared with the parental strains (16,35), but this result is not always observed (42). The capacity to suppress symptom development on immature pear fruits has been a primary assay to evaluate the role of antibiosis in biological control of fire blight (29). Although there is a fair correlation between suppression of symptoms on immature pear fruit in the laboratory and efficacy under field conditions, this laboratory assay has limitations (29,41). Strains that suppress fire blight through competitive exclusion of the pathogen from sites and nutrients on stigmas may not compete well on wounded parenchyma tissues of pear fruit (23). Pear juice is rich in several amino acids including arginine and proline, which can reverse inhibition by some antibiotics produced by strains of *P. agglomerans* (40). Conversely, pear juice has low concentrations of histidine; thus, the immature pear fruit assay may select for strains of *P. agglomerans* that produce histidine-reversible antibiotics or antibiotics insensitive to amino acids (40). Therefore, the immature pear fruit assay may mask or accentuate the relative importance of antibiotics in disease suppression of fire blight on the stigmatic surfaces of blossoms (40).

Two studies have evaluated the role of antibiotics produced by strains of *P. agglomerans* in fire blight suppression on blossoms in laboratory assays. A strain of *Escherichia coli* was transformed with a 12-kb plasmid isolated from *E. herbicola* (4). The transformed *Escherichia coli* produced antibiotics in culture and reduced growth of the pathogen and symptom development on inoculated hawthorn blossoms (4), whereas the nontransformed parental strain did not. In a second study, Wright and Beer (42) and Wright et al. (43) generated mutants of strain Eh318 that were deficient in pantocin A, pantocin B, or both antibiotics. Mutants of Eh318 deficient in production of either one of the two antibiotics provided control of fire blight on greenhouse-grown apple blossoms that was similar to the parental strain. A mutant deficient in both antibiotics suppressed fire blight but was inferior to the parental strain. To date, the role of antibiotic production in sup-

pression of fire blight has not been evaluated in the field. The objective of this study was to compare a wild-type strain of *P. agglomerans* and its antibiotic-deficient mutant for suppression of fire blight in an orchard environment.

MATERIALS AND METHODS

Bacterial strains. The experiments were conducted with *P. agglomerans* Eh252 (syn. *E. herbicola* Eh252) and its antibiotic-deficient derivative, 10:12, which were provided by S. V. Beer, Cornell University. Strain Eh252 produces a single antibiotic called mceEh252 (32,36). The nucleotide sequence of chromosomal genes encoding for synthesis of mceEh252 has not been published but is reported to be similar to *mccB* of *Escherichia coli*, which is involved in the synthesis of microcin C7 (32,33, 35,36). MceEh252 is histidine-reversible, sensitive to proteolysis, heat-stable, insensitive to pH extremes, exhibits a narrow host range of sensitivity (*Erwinia* and *Pantoea* spp.), and is likely a small (<3,000 Da) peptide because it is sensitive to proteolysis and passes through dialysis tubing (35). Strain 10:12 has a single Tn5 insertion in a gene required for synthesis of mceEh252 (32,33,35). Strain 10:12 does not produce mceEh252 but is prototrophic and has a growth rate similar to the parental strain in culture and on immature pear fruit slices (35); polar effects due to the insertion have not been reported. Complementation of 10:12 with cosmids containing mceEh252 biosynthesis genes restores mceEh252 production and suppression of symptoms of fire blight in immature pear fruit laboratory assays (29,33,37).

E. amylovora 153N (Ea153N) was originally isolated from a fire blight canker on an apple tree (cv. Gala) in Milton-Freewater, OR, and a spontaneous nalidixic acid-resistant (100 µg/ml) mutant was selected. Ea153N is similar to the parental strain in growth rate and colony morphology and is sensitive to streptomycin and oxytetracycline. Ea153N has been used as a representative pathogen isolate in numerous field trials (11,22,23,25,26). Each bacterial strain was stored in nutrient broth with 15% glycerol at –80°C.

Experimental design. Field experiments evaluating population dynamics and relative suppressiveness of fire blight by Eh252 and its derivative 10:12 were conducted in 0.5-ha blocks of pear (*Pyrus communis* L. cvs. Bartlett and Bosc) at the Oregon State University, Botany and Plant Pathology Field Laboratory near Corvallis, OR in 1998, 1999, and 2000 and at the Southern Oregon Research and Extension Center near Medford, OR, on cv. Bartlett pear in 1999 (Table 1). An additional efficacy trial was

TABLE 1. Location, cultivar, age, bloom period, treatment, and sampling dates

Year	Location	Cultivar	Year planted	Date of treatments			Sampling dates						Disease assessment period
				First ^v	Second ^w	Pathogen ^x	1	2	3	4	5	6	
1998	Corvallis	Bartlett	1963	4 Apr	8 Apr	9 Apr 10 Apr	9 Apr	16 Apr	18 Apr	20 Apr Petal fall ^y	24 Apr	...	25 Apr to 18 May
1999	Corvallis	Bartlett	1963	17 Apr	19 Apr	20 Apr	21 Apr	23 Apr	26 Apr	30 Apr Petal fall	5 May	...	6 May to 8 Jun
	Corvallis	Bosc	1976	20 Apr	23 Apr	24 Apr	28 Apr	30 Apr	3 May Petal fall	7 May	10 May	14 May	16 May to 17 Jun
	Medford	Bartlett	1975	16 Apr	18 Apr	20 Apr	22 Apr	26 Apr	28 Apr Petal fall	4 May	...	...	08 May to 10 Jun
2000	Corvallis	Bartlett	1963	10 Apr	12 Apr	13 Apr	12 Apr	19 Apr Petal fall	24 Apr	27 Apr	2 May	...	02 May to 8 Jun
	Corvallis	Bosc	1976	14 Apr	17 Apr	19 Apr	18 Apr	21 Apr	26 Apr Petal fall	1 May	4 May	...	06 May to 3 Jun
	Corvallis	Rome	1958	2 May	4 May	5 May	NS ^z	...	...	...	...	...	10 Jun to 17 Jul

^v Date of first application of *Pantoea agglomerans* strains, water, or antibiotics to trees in 30% bloom.

^w Date of second application of *P. agglomerans* strains, water, or antibiotics to trees in 70% bloom.

^x Date of inoculation with *Erwinia amylovora* 153N to trees in full bloom.

^y Approximate date of 100% petal fall in relationship to sampling dates.

^z Trial established for efficacy only and not sampled for bacterial populations.

conducted in a 0.4-ha block of apple (*Malus X domestica* Borkh. cv. Rome Beauty) at the Botany and Plant Pathology Field Laboratory near Corvallis in 2000.

Treatments. Treatments were assigned to individual trees in field plots in a randomized complete block design. Trees were grouped into blocks based on relative bloom density and location in the orchard. Each treatment was applied to five replicate trees, except in the 1999 trial in Medford and the 2000 trial on apple, which contained four replicate trees per treatment. Eh252 and 10:12 were cultured for 4 days at 25°C on nutrient agar (Difco Laboratories, Detroit) 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 & Lomb Spec 20, Rochester, NY), and the concentrated stock solution was transported to the field in an ice chest. In the field plots, bacterial suspensions were diluted with water (local groundwater sources) to 1×10^8 to 3×10^8 CFU/ml prior to spraying. The bacterial cell concentration of the applied suspension was confirmed after treatment by dilution plating.

At sunrise (6:30 to 7:00 a.m.), trees at 30% bloom and again at 70% bloom were treated with Eh252 or 10:12 until near run-off with 12-liter backpack sprayers fitted with adjustable brass nozzles attached to a hand-held spray wand (Table 1). Approximately 2 to 4 liters of the bacterial suspensions was applied to individual trees. Additional trees in each plot were treated with water or streptomycin sulfate (in the 1998 trial: Agristrep 17%, 100 µg/ml a.i., Novartis Crop Protection, Greensboro, NC; in 1999 and 2000 trials: Bac-Master 17%, 100 µg/ml a.i., Pace International, Seattle) as controls for disease suppression. Oxytetra-

cycline (Mycosshield, 200 µg/ml a.i., Novartis Crop Protection) also was included as a disease control treatment in all trials except those conducted on cv. Bosc pear due to a limited number of trees for experimentation.

At full bloom, trees were inoculated with Ea153N. A lyophilized cell preparation ($\approx 1 \times 10^{11}$ CFU/g) of Ea153N was suspended in water in a 1-liter Nalgene bottle for 1 h prior to inoculation. Afterward, the rehydrated pathogen stock solution was added to the 100-liter tank of a motorized sprayer fitted with an agitator and a handgun. The pathogen suspension ($\approx 1 \times 10^5$ to 3×10^5 CFU/ml in pear trials and 3×10^6 CFU/ml in the apple trial) was applied as a mist at sunrise (1999 Medford trial and all 2000 trials) or after sunset (8:00 to 9:00 p.m. in the 1998 trial and 1999 pear trials in Corvallis) under still wind conditions, and spray volumes ranged from 2 to 3 liters per tree depending on tree size (Table 1). In 1998, a hard rain (9.4 mm) occurred after application of the pathogen on 9 April so the pathogen inoculation was repeated the following night. In 1999 and 2000 trials, the streptomycin and oxytetracycline treatments were repeated the day following inoculation of trees with *E. amylovora*.

Recovery of bacteria from blossoms. Open pear blossoms with black mature anthers were harvested after the second treatment application and periodically during bloom until symptoms of fire blight were visible (Table 1). Six blossoms of each replicate tree in each pear trial (except in 1998, eight blossoms) were collected and transported to the laboratory in the individual wells of 12-well sterile plastic plates (Corning Inc., Corning, NY). In the laboratory, the pistil and nectary of each pear blossom were excised and placed into test tubes containing 1 ml of sterile 10 mM potassium phosphate buffer, pH 7.1, and sonicated for 3 min. After sonication, 10 µl of the sample buffer and two 100-fold dilutions were spread onto *Pseudomonas* agar F (PAF, Difco Laboratories) with 50 µg of cycloheximide per ml for enumeration of Eh252 or PAF amended with cycloheximide and 50 µg of kanamycin per ml for selective recovery of 10:12 and CCT medium (7) containing 50 µg of nalidixic acid per ml for recovery of the pathogen. Eh252 was distinguished from indigenous bacteria isolated from blossoms on PAF by colony morphology. Eh252 has distinctive raised yellow mucoid colonies and indigenous strains of *P. agglomerans* are detected rarely on pear blossoms in Oregon (27). The detection limit was 1×10^2 CFU per blossom.

Disease assessment. Blossom clusters with symptoms of fire blight (necrosis, wilting, and/or bacterial ooze) were counted and removed immediately from trees over a 5-week period commencing at the first visible symptoms (Table 1). The cumulative number of fire blight strikes per tree was calculated.

Weather data. Weather data were obtained from AgriMet weather stations (Northwest Cooperative Agricultural Weather Network, Bonneville Power Administration, and the U.S. Bureau of Reclamation, Boise, ID) located within the experimental

Fig. 1. Population sizes of *Pantoea agglomerans* Eh252 (■) and its antibiotic-deficient mutant 10:12 (□) on pear blossoms in **A**, 1998 on cv. Bartlett, **B**, 1999 on cv. Bartlett, **C**, 1999 on cv. Bosc, **D**, 1999 on cv. Bartlett (Medford, OR), **E**, 2000 on cv. Bartlett, and **F**, 2000 on cv. Bosc. All trials were conducted in orchards near Corvallis, OR, except the trial depicted in panel **D**, which was conducted near Medford, OR. The bacteria were applied twice to trees at 30 and 70% bloom. Each point represents the mean log-transformed population size (CFU per blossom) of Eh252 or 10:12 recovered from individual blossoms after the second application of the bacteria. The vertical bars represent one standard error of the mean. The downward arrow represents the approximate day of 100% petal fall in each trial.

Fig. 2. Mean population size at 100% petal fall of *Pantoea agglomerans* Eh252 (■) and its antibiotic-deficient mutant 10:12 (□) from each pear trial regressed on **A**, mean maximal daily temperature (degree Celsius) and on **B**, proportion of days with rain during bloom. Weather in each trial was summarized from time of first application at 30% bloom to the sample date of 100% petal fall. The solid regression lines for Eh252 were statistically similar to the dashed regression lines for 10:12.

field laboratories near Corvallis and Medford. The data are available online.

Data analysis. Mean population size and standard error of bacterial strains on individual blossoms were calculated by averaging the logarithm (base 10) of population values obtained on each sample date. The data are presented with a population size of zero assigned to individual blossoms on which a strain was not detected. Additional statistical analyses were conducted with a value of 99 CFU per blossom (detection limit – 1 CFU) for nondetectable populations, but the statistical outcomes were not different than analyses conducted with the zero value for blossoms without detectable populations. The incidence of detection of populations 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 procedure of Statistical Analysis Systems (SAS Institute, Cary, NC).

To compare differences in growth of bacterial strains on blossoms or to evaluate the effect of treatments on growth of the pathogen, 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 following formula (24)

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

where y is the mean population size of a bacterial strain on the i th sample date and t is the corresponding sample time. Because the length of the sample period varied among trials, the AUPC was converted to the RAUPC by dividing the AUPC by the elapsed number of days during the sampling period (12). The LSD at $P = 0.05$ was used to separate mean RAUPC values obtained for each bacterial treatment of each trial.

The response of populations of *P. agglomerans* Eh252 and 10:12 to weather was evaluated with linear regression. Weather data were summarized from the day of first application of the bacteria to 100% petal fall by calculating the mean maximum daily temperature or the proportion of days with measurable rainfall for each trial (12). The RAUPC of Eh252 and 10:12 at 100% petal fall in each trial were regressed to the corresponding summarized weather data.

The number of diseased blossom clusters per treated tree was converted to a relative disease incidence by dividing the incidence of fire blight on treated trees by the incidence on the water-treated tree within each block. The relative disease incidence data were

TABLE 2. Relative area under the population curve (RAUPC) of Eh252 and 10:12 on pear blossoms

Year	Location	Cultivar	Average RAUPC ^z	
			Eh252	10:12
1998	Corvallis	Bartlett	5.45 a	5.22 a
1999	Corvallis	Bartlett	6.18 a	5.88 a
	Corvallis	Bosc	5.76 b	6.07 a
	Medford	Bartlett	6.78 a	6.91 a
2000	Corvallis	Bartlett	5.88 a	5.90 a
	Corvallis	Bosc	5.89 a	5.79 a
Pooled analyses			5.95 a	5.93 a

^z Trees arranged in a complete randomized block design with four or five replications per treatment were sprayed twice with 1×10^8 CFU/ml of Eh252 or 10:12 at 30 and 70% bloom. The RAUPC of a bacterium was calculated over all sample dates during the bloom period by the equation

$$RAUPC = \left[\sum_{i=1}^n (y_i + y_{i-1} / 2) \times (t_i - t_{i-1}) \right] / (t_n - t_1)$$

where y is the mean population size and t is the day of the sample. Means within a row followed by the same letter are not significantly different according to Fischer’s protected least significant difference at $P = 0.05$.

arcsine square root-transformed before analysis. For analysis of both individual experiments and pooled experiments, Fisher’s protected LSD test at $P = 0.05$ was used to separate treatment means of the transformed relative disease incidence values.

RESULTS

Population dynamics of Eh252 and 10:12 on pear blossoms.

In each experiment, after the initial treatments at 30 and 70% bloom, the incidence of recovery of both Eh252 and 10:12 ranged from 90 to 100% on treated blossoms. Eh252 and 10:12 each established mean population sizes that grew to 10^6 to 10^7 CFU per blossom (Fig. 1). Mean population sizes of Eh252 and 10:12 were greatest prior to 100% petal fall (Fig. 1). The highest mean population sizes were observed on cv. Bartlett in Medford in 1999 and the lowest was recorded on cv. Bartlett in 1998. Weather during early bloom correlated with population sizes established at petal fall. Positive correlations between mean maximal temperature and mean population size of each strain at petal fall were observed and the regression equations for each strain were similar (Fig. 2A). Conversely, a negative correlation existed between proportion of days with rain and mean population size at petal fall (Fig. 2B). Regression equations describing the relationship of the mean population size of each strain with the daily incidence of rainfall were similar for Eh252 and 10:12 (Fig. 2B).

In each trial, the population dynamics of 10:12 on pear blossoms were similar to those of the parental strain (Fig. 1). On occasional sampling dates, the mean population sizes of the strains differed statistically, but the difference was not consistent within or among trials. The RAUPC during the entire sampling

Fig. 3. Population sizes of *Erwinia amylovora* Ea153N on pear blossoms after treatment with water (Δ), Eh252 ($\blacksquare$), or the antibiotic-deficient mutant 10:12 ($\square$) at 30 and 70% bloom in **A**, 1998 on cv. Bartlett, **B**, 1999 on cv. Bartlett, **C**, 1999 on cv. Bosc, **D**, 1999 on cv. Bartlett (Medford, OR), **E**, 2000 on cv. Bartlett, and **F**, 2000 on cv. Bosc. All trials were conducted in orchards near Corvallis, OR, except the trial depicted in panel **D**, which was conducted near Medford, OR. Each point represents the mean log-transformed population size (CFU per blossom) of Ea153N recovered from individual blossoms after inoculation. The detection limit was 1×10^2 CFU per blossom. Nondetectable populations of the pathogen on individual blossoms were assigned a zero population size. The vertical bars represent one standard error of the mean.

period for Eh252 did not differ significantly from that of 10:12 in five of six trials (Table 2). On cv. Bosc blossoms in 1999, the RAUPC of 10:12 was significantly greater than that of Eh252. The similarity in growth patterns of Eh252 and 10:12 on blossoms over a range of environmental conditions support the hypothesis that the Tn5 insertion disrupting *mccEh252* biosynthesis was not detrimental to epiphytic fitness.

Populations of Ea153N on pear blossoms. A low cell density (3×10^5 CFU/ml) of Ea153N was applied to all trees at full bloom. For samples taken within 4 days of inoculation, population sizes of Ea153N on blossoms were generally near or below the limit of detection (1×10^2 CFU per blossom) (Fig. 3). The mean population size of the pathogen increased significantly on water-treated trees during bloom in all trials except cv. Bosc in 1999 (Fig. 3). Populations of Ea153N on individual blossoms greater than or equal to 1×10^5 CFU per blossom, a threshold population size correlated to infection of blossoms (11,22), were first recovered at the initiation of petal fall (data not shown). After this stage, approximately 20% of water-treated blossoms sampled among trials supported populations of Ea153N greater than or equal to 1×10^5 CFU per blossom (Table 3), 58% of water-treated blossoms sampled late in bloom had detectable populations of Ea153N of any size (i.e., $\geq 1 \times 10^2$ CFU per blossom) (Table 3).

In five of six trials, both Eh252 and strain 10:12 significantly reduced the population size of Ea153N on pear blossoms on at least one sample date (Fig. 3). The RAUPC for Ea153N on water-treated trees during late bloom ranged from 0.51 to 3.80, with a mean among trials of 2.46 (Table 3). Compared with water-treated trees, Eh252 significantly reduced the average RAUPC of Ea153N during late bloom in five of six trials (Table 3). Similarly, strain 10:12 significantly reduced the RAUPC of Ea153N in four of six trials (Table 3). Eh252 and 10:12 reduced the RAUPC of the pathogen to a similar degree in all trials, except on cv. Bartlett in Medford in 1999 where Eh252 was more effective than 10:12 in reducing the pathogen population size (Table 3). In an analysis of RAUPC data pooled over all trials, Eh252 and 10:12 were similar in their capacity to suppress growth of Ea153N (Table 3).

Eh252 significantly decreased the proportion of blossoms with detectable populations of Ea153N ($\geq 1 \times 10^2$) in late bloom in five of six trials compared with the incidence of establishment of the pathogen on water-treated trees (Table 3). Strain 10:12 also significantly reduced the incidence of detectable populations of the pathogen in two trials (Table 3). Over all trials, Eh252 significantly reduced the incidence of establishment of detectable populations of the pathogen by 62% compared with the incidence on water-treated trees. Strain 10:12 reduced the incidence of establishment of Ea153N on blossom by 40% over all trials, but this reduction was not significant statistically (Table 3).

When compared with water treatment, Eh252 and 10:12 reduced the proportion of blossoms during late bloom that supported populations of Ea153N greater than or equal to 1×10^5 CFU per blossom in four of six and three of six trials, respectively (Table 3). Eh252 was superior to 10:12 in reducing the incidence of blossoms with populations of Ea153N greater than or equal to 1×10^5 CFU per blossom in only one field trial (cv. Bartlett, Medford 1999) (Table 3). Over all trials, Eh252 reduced the incidence of blossoms supporting high populations by 65% compared with water-treated controls, whereas 10:12 reduced this incidence by 50% (Table 3).

Suppression of fire blight by *P. agglomerans* and commercial antibiotics. Symptoms of fire blight were observed in all experimental plots. The mean number of diseased blossom clusters on inoculated water-treated trees varied among trials from a low of 29 strikes per tree on cv. Bosc in 1999 to a high of 204 strikes per tree on cv. Bosc in 2000 (Table 4). Treatment of trees with Eh252 significantly reduced the incidence of fire blight compared with water-treated trees in all trials except cv. Bosc in 1999 (Table 4). The efficacy of Eh252 varied among trials (Table 4). On cv. Bartlett in Medford in 1999, Eh252 reduced the incidence of fire blight by 88%. In the trials on pear in 2000, Eh252 reduced the incidence of fire blight by 41 and 49% (Table 4). Averaged among trials, Eh252 provided 55% reduction in the incidence of fire blight compared with water-treated trees. The level of disease suppression by Eh252 did not differ statistically from treatment with streptomycin in four of seven field trials but, when analyzed

TABLE 3. Influence of *Pantoea agglomerans* Eh252 and the antibiotic-deficient mutant 10:12 on populations of *Erwinia amylovora* Ea153N on blossoms

Year	Location	Cultivar	Average RAUPC ^x			Incidence of populations of Ea153N $\geq 1 \times 10^2$ CFU per blossom ^y			Incidence of populations of Ea153N $\geq 1 \times 10^5$ CFU per blossom ^z		
			Treatment			Treatment			Treatment		
			Water	Eh252	10:12	Water	Eh252	10:12	Water	Eh252	10:12
1998	Corvallis	Bartlett	3.80 a	2.37 b	3.02 ab	1.00 a	0.68 b	1.00 a	0.27 a	0.14 a	0.16 a
1999	Corvallis	Bartlett	3.00 a	1.54 b	1.48 b	0.33 a	0.15 b	0.17 b	0.31 a	0.14 b	0.16 b
	Corvallis	Bosc	0.51 a	1.01 a	0.22 a	0.20 a	0.20 a	0.04 a	0.01 a	0.01 a	0.03 a
	Medford	Bartlett	2.55 a	0.12 c	1.45 b	0.58 a	0.04 b	0.46 a	0.32 a	0.00 c	0.11 b
2000	Corvallis	Bartlett	1.81 a	0.53 b	0.81 b	0.50 a	0.07 b	0.13 ab	0.16 a	0.07 b	0.08 ab
	Corvallis	Bosc	3.20 a	1.29 b	1.34 b	0.83 a	0.33 b	0.30 b	0.24 a	0.07 b	0.10 b
Pooled analyses			2.46 a	1.17 b	1.38 b	0.58 a	0.22 b	0.35 ab	0.20 a	0.07 b	0.10 b

^x Trees were arranged in a complete randomized block design with four or five replications per treatment and sprayed twice with water or 1×10^8 CFU/ml of Eh252 or 10:12 at 30 and 70% bloom. At full bloom, the pathogen Ea153N was applied at 3×10^5 CFU/ml. The average relative area under the population curve (RAUPC) during late bloom was calculated from mean populations of Ea153N in the final three samples taken during the bloom period by the equation

$$\text{RAUPC} = \left[\sum_{i=1}^n (y_i + y_{i-1} / 2) \times (t_i - t_{i-1}) \right] / (t_n - t_1)$$

where y is the mean population size and t is the day of the sample. Mean RAUPC values within a row followed by the same letter are not significantly different according to Fischer's protected least significant difference at $P = 0.05$.

^y Mean incidence of populations of Ea153N greater than or equal to 1×10^2 CFU per blossom (the detection limit) was calculated from individual blossoms during late bloom. Mean values of populations of Ea153N greater than or equal to 1×10^2 CFU per blossom within a row followed by the same letter are not significantly different according to Fischer's protected least significant difference on the arcsine square root-transformed population incidence data at $P = 0.05$.

^z Mean incidence of populations of Ea153N greater than or equal to 1×10^5 CFU per blossom was calculated from individual blossoms pooled over the final three samples taken during bloom. Mean values of populations of Ea153N greater than or equal to 1×10^5 CFU per blossom within a row followed by the same letter are not significantly different according to Fischer's protected least significant difference on the arcsine square root-transformed population incidence data at $P = 0.05$.

as pooled experiments, streptomycin afforded greater control of fire blight than Eh252 (Table 4).

The non-antibiotic producing mutant 10:12 also reduced the incidence of fire blight compared with water-treated trees in four of seven experiments (Table 4). The suppression of fire blight by 10:12 was significantly less than that obtained with the parental strain Eh252 in three of seven trials. Analysis of pooled data showed that 10:12 reduced the incidence of fire blight by an average of 30%. This level of disease control exceeded that obtained with oxytetracycline treatments but was less than that obtained with the antibiotic-producing parental strain Eh252 or streptomycin (Table 4).

DISCUSSION

The capacity to produce an antibiotic contributed to the suppression of fire blight by the biocontrol agent *P. agglomerans* Eh252. In seven field experiments analyzed together, strain Eh252 was more effective than its antibiotic-deficient derivative 10:12 in suppressing fire blight. These data provide evidence that Eh252 produced the antibiotic mceEh252 on blossoms in a timely manner and in sufficient quantities to affect pathogenesis by *E. amylovora* in orchard environments. Although a role for antibiosis in biological control was demonstrated in this study, mceEh252 was not the sole factor involved in suppression of fire blight by strain Eh252. The antibiotic-deficient mutant strain 10:12 was generally less effective than Eh252 in suppressing fire blight, but it significantly reduced the incidence of fire blight in four of seven field trials when compared with water-treated trees. Therefore, other mechanisms such as competitive exclusion or modification of the blossom habitat are also likely to contribute to the suppression of fire blight by Eh252. Strain Eh252 colonizes similar sites on apple stigmas as the pathogen and was proposed to exclude *E. amylovora* from sites essential for growth (5). *P. agglomerans* may modify habitats or compete for nutrients in sites colonized by *E. amylovora* on plant surfaces (14,29,35,38,41). Additional studies evaluating competition between *P. agglomerans* 10:12 and *E. amylovora* on stigmatic surfaces are needed to assess the roles of competitive exclusion or habitat modification in biological control of fire blight.

Taken together, the data from seven field experiments support the hypothesis that the antibiotic mceEh252 contributes to suppression of fire blight by Eh252 in the orchard environment. Nevertheless, the efficacy of Eh252 and the antibiotic-deficient derivative 10:12 did not differ significantly in four of the seven experiments evaluated independently. Previously in immature pear fruit assays, both Eh252 and 10:12 suppressed fire blight symptoms when large populations of these strains were applied to tissues; antibiosis enhanced disease control only when low populations of these strains were applied (29,35). In the current study on pear blossoms in orchard environments, differential efficacy

between Eh252 and 10:12 was not contingent on population sizes of the antagonist on blossoms (Tables 1 and 4). Instead, in the three trials where control of fire blight was significantly greater with Eh252 than that obtained with 10:12, we noted that the RAUPC of the pathogen on water-treated trees was higher than in other experiments (Table 3). We speculate that rapidly growing populations of the pathogen may be more susceptible to mceEh252. Alternatively, mceEh252 may not be produced uniformly in the diverse field environments evaluated in this study. Future studies evaluating strains of *P. agglomerans* containing reporter gene fusions to antibiotic biosynthesis genes may provide useful information on the factors affecting the temporal and spatial production of antibiotics on blossoms. Knowledge of the stability of mceEh252 on blossoms exposed to the environment and to indigenous microflora may be key to understanding the role of antibiosis in biological control by *P. agglomerans*.

A mutant with an insertion disrupting antibiotic biosynthesis in a biocontrol agent is a useful tool to assess the importance of antibiosis in disease control as long as the mutation does not affect other factors contributing to disease suppression, such as epiphytic fitness. Initially, strain 10:12 was chosen for our experiments because it has a stable Tn5 insertion in a chromosomal gene essential for the production of mceEh252 (32,33,35,36), because its growth rate in culture and on immature pear fruit slices was similar to the wild-type strain Eh252 (35), and complementation of mutants restored antibiosis and disease suppression in immature pear fruit assays (33,37). Furthermore, no polar or indirect effects due to the inserted transposon in 10:12 have been reported. In all pear orchard trials, no evidence was collected to indicate that the epiphytic fitness of 10:12 on the pistillate tissues of pear blossoms was compromised by the Tn5 insertion. Consequently, differences in suppression of fire blight by Eh252 and 10:12 are likely due to differences in antibiosis rather than in colonization of floral tissues.

Following its application to apple and pear blossoms, *P. agglomerans* typically establishes population sizes of 10^4 to 10^6 CFU per blossom in the field (11–13,22,23,25,26). In this study, Eh252 and 10:12 established population sizes within that range, 10^5 to 10^7 CFU per blossom (Fig. 1). The growth dynamics of Eh252 and 10:12 on blossoms was remarkably similar to that of another strain we have studied, *P. agglomerans* C9-1 (9,11,12,22,25,26). Similar to C9-1 (12), population sizes of Eh252 and 10:12 near petal fall were positively correlated with mean temperature and negatively correlated with the incidence of rain during bloom (Fig. 2). As we noted previously (12), low temperatures and frequent rain events are often coincident in Oregon during the spring; thus, these environmental factors should not be considered independently. Models describing the influence of weather on growth and establishment of C9-1 are being developed to optimize the timing of antagonist applications to ensure adequate colonization of blossoms (9). Due to the similarities noted between C9-1 and Eh252,

TABLE 4. Relative incidence of fire blight on trees treated with water, Eh252 or its antibiotic-deficient derivative 10:12, or commercial antibiotics^y

Treatment ^z	1998		1999		2000			Pooled data
	Bartlett	Bartlett	Bosc	Bartlett (Medford)	Bartlett	Bosc	Rome Beauty	
Water	1.00 a (188)	1.00 a (160)	1.00 a (29)	1.00 a (63)	1.00 a (166)	1.00 a (204)	1.00 a (50)	1.00 a
Eh252	0.32 c	0.36 bc	0.88 a	0.12 d	0.59 b	0.51 b	0.16 c	0.45 d
10:12	0.65 b	0.48 b	0.90 a	0.31 c	0.78 ab	0.98 a	0.50 bc	0.70 c
Oxytetracycline	1.65 a	0.51 b	NT	0.62 b	0.69 b	NT	0.66 b	0.84 b
Streptomycin	0.41 c	0.12 c	0.54 b	0.06 d	0.17 c	0.08 c	0.32 bc	0.25 e

^y Experiments were conducted in experimental pear (cvs. Bartlett or Bosc) and apple (cv. Rome Beauty) orchards located near Corvallis, OR, except cv. Bartlett (pear) trial in 1999 that was located in Medford, OR, which is indicated in parentheses. 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 according to Fischer's protected least significant difference at $P = 0.05$. Numbers in parentheses are the average number of fire blight strikes on water-treated trees. NT = not tested.

^z Trees, arranged in a complete randomized block design with four or five replicates per treatment, were sprayed twice with water, Eh252, 10:12, oxytetracycline, or streptomycin at 30 and 70% bloom. At full bloom, the pathogen Ea153N was applied at 3×10^5 CFU/ml. In all trials, except in 1998, antibiotic treatments were repeated within 36 h after inoculation with the streptomycin- and oxytetracycline-sensitive pathogen *Erwinia amylovora* 153N.

we speculate that models describing the influence of the environment on growth of *P. agglomerans* C9-1 will be applicable to the species rather than limited to a single strain.

Epiphytic growth of *E. amylovora* on blossoms is an indicator of subsequent disease severity. In our earlier studies, the proportion of blossoms supporting populations of *E. amylovora* greater than or equal to 1×10^5 CFU per blossom and the incidence of diseased blossom clusters were correlated (11,22). This observation has guided biocontrol research on fire blight to identify treatments that suppress the epiphytic population development of *E. amylovora* on blossoms (e.g., treatments that reduce the number of blossoms with populations of *E. amylovora* greater than or equal to 10^5 CFU per blossom). In this study, we found that, compared with water treatment, both Eh252 and 10:12 significantly reduced the mean population size of Ea153N on pear blossoms, the RAUPC of the pathogen during late bloom, and the proportion of blossoms supporting high populations of Ea153N. Eh252 also significantly decreased the incidence of detectable populations of the pathogen ($\geq 10^2$ CFU per blossom) compared with that on water-treated trees in late bloom, but the reduction was not significantly different from treatment with 10:12 over all trials (Table 3). We speculate that our sample size of six to eight blossoms per tree may not have been adequate to detect significant differences in population sizes of the pathogen in some trials in which differences in disease severity were detected. Because both Eh252 and 10:12 suppressed populations of *E. amylovora* on blossoms, the present study neither demonstrates a clear role for mceEh252 production in suppression of epiphytic populations of the pathogen on blossoms nor excludes a role for mceEh252 in subsequent steps of the disease process.

Strain Eh252 was an effective biocontrol agent for fire blight in Oregon, reducing the incidence of fire blight on pear and apple by an average of 55% compared with water-treated trees (Table 4). Disease suppression provided by Eh252 varied from 12 to 88% among trials, but control was significant in six of seven trials (Table 4). The range of disease suppression reported here is similar to the efficacy of Eh252 and other strains of *P. agglomerans* reported previously. Eh252 reduced the incidence of fire blight on apple and Asian pear by 40 to 60% in trials conducted in New York, France, and New Zealand (1,29–31,34). Strains EhWHF18, EhWHF9, EhNL18, and EhNL19 reduced fire blight of Perry pear in orchards by 15 to 52% (21). Eh1087 reduced fire blight of apple in a glasshouse trial by 70 to 80% (13). Eh318 provided a 65% reduction in fire blight in an apple orchard (6). C9-1 reduced the incidence of fire blight on apple by 33 to 48% in trials conducted over 2 years (20). Clearly, treatment of blossoms with biocontrol strains of *P. agglomerans* results in suppression of fire blight rather than absolute control of the disease. Nevertheless, disease suppression by Eh252 exceeded that provided by oxytetracycline and was similar to that provided by streptomycin in several of the field trials described in this study (Table 4). Therefore, we propose that biological agents such as *P. agglomerans* Eh252 provide useful alternatives or complements to antibiotics for management of fire blight, especially in areas where streptomycin has lost effectiveness due to resistance of the pathogen (17–19,28).

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