Antibiosis and Acidification by *Pantoea agglomerans* Strain E325 May Contribute to Suppression of *Erwinia amylovora*

P. L. Pusey, V. O. Stockwell, and D. R. Rudell

First and third authors: U.S. Department of Agriculture-Agricultural Research Service, Tree Fruit Research Laboratory, Wenatchee, WA 98801; and second author: Department of Botany and Plant Pathology, Oregon State University, Corvallis 97330.
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ABSTRACT

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Pantoea agglomerans strain E325, a commercially available antagonist for fire blight of apple and pear, was originally selected through screening based on suppression of *Erwinia amylovora* on flower stigmas, but specific mechanisms of antagonism were unknown. Bacterial modification of pH was evaluated as a possible mechanism by analyzing stigma exudates extracted from 'Gala' apple stigmas. The pH values for field samples were only slightly lower than controls, but indicated a range (pH 5 to 6) conducive for antibiotic activity according to subsequent assays. Under low-phosphate and low-pH conditions, an antibacterial product of E325 with high specificity to *E. amylovora* was effective at low concen-

trations. A minimum of 20 to 40 ng of a ninhydrin-reactive compound purified using RP-HPLC caused visible inhibition in assays. Activity was heat stable and unaffected by amino acids, iron, or enzymes known to affect antibiotics of *P. agglomerans*. Antibiosis was diminished, however, under basic conditions, and with increasing phosphate concentrations at pH 6 and 7. Inhibition was not observed in media containing phosphate concentrations commonly used in antibiosis assays. We propose that E325 suppresses the fire blight pathogen not only by competing for nutrients on the stigma, but by producing an antibiotic specific to *E. amylovora*. Further work is necessary to substantiate that the compound is produced and active on flower stigmas.

Additional keywords: *Malus pumila*.

Fire blight, a major disease of apple and pear trees, is most commonly initiated by epiphytic populations of *Erwinia amylovora* residing on blossoms (42,44). Under dry climatic conditions, bacteria colonize flower stigmas, and subsequent rain or heavy dew facilitates movement to the floral cup (hypanthium) where infection generally occurs (41). Management programs have emphasized risk assessment (6) and suppression of *E. amylovora* on floral parts with antibiotics (34). The reliance on streptomycin, in particular, led to the development of pathogen resistance in many production areas (24,26).

Suppression of *E. amylovora* on flowers with microbial antagonists is a viable alternative (16,17) or complementary measure to antibiotics (23,39). In 1996 *Pseudomonas fluorescens* strain A506, initially selected based on its inhibition of an ice-nucleating strain of *P. syringae* on corn leaves (22), became the first commercially available antagonist for fire blight management (BlightBan A506; Nufarm Americas Inc., Burr Ridge, IL) (16). Products consisting of strains of *Pantoea agglomerans* (syn. *E. herbicola*) include one in commercial use in New Zealand since 2001 (45) and two others recently approved in North America (8,9,37).

Methods used for screening and selection of microbial antagonists of *E. amylovora* have evolved from the use of artificial media (3,38) to laboratory procedures with immature pear fruit (4,47,52) and later to bioassays involving detached flowers (27,29). Media- and fruit-based assays have been useful for identifying and studying antagonist strains that produce anti-

biotics inhibitory to *E. amylovora*, but results often do not correlate with suppression of the pathogen on blossoms (48,49). Direct evaluation of microbial strains on flowers, though more labor intensive than other methods, is more likely to identify antagonists effective on floral surfaces (2,17).

The selection of *P. agglomerans* strain E325, the active ingredient in Bloomtime Biological (Northwest Agricultural Products, Pasco, WA), was selected from more than 1,000 isolates of bacteria and yeasts collected from apple and pear blossoms and evaluated on flowers for potential use in fire blight management (P. L. Pusey, *unpublished data* [30]). In screening assays with detached crab apple blossoms (29), strain E325 showed exceptional suppressive activity toward *E. amylovora* on stigmatic surfaces and later proved effective in reducing blossom blight on mature apple trees (31). Like other microbial strains studied (17), E325 is an excellent colonizer of the stigma (31,32) and likely competes with the pathogen for space and nutrients. Given its high efficacy on flowers, we suspected antibiotic production as another mechanism of antagonism, but detection was elusive. When testing for antibiosis using methods and media of other researchers, inhibition zones either did not appear, or their appearance could not be reproduced in assays. To investigate mechanisms of strain E325 and other antagonists on flower stigmas, stigma exudates from pomaceous flowers were chemically analyzed (33), and free sugars and amino acids detected in these analyses were incorporated into a partial stigma-based medium (PSBM). The phosphate-buffer concentration in PSBM was relatively low, and suppression of *E. amylovora* by strain E325 was correlated with acidification of the medium and the presence of inhibition zones on solid PSBM (*unpublished data*).

Early fire blight researchers proposed that bacteria fitting the description of *P. agglomerans* inhibited *E. amylovora* on apple and pear trees in the orchard environment by producing acid (11,12), and Riggle and Klos (35) suggested the acidification

Corresponding author: P. L. Pusey; E-mail address: larry.pusey@ars.usda.gov

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occurs on blossoms. Wodzinski et al. (51) found that strains of *P. agglomerans* differing in ability to protect pear fruit from infection by *E. amylovora* did not cause a corresponding effect on pH in fruit tissue or in a complex medium, and thus, concluded that acid production is an unlikely mechanism of inhibition in planta. At present, the most important mechanisms of antagonism on blossoms are thought to be competitive exclusion and antibiotic production (17,43).

The following describes recent efforts to evaluate pH changes on flower stigmas inoculated with strain E325, and to partially characterize an inhibitory product of this antagonist active against *E. amylovora* under low-phosphate and low-pH conditions.

MATERIALS AND METHODS

Bacterial strains. *P. agglomerans* strain E325 was isolated from 'Gala' apple blossoms near Wenatchee, WA, in 1994 (29). The primary pathogen used was *E. amylovora* strain Ea153, obtained from K. Johnson (Oregon State University, Corvallis). It was originally isolated from cankers of Gala apple in Oregon and later used in studies with apple and pear (18,19). Both antagonist and pathogen are spontaneous antibiotic-resistant derivatives generated at the source laboratories. Strain E325 is resistant to rifampicin and streptomycin, and strain Ea153 is resistant to nalidixic acid. Original strain codes (i.e., without added letters indicating specific derivatives) are used in this article. Other bacteria were used to test the activity spectrum of an antibiotic product of E325. Those that lacked established identification in the literature, were identified to species by at least two methods: (i) fatty acid methyl ester analysis with the Microbial Identification System (Microbial ID, Inc., Newark, DE) and (ii) 16S rRNA gene sequence analysis (1). The latter method involved reaction conditions described by Tewoldemedhin et al. (40), analysis with a CEQ 8000 Genetic Analysis System (Beckman Coulter, Fullerton, CA), and BLAST searches on GenBank.

Bacteria were generally stored on silica gel at -20°C (36) and cultured initially on nutrient yeast dextrose agar (NYDA; 8 g of nutrient broth, 5 g of yeast extract, 5 g of dextrose, and 15 g of agar in 1 liter of deionized water) for 24 h at 24°C . Cell suspensions were prepared from these cultures for inoculating flower stigmas or an agar medium used for producing and detecting the E325 antibiotic. For stigma inoculations, suspensions were adjusted to 0.1 optical density at 600 nm using a spectrophotometer (Spectronic 20; Milton Roy Co., Rochester NY) to approximate a cell density of 1×10^8 colony forming units (CFU)/ml. This concentration or a one-tenth dilution was used in the experiments.

Evaluation of pH on inoculated stigmas. To study the effect of *P. agglomerans* E325 on the pH of blossom stigmas, exudates were extracted from inoculated stigmas of Gala apple (*Malus pumila*) and evaluated for pH. On three dates in 2005 (26, 27, and 28 April), 150 newly opened flowers were detached from trees located in a research orchard at Columbia View, north of Wenatchee, WA. The cut end of each flower pedicel was submerged in 10% sucrose contained in a 2-ml vial (29). Holding vials were supported in plastic tube racks inside 4-liter plastic containers, which were transported to the laboratory. One group of 50 flowers was inoculated on stigmas with E325 by applying 1 to 2 μl of suspension (10^8 CFU/ml in 0.03% Tween-20 and 0.01 M phosphate buffer, pH 7) per flower by touching a droplet to each stigma (normally five per flower) to form a thin film of moisture. A second group of 50 was likewise inoculated with *E. amylovora* Ea153, and the third group was not inoculated. After 24 h at 24°C and 90% relative humidity, petals and anthers were removed, and stigma tips of each group of 50 flowers were submerged, one flower at a time, in 600 μl of water in a microfuge tube, while sonicated for 10 s per flower. Pollen, debris, and microorganisms were removed from the aqueous solution by

centrifugation at $16,000 \times g$ for 5 min and by subsequent filtration of supernatant in syringe-driven unit with polyvinylidene difluoride (PVDF) membrane, 4-mm diameter and 0.22- μm pore size (Millex-GV; Millipore Corp., Billerica, MA). The pH was measured in each 600- μl sample representing 50 flowers. To evaluate pH in exudate concentrations more comparable to those in situ, samples were dried in a vacuum evaporator and redissolved in reduced volumes of water to increase concentrations by 10 and 50 $\times$. The pH in these low volumes (60 and 12 μl) was measured with an Accumet AP63 pH meter (Fisher Scientific, Pittsburgh, PA) using electrodes designed for use in a minimum liquid depth of 3 mm (Accumet Combination MicroProbe, 13-620-96; Fisher Scientific) or 1 mm (Orion 98-10, Micro-Combination Electrode; Thermo Electron Corp., Beverly, MA).

To evaluate whether bacteria alter stigma pH in the field, an experiment was conducted with Gala apple at Columbia View in 2006 and 2007. Branches were tagged for use, and new flowers were isolated by removing all opened flowers on 1 day and removing all unopened flowers the following day, leaving a minimum of 120 new flowers per tree. Inoculations were made on two dates, i.e., when flowers were newly opened and 2 days later (27 and 29 April 2006; 24 and 26 April 2007), by applying suspensions of *P. agglomerans* E325 (10^8 CFU/ml) or *E. amylovora* Ea153 (10^7 CFU/ml) in 0.03% Tween-20 to stigmas with an artist paint brush. Flowers were inoculated late in the day, between 1730 and 1930 h, to limit exposure to sunlight during early bacterial establishment. One exception to this was inoculation between 1500 and 1600 h on 26 April 2007 when the sky was overcast. Treatments were: (i) noninoculated control, (ii) Ea153 applied first date, (iii) Ea153 applied second date, (iv) E325 applied first date, and (v) E325 applied first date followed by Ea153 on second date. Four single-tree replicates were used per treatment in a completely randomized block in 2006, and six replicates were used in 2007.

Flowers were sampled for pH measurement and for estimation of bacterial population sizes just prior to treatment on the second inoculation date, then again 2 and 4 days later. For each treatment on each tree, 20 flowers were sampled for pH measurement and 10 flowers were sampled for estimating population sizes. Stigma exudates were extracted as already described, except 20 rather than 50 flowers were combined per 600- μl sample, and samples were concentrated by 50 $\times$ prior to pH measurement. To determine population size, stigmas of individual flowers were placed, along with stylar tissue, in sterile microcentrifuge tubes containing 1 ml of sterile buffer (10 mM potassium phosphate, pH 7). Tubes were vortexed briefly and placed in a sonication bath for 60 s. Samples were again vortexed, and serial dilutions spread on Kings medium B (21) amended with rifampicin (25 $\mu\text{g}/\text{ml}$) and cycloheximide (50 $\mu\text{g}/\text{ml}$) for detection of strain E325 and on CCT medium (defined by Ishimaru and Klos, 13) amended with nalidixic acid (100 $\mu\text{g}/\text{ml}$) for detection of strain Ea153. Dilutions were also spread on NYDA amended with cycloheximide (50 $\mu\text{g}/\text{ml}$) to enumerate indigenous culturable bacteria.

Weather data within the orchard were collected with a WatchDog Data Logger (Model 450; Plainfield, IL). The pH data were subjected to analysis of variance using SAS version 9.1 (SAS Institute, Cary, NC) and means were separated according to least significant difference (LSD) test ($P \leq 0.05$). A value of zero was assigned to bacterial populations below the detection limit of 10^2 CFU/flower. Population sizes for each single-tree replicate were averaged before calculating mean and standard error.

Production and assay of antibacterial compound. Observation of inhibition zones on solid PSBM, a medium partially based on sugars and amino acids identified in stigma exudates (33), were followed by attempts to characterize the inhibitory substance. Ingredients of PSBM by weight in 1 liter of water were: 25 g of glucose, 25 g of fructose, 0.2 g of amino acid mix (proline, asparagine, glutamine, and serine in ratio of 3:2:2:1),

0.8 g of NH_4Cl , 0.3 g of K_2HPO_4 , 0.1 g of NaH_2PO_4 , 0.4 g of MgSO_4 , 1.2 mg of nicotinic acid, and 15 g of agar (from Sigma-Aldrich; St. Louis, MO). To produce the active compound, a 0.1-ml suspension of *P. agglomerans* E325 (10^8 CFU/ml in water prepared from NYDA culture) was spread over the entire surface of PSBM in a single plate (25 ml of medium per standard 9-cm petri plate). After incubation for 24 h at 28°C, growth was removed with a razor blade and the agar from one plate was transferred as sections to a 125-ml flask containing 20 ml of water. Noninoculated PSBM agar was similarly transferred as a negative control. The flask was placed on a rotary shaker at 80 rpm for 1 h, and the liquid decanted. The 20-ml water extraction of agar was repeated twice, and the decanted liquid (≈ 60 ml) was pooled and filter sterilized. For partial purification and concentration, 50 ml of crude filtrate was adsorbed onto a C-18 Plus Sep-pak (Waters, Millford, MA), the column was rinsed with 20 ml of water followed by 20 ml 5% MeOH, then the active component was eluted with 20 ml 20% MeOH. The active fraction was dried in a rotary evaporator after adding 50 ml of isopropyl alcohol, then reconstituted in 500 μl water. The crude extract and partially purified material was stored at 4°C for up to 7 days or at -20°C for periods up to 8 weeks prior to characterization tests or further purification. Activity assays were performed by pipetting 10 to 12 μl of a sample into wells (1.5-mm diameter) in PSBM. After the aqueous sample diffused into the agar (15 to 45 min), the medium was overlaid with 4 ml of soft PSBM containing 0.8% agar inoculated with 100 μl of $\approx 10^9$ CFU/ml of *E. amylovora* Ea153 (from NYDA culture). The plates were incubated overnight at 28°C and then examined for inhibition zones surrounding wells.

Characterization of antimicrobial activity. Crude aqueous agar extract was subjected to varying temperatures, pH values, and phosphate concentrations before assaying for antibacterial activity as described previously. To test heat stability, the extract was placed in microcentrifuge tubes (500 μl per tube) and exposed for 1 h to 85, 95, 105, and 115°C, or for 72 h to 25, 30, 37, 50, and 70°C. An oven or incubator was used depending on heat treatment, and control samples were held at 4°C. To test pH sensitivity, the crude extract, with a pH between 3.5 and 4.0, was adjusted to pH values ranging from 2 to 11 with 1 M HCl or 1 M NaOH, then held for 24 h at 20°C before adjusting to pH 7 and performing the inhibition assay. The effect of phosphate was evaluated by preparing K_2HPO_4 and NaH_2PO_4 in varying proportions and concentrations and combining the buffer solutions with crude extract (1:1, vol/vol). Phosphate levels, thus, were varied from 0 to 96 mM at pH 5, 6, and 7. Samples representing each phosphate/pH treatment were held at 28°C for 1, 6, and 18 h prior to assaying for activity.

Sensitivity of antibacterial activity to amino acids, enzymes, and iron was determined by incubating these substances with the partially purified E325 product (solutions of each were combined 1:1) and then assaying for activity as already described. Each of 20 standard amino acids plus cysteine and hydroxyproline (Amino Acid Standard Kit 22; Pierce, Rockford, IL) were incubated with the antibacterial compound at a concentration of 10 mM for 2 h at 28°C. The following enzymes from Sigma-Aldrich were used: β -amylase II-B (Catalog No. A7130), bromelain (No. B4882), chymotrypsin (No. C4129), lipase (No. L3001), papain (No. P4762), penicillinase IV (No. P4524), proteinase K (No. P6556), pepsin (No. P7000), and trypsin (No. T303). Solutions of enzymes were prepared with consideration to approximate molecular weight and mass of antibacterial compound and parameter specifications of the manufacturer. Thus, all enzymes were prepared at 1 mg/ml in partially purified antibiotic solution, except papain was prepared at 10 mg/ml. The pH was adjusted to 5.0 with 24 mM phosphate buffer, and solutions were incubated for 1.0 h at 28°C, with the exception of lipase, which was incubated with antibiotic sample at 37°C. The effect of iron

on antibacterial activity was tested by incorporating FeCl_3 at 10 mM and incubating for 2 h at 28°C.

The antimicrobial spectrum of the partially purified E325 compound was evaluated by testing the sensitivity of 73 microbial isolates representing 10 genera (*Erwinia*, *Pectobacterium*, *Pantoea*, *Enterobacter*, *Escherichia*, *Klebsiella*, *Burkholderia*, *Pseudomonas*, *Bacillus*, and *Microbacterium*). To prepare inocula, each strain was initially cultured in 4 ml of nutrient yeast dextrose broth (same as NYDA, but without agar) at 28°C and 125 rpm for 24 h. Assay plates were prepared by cutting eight wells in PSBM agar spaced approximately 1.0 cm apart in a line passing through the plate center. A 10- μl volume of the partially purified antibacterial product of E325 (obtained using C-18 Plus Sep-pak as already described) was added to each well and allowed to diffuse into the agar. A 1:10 dilution of each test strain was then transferred with loop and spread in a line (>5.0 cm long) perpendicular to the row of eight wells and passing directly over one of the wells. *E. amylovora* Ea153 and *P. fluorescens* A506 were included on each plate as positive and negative controls, respectively. Plates were incubated at 28°C for 48 to 72 h and then evaluated for inhibition zones extending outward from the wells.

Further purification and characterization of antibacterial compound. For preparative high-performance liquid chromatography (HPLC), 100 μl of partially pure extract was injected into a Waters modular HPLC (Milliford, MA) equipped with two Waters 510 binary pumps, a Zorbax ODS (9.4 $\times$ 250mm) column, a Waters TCM column heater, and a Waters automated fraction collector. Elution solvents used for a linear gradient were (A) methanol/water/formic acid (50:49.8:0.2, vol/vol) and (B) methanol/water/formic acid (99.8:0.2, vol/vol). The flow rate was maintained at 2 ml/min and the column temperature was 30°C. Following injection, solvent A comprised the entire flow for 2 min followed by a linear increase of solvent B until 22 min where it was held for 8 min. One-milliliter fractions were collected within the retention time range of detected activity, i.e., from 10 to 25 min. Activity of the fractions was evaluated and dilution end point of the most active fraction determined by bioassay on PSBM, as already described, prior to characterization using high-performance liquid chromatography-UV/visible-mass spectrometry and analytical thin-layer chromatography (TLC).

Ten-microliter samples of purified fractions were injected into a Series 1100 HPLC system (Agilent Technologies, Palo Alto, CA) equipped with a 5- μm Agilent Hypersil ODS (4.0 $\times$ 125 mm) reverse-phase column, a G1315B diode array detector (DAD), and a G1946D mass selective detector (MSD), coupled with an atmospheric pressure chemical ionization (APCI) source. Elution solvents used for the linear gradient were (A) methanol/water/formic acid (0:99.8:0.2, vol/vol) and (B) methanol/water/formic acid (50:49.8:0.2, vol/vol). The column temperature and mobile phase flow rate were 20°C and 0.5 ml/min, respectively. The mobile phase was comprised entirely of solvent A for the initial 2 min after sample injection, followed by a linear gradient of solvent A plus B until reaching 100% B at 20 min, and then remaining entirely solvent B until 25 min. The DAD continuously monitored and recorded spectra (200 to 700 nm) for the entire analysis. The APCI spray chamber conditions were drying gas (N_2) flow 5 L/min, drying gas temperature 350°C, nebulizer pressure 414 kPa, vaporizer temperature 425°C, and coronal discharge 4 μA . The fragmentor and capillary potentials were 130 and 4,000 V, respectively. The MSD was adjusted to monitor positive ions in the scanning mode, continuously monitoring and recording entire mass spectra within a 100 to 2,500 m/z range.

For TLC analysis, lyophilized partially purified extract was dissolved in 1 ml 90% methanol, and a 10- μl aliquot was adsorbed on Whatman LHPK silica gel 60A plates. Chromatographic separation was carried out in chloroform/methanol/acetic acid/water (65:25:3:4, vol/vol) until the solvent front reached 8 cm. Components were visualized using ninhydrin

reagent, which consisted of 25 ml 0.2% (wt/vol) ninhydrin in 1-butanol added to 1 ml 10% (vol/vol) acetic acid in water. Plates sprayed with ninhydrin reagent were dried for 2 min at 120°C. Components also were tested for pentoses using Orcin reagent, prepared by dissolving 0.1 g of 3,5-dihydroxytoluene and 0.05 g of FeCl₃ in 50 ml of 12N HCl. Plates were sprayed with Orcin reagent and dried for 5 min at 150°C.

RESULTS

Evaluation of pH on inoculated stigmas. When stigma exudates were extracted from Gala apple blossoms inoculated in the laboratory on different dates, pH values for extracts were always numerically highest for flowers inoculated with *E. amylovora* strain Ea153 and lowest for flowers inoculated with *P. agglomerans* strain E325. Reduction of extract concentration to volumes that are closer to the original fluid volumes on stigmas revealed even greater differences in pH. On each date, pH values for the Ea153 treatment were always numerically higher (pH 6.5 to 6.9) than those of the noninoculated control (pH 5.6 to 6.2), which were always numerically higher than those for E325 (4.8 to 5.1). Analysis of data collected on different dates as replicates showed that the mean of the pH of concentrated extracts for the three treatments were all different ($P \leq 0.05$) (Fig. 1).

In the experiment designed to evaluate pH changes on stigmas in the orchard, weather conditions were typical for central Washington State. Average temperature during the 7-day period from first inoculation to final sampling was 14.0°C in 2006 and 13.8°C in 2007. No precipitation occurred and little or no vegetative wetness was recorded. Results indicated a similar trend to that indicated in the laboratory; however, statistical differences ($P \leq 0.05$) were indicated only for the final sampling date in 2007 (Fig. 2). Mean pH values for samples increased over time and were in a narrower range (between 5.0 and 5.8 overall, or within 0.4 pH units on any sampling date) than those of the laboratory test. Populations of bacteria applied to flowers were at the expected levels (Fig. 3) and preinoculation of stigmas with strain E325 resulted in lower populations of *E. amylovora*. With the detection limit at 10² CFU/flower, indigenous culturable organisms were detected at log CFU of <3.0 on fewer than 30% of control blossoms.

Characterization of antimicrobial activity. When 10 to 12 µl of crude extract from solid PSBM culture of strain E325 was

applied to wells cut in PSBM agar and overlaid with a soft agar suspension of *E. amylovora*, clear inhibition zones typically 10 to 12 mm in diameter resulted; no inhibition was observed with extracts from noninoculated PSBM agar. Crude extract samples subjected to various temperatures for 1 h retained activity at 85, 95, and 105°C, but not at 115°C; samples held for 72 h retained activity at 25, 30, 37, and 50, but not at 70°C. Adjustment of pH of the extract to values from 2 to 11, followed by a 24-h incubation and then readjustment to neutral pH prior to bioassay, showed that there was no change in inhibitory activity when extract was held at pH range from 2 to 7, but inactivation resulted from exposure to pH values from 8 to 11. Inactivation under alkaline conditions was not reversed even when readjusted to acid conditions (pH between 3 and 6). When phosphate concentration was increased from 2.4 to 96 mM, and pH and incubation period also were varied, inhibitory activity remained stable at pH 5. At pH 6 after 18 h, activity was stable in the presence of 0 to 6 mM phosphate, but diminished when phosphate concentrations were at 12 to 96 mM (Fig. 4). At pH 7, activity was undetectable after 6 h in solutions of 24 to 96 mM phosphate, and only slightly detectable after 18 h in 2.4 mM phosphate. Activity of the partially purified E325 product toward *E. amylovora* was unaffected by the addition of amino acids, selected enzymes, or iron. Of 73 microbial isolates tested for sensitivity to the E325 compound, only the 27 isolates of *E. amylovora* tested were sensitive (Table 1). Bacteria representing *Erwinia rhapsodici* and seven other species in Enterobacteriaceae were not inhibited by the compound.

Fig. 1. Effect of *Pantoea agglomerans* E325 (Pa) and *Erwinia amylovora* Ea153 (Ea) on pH of Gala apple stigmas in laboratory inoculations, as determined by measuring pH in extracted exudates. The detached flowers were inoculated and incubated at 24°C for 24 h prior to extraction. Vertical lines represent standard error. Within each bar group, means with unlike letters were different according to least significant difference ($P \leq 0.05$).

Fig. 2. Effect of *Pantoea agglomerans* E325 (Pa) and *Erwinia amylovora* Ea153 (Ea) on pH of Gala apple stigmas in field inoculations in **A**, 2006 and **B**, 2007 as determined by measuring pH in extracted exudates. Inoculations were performed on two dates (1 and 2 in key), i.e., 2 days before the first sampling shown and a few hours after first sampling. Vertical lines represent standard error. Means with unlike letters were different according to least significant difference ($P \leq 0.05$).

Nonenteric bacteria in the genera *Burkholderia*, *Pseudomonas*, *Bacillus*, and *Microbacterium* were likewise unaffected.

Further purification and characterization of antibacterial compound. Analyses of the purified active substance yielded a single total ion chromatogram peak (Fig. 5A). The UV-vis signal in the monitored range was weak and indicated, in conjunction with the TIC, that no other peaks were associated within the active fractions. Peaks from active fractions had mass spectra with prominent peaks at 556 *m/z* (Fig. 5B), indicating a molecular weight of approximately 555. Dilution-end-point assays with material derived from active fractions indicated inhibitory activity toward *E. amylovora* with a minimum of 20 to 40 ng of the purified compound (or approximately 3.6 to 7.2 μ M concentration in 10 μ l added to agar well). Following TLC, reaction with ninhydrin reagent produced a reddish blue band at *R_f* = 0.3, revealing the presence of free amines. Orcin reagent did not react, thus sugar residues were not detected in the purified preparation.

DISCUSSION

Early fire blight researchers hypothesized based on in vitro studies that bacterial antagonists inhibit *E. amylovora* by producing acid (11,12,35), but workers now generally believe competitive exclusion and antibiotic production are the main mechanisms of antagonism on blossoms (17,43). Prior to the current study, pH modification by bacteria on flower stigmas, the primary site of epiphytic establishment by the pathogen, had not been evaluated. Under ideal laboratory conditions for bacterial colonization of flowers, we found that *P. agglomerans* strain E325

exhibited a capacity to reduce the pH on stigmas to levels that could reduce growth of *E. amylovora*. Conversely, an increase in pH was observed on flowers inoculated with *E. amylovora* alone and was comparable to previous results with pear fruit tissue inoculated with the pathogen (51). A similar trend was indicated in the field on the final sampling date, with mean pH values numerically higher for pathogen-inoculated flowers and lower for the antagonist-inoculated flowers. However, statistical differences were shown only in 2007, when the pH for antagonist-inoculated flowers, whether they were challenged with *E. amylovora* or not, was slightly lower ($P \leq 0.05$) than treatments with the pathogen alone. These results did not implicate pH modification as an important mode of antagonism toward *E. amylovora*, but the possibility still exists that increased acidity caused by E325 may reduce the growth rate of *E. amylovora*, thereby contributing to antagonism and suppression of the pathogen. The optimum pH for *E. amylovora* is between 6 and 7 or higher (42) and pH values below 6.0 have been shown to reduce growth (12,51). It is questionable, however, whether pH in extracts from flowers was representative of the pH of the intercellular spaces on stigmas where bacteria reside and not artifactual, so conclusions related to stigma pH must await the development and application of more direct methods of pH measurement on stigmas.

If pH in exudates extracted from orchard blossoms reflects approximate hydrogen ion concentrations on stigmatic surfaces, the data indicate a pH range (between 5 and 6) conducive for strain E325 to inhibit *E. amylovora* through antibiosis as demonstrated in vitro. Strong antibacterial activity was shown under acid or neutral pH. The activity became unstable at pH 6 or 7 when

Fig. 3. Population size of *Pantoea agglomerans* E325 (Pa) and *Erwinia amylovora* Ea153 (Ea) on Gala apple stigmas in field experiment in **A**, 2006 and **B**, 2007 designed for evaluating bacterial modification of pH. Data are presented for the final sampling date. Vertical lines represent standard error.

Fig. 4. Results of assays in which phosphate concentration in crude antibacterial extract from culture of *Pantoea agglomerans* E325 grown on partial stigma-based medium (PSBM) was increased by 0, 2.4, 6, and 12 mM (top row of each panel) or 24, 36, 48, and 96 mM (bottom row of each panel) at varying pH (left); concentrations are shown in top panel only. Solutions were incubated at 28°C for 18 h before assaying for activity in PSBM-agar wells overlaid with agar suspension of *Erwinia amylovora* Ea153.

phosphate was increased beyond the initial level in PSBM (2.4 mM), and quickly disappeared at pH 8 even under low phosphate conditions. It is not known whether production of the E325 antibiotic is pH dependent. The antibiotic was produced under neutral to acidic conditions in low phosphate media, but production under alkaline conditions would not be detected in our assays because the antibiotic activity is irreversibly eliminated after exposure to basic pH conditions.

The phosphate concentration in PSBM was below that of most media used by researchers to test strains of *P. agglomerans* for production of antibiotics inhibitory to *E. amylovora* (20,46, 50,53). For instance, Wodzinski et al. (50) tested 346 putative strains of *P. agglomerans* on a glycerol-ammonium medium containing approximately 100 mM phosphate. Conversely, a glucose-asparagine medium used by Ishimaru et al. (14) for antibiotic production by *P. agglomerans* strain C9-1 contained only 1.3 mM phosphate and was buffered to neutrality with morpholino-propane sulfonic acid. Strain E325 did not inhibit *E. amylovora* on this medium (P. L. Pusey, unpublished data), perhaps because the buffering by morpholino-propane sulfonic acid (initially pH 7.4) prevents the development of acidic conditions required for consistent antibiosis in the bioassay. Phosphates can regulate antibiotic production by some microorganisms, suppressing biosynthesis when concentrations are above 10 mM (25); in our study with E325, however, we demonstrated that phosphate deactivated an antibacterial compound after it was produced. Although phosphates are transported in plants and detected in xylem exudates (5), it is unknown whether they are present in

stigma exudates or occur at levels that may affect the antibiotic synthesis or activity of E325.

The antibacterial activity of the E325 product has so far exhibited characteristics distinguishing it from other antibiotics of *P. agglomerans* that are inhibitory to *E. amylovora* (10,17,43). Unlike many antibiotics of this group, activity of the E325 compound was unaffected by amino acids. Wodzinski and Paulin (50) found that all but six of a group of 90 strains of *P. agglomerans* produced antibiotics that were inactivated by certain amino acids, and distinguished 13 antibiotic classes based on amino acid sensitivity. Antibiosis in their study was most commonly reversed by histidine. The antibiotic activity of E325 also was unaffected by enzymes reported to inactivate some antibiotics of *P. agglomerans*. For example, unlike the E325 compound, the antibiotic of strain Eh252 was inactivated by proteases (46). The high specificity of the E325 compound to *E. amylovora* is notable, given that other antibiotics of *P. agglomerans* have reportedly shown a broader spectrum of activity (14,20,46,53) affecting other enteric bacteria and frequently also nonenteric bacteria. The specificity of the antibiotic of E325 to *E. amylovora* may be advantageous for incorporating E325 in mixtures of complementary antagonists, an approach proposed for maximizing biological control of fire blight (17). Other unique properties of the E325 antibiotic include its inactivity above pH 7 and its instability in the presence of high phosphate levels. The sensitivity to phosphate at concentrations typically used in media for antibiosis assays likely explains why antibiosis due to E325 was previously undetected or inconsistent. These findings are congruous with

TABLE 1. Sensitivity of microbial strains to inhibitory activity of the isolated compound produced by *Pantoea agglomerans* E325 in partial stigma-based medium

Indicator species ^a	Isolate source ^b	No. of isolates	
		Total tested	Sensitive
<i>Erwinia amylovora</i>	J,Mc,P8,R15,Su,T	27	27
<i>Erwinia rhapontici</i>	S	1	0
<i>Pectobacterium carotovora</i>	S3	3	0
<i>Pantoea agglomerans</i>	B2,I,P7,R2,S3,T2	17	0
<i>Pantoea ananatis</i>	T	1	0
<i>Enterobacter cloacae</i>	Mc	1	0
<i>Enterobacter hormaechei</i>	P	1	0
<i>Escherichia coli</i>	G	1	0
<i>Klebsiella pneumoniae</i>	Mc	1	0
<i>Burkholderia cepacia</i>	A25416	1	0
<i>Pseudomonas aureofaciens</i>	A15926	1	0
<i>Pseudomonas fluorescens</i>	A13525,A17518, L,M,P,S	6	0
<i>Pseudomonas putida</i>	A12633,A17470, M2	4	0
<i>Pseudomonas syringae</i>	P	1	0
<i>Bacillus pumilus</i>	P	1	0
<i>Bacillus subtilis</i>	M, P3	4	0
<i>Microbacterium</i> sp.	P2	2	0

^a Identification, unless established in literature or by American Type Culture Collection (ATCC; Manassas, VA) was determined using at least two methods: (i) fatty acid methyl ester analysis with Microbial Identification System (Microbial ID, Inc., Newark, DE), and (ii) 16S rRNA gene sequence analysis (1). In addition to ATCC strains, isolates previously referred to in the literature are *E. amylovora* Ea153 (18,19); *Pantoea agglomerans* strains C9-1 (14,19), Eh252 (46), and Eh318 (7,53); *E. coli* C600-2Sm (28); and *Pseudomonas fluorescens* A506 (18,23).

^b Isolates were obtained from S. V. Beer (B), K. Geider (G), C. A. Ishimaru (I), K. B. Johnson (J), S. E. Lindow (L), M. Mazzola (M), R. J. McLaughlin (Mc), P. L. Pusey (P), R. G. Roberts (R), V. O. Stockwell (S), G. W. Sundin (Su), and T. N. Temple (T). When more than one isolate was obtained from the same source, the letter(s) representing source is followed by number of isolates. ATCC strains are indicated with "A" followed by the ATCC number. All isolates of *E. amylovora* came from apple or pear in Washington or Oregon, except one isolate from apple in Michigan (Su). All other bacteria were from pome orchard environments, mostly aerial plant parts, with the exception of *Escherichia coli*, *Pectobacterium carotovora*, and ATCC strains.

Fig. 5. Results of liquid chromatography-UV/visible-mass spectrometry analysis presented as A, total ion chromatogram indicating single peak at retention time also yielding a fraction from culture of *Pantoea agglomerans* strain E325 highly inhibitory to *Erwinia amylovora* and B, positive atmospheric pressure chemical ionization (APCI) mass spectra of inhibitory compound produced by E325. Arrows indicate top of bars conjoined to other bars.

recent polymerase chain reaction (PCR) analyses of strain E325 that failed to detect *paab* (V. O. Stockwell, *unpublished data*), a gene in the operon for synthesis of pantocin A (15), a common antibiotic of strains of *P. agglomerans* with efficacy against fire blight.

Two separate data sets, one dealing with pH on the stigma and the other with antibiotic production in a partial stigma-based medium, were not shown to be directly related, but both have possible implications in biological control of fire blight with *P. agglomerans* strain E325. Analysis of exudates from inoculated stigmas indicated that strain E325 may lower the pH on stigmas, which to some degree, may reduce growth of *E. amylovora*. Secondly, assuming pH values are close to those actually occurring on flower stigmas, results indicate a pH range on the stigma favorable for antibiotic activity, which was found in vitro to be pH sensitive. Work is in progress to further investigate acidification and antibiosis and their possible roles and inter-relationship in the suppression of *E. amylovora* by *P. agglomerans* E325 on stigmatic surfaces of apple and pear.

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