Effect of Nectar on Microbial Antagonists Evaluated for Use in Control of Fire Blight of Pome Fruits

P. L. Pusey

United States Department of Agriculture, Agricultural Research Service, Tree Fruit Research Laboratory, 1104 N. Western Avenue, Wenatchee, WA 98801.

Accepted for publication 28 August 1998.

ABSTRACT

Pusey, P. L. 1999. Effect of nectar on microbial antagonists evaluated for use in control of fire blight of pome fruits. *Phytopathology* 89:39-46.

Under warm, dry conditions, *Erwinia amylovora* can become established in relatively high populations on apple (*Malus domestica*) or pear (*Pyrus communis*) flower stigmas, and subsequent wet conditions facilitate its movement to the flower hypanthium where infection generally is initiated through the nectarthodes. Research on biological control of fire blight has focused mainly on the flower stigma, and knowledge is lacking regarding the effect of nectar on microbial antagonists in the flower hypanthium. The biocontrol agents *Pseudomonas fluorescens* strain A506 and *Pantoea agglomerans* strain C9-1 were cultured in a basal liquid medium with various concentrations (0 to 50% total sugar) of sucrose or synthetic nectar (sucrose/glucose/fructose, 2:1:1). Strain A506

showed less growth and lower survival than strain C9-1 at high sugar levels, and A506 was less effective than C9-1 as a preemptive antagonist of *E. amylovora* in high-sugar media. Both antagonist strains were less tolerant to high sugar levels than *E. amylovora* (strain Ea153). The same bacteria were cultured in a medium with 25% total sugar consisting of various proportions of sucrose, glucose, and fructose, and growth response correlated strongly with solute potential. When 28 microbial strains were cultured in synthetic nectar (25% total sugar) and ranked based on growth, strains clustered according to taxonomic group. Yeasts were most osmotolerant, followed by strains of *E. amylovora*, *Pantoea agglomerans*, *Bacillus* spp., and *Pseudomonas* spp. Further studies done in planta are necessary to determine whether osmotolerance of antagonists is advantageous in the biological control of fire blight.

Fire blight is a major limiting factor in the production of pome fruit. It is most often initiated by epiphytic populations of *Erwinia amylovora* that develop on blossoms (1,31). Control programs have emphasized antibiotic suppression of *E. amylovora* on floral parts; however, pathogen resistance to streptomycin has developed in many areas (20,21), and the less effective antibiotic oxytetracycline has been substituted. Biological control of the blossom blight phase of fire blight with bacterial antagonists has been demonstrated as an alternative to antibiotics (7,14,33) or as a measure that could complement the use of antibiotics (19,28). Recently, the commercial product BlightBan A506 (a.i., *Pseudomonas fluorescens* strain A506) produced by Plant Health Technologies (Boise, ID) became available. Future improvement of fire blight control with biological agents may be accomplished with the discovery of more effective microbial antagonists (23,25) or through genetic enhancement of known antagonists. Also, there has been a growing interest in the use of microbial mixtures employing complementary biocontrol mechanisms and to increase product activity under a broad range of environmental conditions (14,33,34).

Under the relatively dry climatic conditions in the western United States, colonization by *E. amylovora* occurs predominantly on flower stigmas, and infrequent rain or heavy dew facilitates its movement to the inner part of the cup-shaped body formed by the conjoined sepals, petals, and stamens (hypanthium) where invasion generally occurs through the nectarthodes (30,32,40). If nectar is present, an influx of water will cause a decrease in osmotic pres-

sure and may increase the suitability of the hypanthium for microbial colonization (9,12,29). Nectar production of apple and pear flowers is dependent on environmental conditions, and the sugar concentration of nectar fluctuates widely as influenced by relative humidity and free moisture (3,12,29). The ratio of constituent sugars in nectar (i.e., sucrose, glucose, and fructose) varies among species and cultivars of pomaceous plants (5,24,41). Early studies indicated that *E. amylovora* can tolerate relatively high sugar concentrations, because it grew in synthetic nectar solutions with total sugar levels up to 30 or 35% and survived for several days in these mixed sugars at much higher concentrations (9, 12). The pathogen reportedly grew minimally or survived for extended periods in sucrose solutions of 58 or 60% (9,29). Infection was found to occur when nectar was sufficiently diluted, thereby allowing *E. amylovora* to multiply and invade the flower tissue (12).

Research on biological control of fire blight has focused mainly on the flower stigma (7,13,33,38). The ability of an antagonist to colonize the stigma and suppress stigmatic populations of *E. amylovora* through a combination of mechanisms is of primary importance, particularly in arid or semi-arid production areas (30, 37-39). With this characteristic, an antagonist presence will be maintained, and pathogen inoculum levels will be held to a minimum during dry periods before conditions become favorable for pathogen spread to the hypanthium and infection via the nectarthodes. The hypanthium may be important as a second line of defense; however, few studies have given attention to antagonist-pathogen-host interactions on this flower part. Wilson and Lindow (39) reported that *P. fluorescens* strain A506 reduced colonization of pear nectaries by *E. amylovora* and suggested that the mechanisms involved may have included induced cessation of nectar secretion or accumulation of a host toxin. Klopmeier and Ries (17) found that *E. herbicola* (*Pantoea* sp.) was attracted chemotactically to Jonathan apple nectar extract. No reports indicate the effect of nectar

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

Publication no. P-1998-1030-01R

This article is in the public domain and not copyrightable. It may be freely reprinted with customary crediting of the source. The American Phytopathological Society, 1999.

concentration on the growth, survival, or antagonism of microorganisms used for control of fire blight.

The establishment of a relationship of nectar to biocontrol activity on the hypanthium could lead to the selection of superior antagonists or more effective antagonist communities, perhaps consisting of components more adaptive to the sugar-rich milieu of the hypanthium. The current paper reports the effect of total sugar concentration and constituent sugar composition of synthetic nectar on the ability of two known antagonists (*P. fluorescens* strain A506 and *Pantoea agglomerans* (syn. *E. herbicola*) strain C9-1) to grow, survive, and suppress *E. amylovora*. Also presented are results indicating the relative osmotolerance of a number of bacteria and yeasts representing taxa of interest for fire blight control.

MATERIALS AND METHODS

Microbial strains. Bacteria used in all experiments were a nalidixic acid-resistant derivative of *E. amylovora* strain Ea153, rifampicin-resistant *P. fluorescens* strain A506, and a rifampicin-resistant derivative of *Pantoea agglomerans* strain C9-1. Original strain codes, without letters previously added to indicate specific derivative types, are used in this report. Strain Ea153, obtained from K. Johnson (Oregon State University, Corvallis), was isolated from cankers on Gala apple in Oregon and later used in studies of biocontrol in the field (13,14,28). Strain A506, originally from S. Lindow (University of California, Berkeley), was isolated from pear (*Pyrus communis* L.) in California and reduced the incidence of fire blight (14,19,34). Strain C9-1, originally from C. Ishimaru (Colorado State University, Fort Collins), was isolated from Jonathan apple (*Malus domestica* Borkh.) fruit in Michigan (11) and the rifampicin-resistant derivative was later combined with strain A506 to reduce fire blight of pear (14).

Additional microbial strains were employed in one experiment to assess the range of tolerance to high sugar levels among apple and pear epiphytes representing different genera and species (Table 1). Organisms not referenced to other reports were identified based on fatty acid methyl ester analysis of whole-cell fatty acids using gas chromatography and profile analysis with the Microbial Identi-

fication System (Microbial ID, Inc., Newark, DE), carbon utilization with the Micro Station System (Biolog, Inc., Hayward, CA), and use of polymerase chain reaction (PCR) primers to distinguish phenazine-producing *Pseudomonas* spp. (26).

Effect of sugar concentration and composition. To test the effect of total sugar concentration, solutions of sucrose or synthetic nectar (composed of sucrose, glucose, and fructose in the ratio 2:1:1, respectively) were prepared at concentrations of 0, 5, 10, 15, 20, 25, 30, 40, and 50% in a basal medium (7 g of K_2HPO_4 , 2 g of KH_2PO_4 , 0.5 g of sodium citrate, 0.1 g of $MgSO_4 \cdot 7H_2O$, 1 g of $(NH_4)_2SO_4$, 0.5 g of niacin, and 1 liter of deionized water). Solutions were transferred to 96-well Micro Test III plates with flat-bottom wells (Becton Dickinson Labware, Oxnard, CA), 250 μ l per well. Bacterial inoculum was prepared by culturing strains Ea153, A506, and C9-1 for 24 h at 24°C on 8 g of nutrient-yeast-dextrose agar consisting of 8 g of nutrient broth (Difco Laboratories, Detroit), 5 g of yeast extract (Difco Laboratories), 5 g of dextrose, 15 g of agar (BBL Microbiology Systems, Cockeysville, MD), and 1 liter of deionized water, and then suspending cells at 10^6 CFU/ml in 10 mM potassium phosphate buffer (pH 7.0) and 0.03% Tween-20. Bacterial suspension (10 ml) was added to the 250 μ l of sugar medium in each well. The solute potential (ψ_s) of each sugar medium plus buffer and Tween-20 in the above proportions was determined with a model 5500 vapor pressure osmometer (Wescor Instruments, Logan, UT). The 96-well plates were covered with lids and placed in 4-liter plastic containers (Rubbermaid, Twinsburg, OH) with two moist paper towels on the bottom (15 ml of water added) and incubated on a rotary shaker (Orbit Environ-shaker; Labline Instruments, Melrose Park, IL) at 125 rpm at 26°C. After 24 and 72 h, bacterial population size in each of three wells per treatment (separate wells for different time periods) were determined by the dilution-plating method of Harris and Sommers (8). CCT medium (sucrose, 100 g; sorbitol, 10 g; 1% aqueous solution of tergitol anionic 7, 30 ml; 0.1% crystal violet in absolute ethanol, 2 ml; nutrient agar, 23 g; and deionized water, 970 ml [10]) amended with nalidixic acid (50 μ g/ml) was used for detection of strain Ea153, and Kings' medium B (16) amended with rifampicin (25 μ g/ml) and cycloheximide (100 μ g/ml) was used for

TABLE 1. Microorganisms cultured in synthetic nectar medium to test tolerance to 25% total sugar

Organism	Strain	Plant	Site ^a	Source	Reference
<i>Aureobasidium pullulans</i>	E19	Pear flower	Washington	R. J. McLaughlin	
<i>Aureobasidium pullulans</i>	E6	Pear flower	Washington	P. L. Pusey	
<i>Bacillus megaterium</i>	F113	Apple flower	Washington	P. L. Pusey	
<i>Bacillus pumilis</i>	F207	Apple flower	Washington	P. L. Pusey	
<i>Bacillus thuringiensis</i>	F468	Apple flower	Washington	P. L. Pusey	
<i>Bacillus subtilis</i>	E198	Apple flower	Washington	P. L. Pusey	
<i>Candida sake</i>	E583	Apple flower	Washington	P. L. Pusey	
<i>Erwinia amylovora</i>	Ea153	Apple branch	Oregon	K. B. Johnson	13, 14
<i>Erwinia amylovora</i>	PD494	Apple or pear	Netherlands	K. Geider	2
<i>Erwinia amylovora</i>	87-66	Pear branch	Washington	R. G. Roberts	
<i>Erwinia amylovora</i>	87-70	Apple branch	Washington	R. G. Roberts	
<i>Erwinia amylovora</i>	87-80	Apple branch	Washington	R. G. Roberts	
<i>Erwinia amylovora</i>	88-38	Apple branch	Washington	R. G. Roberts	27
<i>Pantoea agglomerans</i>	C9-1	Apple fruit	Michigan	C. Ishimaru	11
<i>Pantoea agglomerans</i>	Eh252	Apple	New York	S. V. Beer	35
<i>Pantoea agglomerans</i>	87-167	Apple fruit	Washington	R. G. Roberts	
<i>Pantoea agglomerans</i>	C106	Pear flower	Oregon	R. J. McLaughlin	
<i>Pantoea agglomerans</i>	E325	Apple flower	Washington	P. L. Pusey	
<i>Pantoea agglomerans</i>	E873	Apple flower	Washington	P. L. Pusey	
<i>Pantoea agglomerans</i>	F257	Apple flower	Washington	P. L. Pusey	
<i>Pseudomonas chlororaphis</i>	F516	Apple flower	Washington	P. L. Pusey	
<i>Pseudomonas chlororaphis</i>	E326	Apple flower	Washington	P. L. Pusey	
<i>Pseudomonas chlororaphis</i>	F224	Apple flower	Washington	P. L. Pusey	
<i>Pseudomonas fluorescens</i>	A506	Pear leaf	California	S. E. Lindow	39
<i>Pseudomonas fluorescens</i>	F395	Apple flower	Washington	P. L. Pusey	
<i>Pseudomonas putida</i>	E300	Apple flower	Washington	P. L. Pusey	
<i>Pseudomonas putida</i>	F143	Apple flower	Washington	P. L. Pusey	
<i>Pseudomonas syringae</i>	F301	Apple flower	Washington	P. L. Pusey	

^a State within the United States or country from which strains were isolated.

detection of strains A506 and C9-1. Plates were incubated 3 to 4 days at 24°C before counting colonies.

To test the effect of constituent sugar composition on growth of strains Ea153, A506, and C9-1, procedures were the same as above, except that the total sugar concentration was always at 25% and the proportion of disaccharide (sucrose) to monosaccharide (equal parts of glucose and fructose) was 1:0, 2:1, 1:1, 1:2, or 0:1. Populations were determined after 24 and 48 h.

Interaction of antagonist and pathogen at various sugar levels. Methods were the same as described above unless otherwise indicated. Synthetic nectar (sucrose/glucose/fructose, 2:1:1) was prepared at concentrations of 0, 5, 10, 15, 20, and 25%. The sugar media in plate wells (250 µl per well) were preinoculated in separate tests with strains A506 or C9-1 (10 µl of a 10⁷ CFU/ml suspension) 24 h before being inoculated with strain Ea153 (10 µl of a 10⁷ CFU/ml suspension). For controls, media were also inoculated

with only the antagonist or strain Ea153. Three wells were used per treatment per time period. Plates were incubated for a total of 48 or 72 h (24 or 48 h after inoculation with strain Ea153) before determining population size.

Growth response of various microbial strains at high sugar level. To assess the variability in sugar tolerance among microbial strains within taxa that are of interest for biocontrol of fire blight, strains listed in Table 1 were tested for growth response in synthetic nectar (sucrose/glucose/fructose, 2:1:1) with a total sugar level of 25%. Other methods were the same as above. Each strain was suspended at 10⁶ CFU/ml and 10 µl of the suspension was added to 250 µl of medium in each of three plate wells. Plates were incubated for 24 or 72 h before estimating populations.

Statistical analyses. For all experiments, log-transformed data from two separate trials were tested for homogeneity according to Hartley's *F*-max test. The data were then pooled and analyzed using

Fig. 1. Effect of the concentration of sugar on growth and survival of *Pantoea agglomerans* strain C9-1, *Pseudomonas fluorescens* strain A506, and *Erwinia amylovora* strain Ea153. **A and B,** Population sizes in liquid medium with various concentrations of sucrose after 24 and 72 h, respectively. **C and D,** Population sizes in medium with various concentrations of synthetic nectar after 24 and 72 h, respectively. Synthetic nectar was composed of sucrose, glucose, and fructose in the ratio of 2:1:1, respectively. Horizontal dotted line indicates approximate size of starting population at time of inoculation. For each concentration, data were compared according to least significant difference test ($P \leq 0.05$) and results were indicated with symbols along the upper side of the x axis. x = highest and lowest values different; + = two closest values not different from each other, but different from third value; and * = all values different from each other.

SAS (Statistical Analysis Systems version 6.1; SAS Institute, Cary, NC) analysis of variance (ANOVA), and means were separated according to least significant difference test ($P \leq 0.05$).

RESULTS

Effect of sugar concentration and composition. Actual sugar concentrations were slightly lower than percentages given below due to the addition of inoculum suspensions after the sugar media were prepared. The ψ_s values for 0, 5, 10, 15, 20, 25, 30, and 40% sucrose, as determined for the final solution after inoculation, were -0.40 , -0.90 , -1.27 , -1.78 , -2.31 , -2.90 , -3.86 , and -5.95 MPa, respectively. The ψ_s values for 0, 5, 10, 15, 20, 25, and 30% total sugar in synthetic nectar media were -0.40 , -0.97 , -1.59 , -2.26 , -3.02 , -3.91 , and -5.45 MPa, respectively. The ψ_s of media with sugar levels higher than those indicated above could not be determined accurately with the osmometer. The optimum level of total sugar for growth of all bacterial strains tested was generally in the range from 5 to 20% (Fig. 1). At 40 or 50% total sugar, the population size of these bacteria after 24 or 72 h was close to or below the initial population at the time of inoculation. After 24 h at 0% sucrose, the population size of strain A506 was greater than that of strains C9-1 and Ea153 ($P \leq 0.05$), but at sucrose levels of 15% and higher, it was lower than that of the other two bacteria (Fig. 1A). After 24 h, population size of strain C9-1 was greater than

that of strain Ea153 at sucrose concentrations from 0 to 30%, but was lower compared with Ea153 at 40 and 50%. After 72 h, populations of strain A506 were larger than those of strains C9-1 and Ea153 at 0 to 10% sucrose, but smaller compared with the other bacteria at levels from 25 to 50% (Fig. 1B). At 40 and 50% sucrose, strain A506 was barely detectable. The population size of strain C9-1 exceeded that of strain Ea153 at 0, 25, and 30% sucrose, but was much smaller than that of Ea153 at 50% sucrose (Fig. 1B). At 40% sucrose, the strain C9-1 population size was about the same as the starting population and not different from that of strain Ea153. In tests with synthetic nectar, the population size of strain A506 after 24 h was greater than that of strains C9-1 and Ea153 at 0% sugar, but was lower compared with the other bacteria at total sugar levels of 5% and higher (Fig. 1C). Strain C9-1 populations were larger than strain Ea153 populations at sugar levels from 0 to 15%, but smaller in comparison from 20 to 50%. After 72 h, populations of strain A506 were larger at 0% synthetic nectar compared with the other bacteria, but smaller than that of strain C9-1 from 15 to 30% total sugar and smaller than that of strain Ea153 from 15 to 50% sugar (Fig. 1D). Strain C9-1 populations were larger than strain Ea153 populations at sugar levels from 5 to 15%, but smaller in comparison from 25 to 50%. The differences in tolerance among the bacterial strains to high sugar levels was particularly evident at 72 h and an initial nectar concentration of 25% (Fig. 1D). At this level, strain Ea153 had a population size nearly 3 log units higher than that of the starting population, strain C9-1 had a population size more than 1 log unit smaller than that of the starting population, and strain A506 was undetectable.

When the above bacteria were cultured in media consisting of various proportions of constituent sugars totaling 25%, population size generally decreased as the disaccharide portion relative to the monosaccharide portion was decreased (Fig. 2). The ψ_s of the 25% sucrose medium (1:0) was -2.90 MPa, and the ψ_s of the 25% hexose sugar medium (0:1) was -4.92 MPa. The ψ_s values for disaccharide to monosaccharide ratios of 2:1, 1:1, and 1:2 were -3.45 , -3.91 , and -4.21 MPa, respectively. After 24 and 48 h, ψ_s correlated strongly with growth of strain C9-1 ($P = 0.0001$; $R = 0.93$ and 0.94 , respectively), strain A506 ($P = 0.0001$; $R = 0.75$ and 0.70 , respectively), and strain Ea153 ($P = 0.0001$; $R = 0.90$ and 0.88 , respectively). After 24 h in the medium prepared with only sucrose and no monosaccharides, the population size of strain C9-1 was greater than that of strain Ea153 ($P \leq 0.05$) (Fig. 2A); however, when the sugar medium included monosaccharide sugars, the strain C9-1 population size was either not different from or was smaller than that of strain Ea153. When the disaccharide portion was larger than the monosaccharide portion, the strain C9-1 population was 2 to 3 log units smaller than the strain Ea153 population. Regardless of sugar composition, the population size of strain A506 after 24 h was always lower than that of strains Ea153 and C9-1 (Fig. 2A). From 24 to 48 h, bacterial populations in sugar composed totally of sucrose (1:0) had increased significantly, but they greatly decreased in sugar composed totally of monosaccharides (0:1) (Fig. 2B). In the latter case, only strain Ea153 was detectable. Comparisons among bacterial strains at 48 h were similar to those shown at 24 h. After 48 h, populations of strain A506 were measured at nearly 10^6 CFU/ml in sugar composed only of sucrose, and were barely detected or undetected in all other media with sugar composed partly or wholly of monosaccharides.

Interaction of antagonist and pathogen at various sugar levels.

When synthetic nectar at various concentrations was preinoculated with strain A506 before being inoculated with strain Ea153, the population size of strain A506 alone in the experiment was affected by total sugar levels as previously shown (Fig. 1C and D), but was not influenced by the presence of strain Ea153 according to statistical comparisons at $P \leq 0.05$ (Fig. 3A and B). At 24 and 48 h after inoculation with strain Ea153, the population

Fig. 2. Effect of constituent sugar composition on growth and survival of *Pantoea agglomerans* strain C9-1, *Pseudomonas fluorescens* strain A506, and *Erwinia amylovora* strain Ea153. **A and B,** Population sizes in liquid medium with 25% total sugar composed of different proportions of disaccharide (sucrose) to monosaccharide (equal parts of glucose and fructose) after 24 and 48 h, respectively. For each bar group, means with the same letter were not different according to the least significant difference test ($P \leq 0.05$).

size of strain Ea153 was reduced in the presence of strain A506 at 0, 5, and 10% total sugar, but not at 15, 20, or 25% sugar (Fig. 3C and D). Likewise, strain C9-1 populations were affected by sugar levels as already shown (Fig. 1C and D), but not influenced by strain Ea153 (Fig. 4A and B). After 24 h, strain Ea153 populations were reduced by strain C9-1 at sugar levels from 5 to 15% (Fig. 4C); after 48 h, suppression occurred from 5 to 20% sugar (Fig. 4D).

Growth response of various microbial strains at high sugar level. A range of growth responses was observed for 28 microbial strains cultured in synthetic nectar with a total sugar concentration of 25% (Fig. 5). After 24 h, yeast or yeast-like strains increased the most in population; population sizes of *Candida sake* strain E583 and *Aureobasidium pullulans* strain E6 were ranked first and second, respectively, and were significantly greater than any of the other strains ($P \leq 0.05$) (Fig. 5A). A general ranking of bacterial strain groups indicated that population sizes for *E. amylovora* were highest, followed in order by *E. amylovora*, *Pantoea agglomerans*, *Bacillus* spp., and *Pseudomonas* spp. After 72 h, ranking by taxonomic group was less discernible (Fig. 5B); however, the two organisms with the largest population sizes were both strains of *E. amylovora*. Values for these strains were not significantly different from *C. sake* strain E583, which ranked third. Population sizes for certain strains of *Pantoea agglomerans* were greater than that of some strains of *E. amylovora* ($P \leq 0.05$). The strain of *E. amylovora* that ranked lowest was PD494, a strain previously shown to be deficient in levan and amylovoran synthesis (2). The seven lowest-ranked strains according to population size were all pseudomonads; three of these were undetectable.

Two biocontrol agents used for fire blight, *P. fluorescens* strain A506 and *Pantoea agglomerans* strain C9-1, were repeatedly shown to be less adapted to high concentrations of sugars than was the pathogen *E. amylovora* strain Ea153. Also, strain A506 was much less tolerant than strain C9-1 to high-sugar conditions. Because the pathogen generally grows or survives in the often sugar-rich environment of the flower hypanthium before invading through the nectarthodes, results appear to have relevance to the biological control of fire blight. Although it is of primary importance that antagonists preemptively colonize the stigma to exclude *E. amylovora* (30,37–39), the hypanthium could be important as a second line of defense during and after wet periods. It may be advantageous to select biocontrol agents for fire blight based partly on their adaptability to the sugar-rich environment of the hypanthium. One possible approach would be to include an osmotolerant strain or strains as part of a mixture of microbial antagonists with different adaptive characteristics.

The above study may apply more to apple than to pear, because generally, apple nectar is more frequently present and its sugar concentration higher than that of pear nectar (5,29,36). Under conditions of low humidity, sugar levels in apple nectar commonly range from 35 to 55% (5,12,36). Early studies with pear in California indicated that nectar was undetectable or contained relatively low concentrations of sugar, with maximum levels from 17 to 22% (29,36); however, in Wisconsin, when pear nectar was present in amounts large enough for sampling, sugar levels ranged from 35 to 61% (10,15). The proportions of sugar constituents in nectar varies among apple cultivars, but the average ratio is approximately one

Fig. 3. Effect of sugar concentration on the ability of *Pseudomonas fluorescens* strain A506 to inhibit *Erwinia amylovora* strain Ea153 when synthetic nectar medium was preinoculated with A506 24 h in advance of Ea153. **A and B,** Population size of A506 alone and in the presence of Ea153 in liquid medium with various levels of total sugar (sucrose/glucose/fructose, 2:1:1) at 24 and 48 h, respectively, after inoculation with Ea153. **C and D,** Population size of Ea153 alone and in the presence of A506 in medium with various sugar levels after 24 and 48 h, respectively. An asterisk along the upper side of the x axis signifies that the two means at the concentration indicated were different according to the least significant difference test ($P \leq 0.05$).

part disaccharide to one part monosaccharide (3,5); thus, a 1:1 ratio was used for synthetic nectar in tests described above. According to the limited information pertaining specifically to pear, the monosaccharide portion of pear nectar is generally greater than the disaccharide portion (4,41).

The negative effect of high concentrations of sugar on bacterial growth and survival was apparently due mainly to the sensitivity of cells to osmotic pressure. When bacteria were incubated in media with different proportions of constituent sugars totaling 25% by weight, the bacterial population size decreased as ψ_s became more negative (Fig. 2). The effects of sucrose and synthetic nectar over a range of sugar concentrations were more similar when examined in relation to ψ_s (Fig. 1). However, differences in growth response to these sugar media could not be entirely explained based on ψ_s . For instance, after 24 h, the population size of strain A506 cultured in the 15% sucrose medium, with ψ_s measured at -1.78 MPa, was more than 1 log unit greater than that of the starting population; but in the synthetic nectar medium with 10% total sugar and a ψ_s of only -1.59 MPa, it was nearly 2 log units smaller than at the start. Also, after 72 h, the population size of strain C9-1 grown in the 40% sucrose medium, measured at -5.95 MPa, was slightly greater than that of the starting population; but in the synthetic nectar medium at 25% and -3.91 MPa, it decreased from the start by more than a log unit. Thus, besides the differential effect due to ψ_s , sucrose favored bacterial growth more than did the hexose sugars.

The lower tolerance of strain A506 than strain C9-1 to relatively high sugar concentrations based on growth response corresponded to a lower efficacy of strain A506 than strain C9-1 as a preemptive antagonist of strain Ea153 under these conditions (Figs. 3 and 4).

This may explain why strain A506 was equal to strain C9-1 in its capacity to colonize stigmas of crab apple flowers, but was less effective than strain C9-1 in colonizing hypanthia and preventing disease development in blossoms (25). The low osmotolerance of strain A506 may also be the reason why hypanthial populations of this bacterium only declined following inoculation of pear flowers, even though stigmatic populations dramatically increased in size (39).

When a range of microorganisms previously collected from apple or pear surfaces were incubated in 25% total sugar and ranked based on growth, strains tended to cluster according to taxonomic group (Fig. 5). It was previously established that strains of *E. amylovora* could grow in media consisting of relatively high concentrations of sugar (9,12). This may be partly related to their production of extracellular polysaccharides, because strain PD494, which lacks the ability to produce amylovoran or levan (2), was shown after 72 h in media with 25% sugar to have a much smaller population size than any of the other strains of *E. amylovora* (Fig. 5B). As a group, strains of *Pantoea agglomerans* were less tolerant to the high sugar level than were strains of *E. amylovora*, but more tolerant than strains of *Bacillus* spp. or *Pseudomonas* spp. Results were consistent with previous studies indicating, in general, that yeasts are more osmotolerant than bacteria and that *Bacillus* spp. are more osmotolerant than *Pseudomonas* spp. (6,18,22). Among all microbial strains tested, yeast or yeast-like organisms were shown to have the largest population sizes after 24 h, but not 72 h. However, yeast cells are much larger in size than bacterial cells, and the biomass of the yeasts based on dry weight per CFU was approximately 100-fold that of the bacteria (P. L. Pusey, unpublished data). It is assumed that the yeasts depleted nutrients in

Fig. 4. Effect of sugar concentration on the capacity of *Erwinia herbicola* strain C9-1 to suppress *Erwinia amylovora* strain Ea153 when synthetic nectar medium was preinoculated with C9-1 24 h in advance of Ea153. **A and B**, Population size of C9-1 alone and in the presence of Ea153 in liquid medium with various levels of total sugar (sucrose/glucose/fructose, 2:1:1) at 24 and 48 h, respectively, after inoculation with Ea153. **C and D**, Population size of Ea153 alone and in the presence of C9-1 in medium with various sugar levels after 24 and 48 h, respectively. An asterisk along the upper side of the x axis signifies that the two means at the concentration indicated were different according to the least significant difference test ($P \leq 0.05$).

the medium sooner and reached a growth peak earlier than did the bacteria.

Studies conducted in planta and with natural nectar will be necessary to determine conclusively whether the osmotolerance of an-

tagonists is advantageous or of critical importance in the biological control of fire blight of apple and pear. Possibly overall disease control could be improved by establishing antagonist communities that include bacteria or yeasts with a relatively high tolerance to sugar levels frequently present in flower hypanthia.

LITERATURE CITED

1. Aldwinckle, H. S., and Beer, S. V. 1979. Fire blight and its control. *Hortic. Rev.* 1:423-474.
2. Bereswill, S., Jock, S., Aldridge, P., Janse, J. D., and Geider, K. 1997. Molecular characterization of natural *Erwinia amylovora* strains deficient in levan synthesis. *Physiol. Mol. Plant Pathol.* 51:215-225.
3. Beutler, R. 1930. Biologisch-chemische untersuchungen am nektar von immenblumen. *Z. Vergleich Physiol.* 12:72-176.
4. Bieleski, R. L., and Redgwell, R. L. 1980. Sorbitol metabolism in nectaries from flowers of Rosaceae. *Aust. J. Plant Physiol.* 7:15-25.
5. Campbell, R. J., Fell, R. D., and Marini, R. P. 1990. Characterization of apple nectar sugars in selected commercial and crab apple cultivars. *Fruit Var. J.* 44:136-141.
6. Christian, J. H. B. 1981. Specific solute effects on microbial water relations. Pages 825-854 in: *Water Activity: Influences on Food Quality*. L. B. Rockland and G. F. Stewart, eds. Academic Press, New York.
7. Epton, H. A. S., Wilson, M., Nicholson, S. L., and Sige, D. C. 1994. Biological control of *Erwinia amylovora* with *Erwinia herbicola*. Pages 335-352 in: *Ecology of Plant Pathogens*. J. P. Blakeman and B. Williamson, eds. CAB International, Wallingford, United Kingdom.
8. Harris, R. F., and Sommers, L. E. 1968. Plate-dilution frequency technique for assay of microbial ecology. *Appl. Microbiol.* 16:330-334.
9. Hildebrand, E. M., and Phillips, E. F. 1936. The honeybees and the beehive in relation to fire blight. *J. Agric. Res.* 52:789-810.
10. Ishimaru, C., and Klos, E. J. 1984. New medium for detecting *Erwinia amylovora* and its use in epidemiological studies. *Phytopathology* 74:1342-1345.
11. Ishimaru, C. A., Klos, E. J., and Brubaker, R. R. 1988. Multiple antibiotic production by *Erwinia herbicola*. *Phytopathology* 78:746-750.
12. Ivanoff, S. S., and Keitt, G. W. 1941. Relations of nectar concentration to growth of *Erwinia amylovora* and fire blight infection of apple and pear blossoms. *J. Agric. Res.* 62:733-743.
13. Johnson, K. B., Stockwell, V. O., Burgett, D. M., Sugar, D., and Loper, J. E. 1993. Dispersal of *Erwinia amylovora* and *Pseudomonas fluorescens* by honey bees from hives to apple and pear blossoms. *Phytopathology* 83:478-484.
14. Johnson, K. B., Stockwell, V. O., McLaughlin, R. J., Sugar, D., Loper, J. E., and Roberts, R. G. 1993. Effect of antagonistic bacteria on establishment of honey bee-dispersed *Erwinia amylovora* in pear blossoms and on fire blight control. *Phytopathology* 83:995-1002.
15. Keitt, G. W., and Ivanoff, S. S. 1941. Transmission of fire blight by bees and its relation to nectar concentration of apple and pear blossoms. *J. Agric. Res.* 62:745-753.
16. King, E. O., Ward, M. K., and Raney, D. E. 1954. Two simple media for the demonstration of pyocyanin and fluorescein. *J. Lab. Clin. Med.* 44:301-307.
17. Klopmeier, M. J., and Ries, S. M. 1987. Motility and chemotaxis of *Erwinia herbicola* and its effect on *Erwinia amylovora*. *Phytopathology* 77:909-914.
18. Leistner, L., Rodel, W., and Krispien, K. 1981. Microbiology of meat and meat products in high- and intermediate-moisture ranges. Pages 855-916 in: *Water Activity: Influences in Food Quality*. L. B. Rockland and G. F. Stewart, eds. Academic Press, New York.
19. Lindow, S. E., McGourty, G., and Elkins, R. 1996. Interactions of antibiotics with *Pseudomonas fluorescens* strain A506 in the control of fire blight and frost injury to pear. *Phytopathology* 86:841-848.
20. Loper, J. E., Henkels, M. D., Roberts, R. G., Grove, G. G., Willet, M. J., and Smith, T. J. 1991. Evaluation of streptomycin, oxytetracycline, and copper resistance of *Erwinia amylovora* isolated from pear orchards in Washington state. *Plant Dis.* 75:287-290.
21. McManus, P. S., and Jones, A. L. 1994. Epidemiology and genetic analysis of streptomycin-resistant *Erwinia amylovora* from Michigan and evaluation of oxytetracycline for control. *Phytopathology* 84:627-633.
22. Measures, J. C. 1975. Role of amino acids in osmoregulation in non-halophilic bacteria. *Nature (Lond.)* 257:398-400.
23. Mercier, J., and Lindow, S. E. 1996. A method involving ice nucleation for the identification of microorganisms antagonistic to *Erwinia amylovora* on pear flowers. *Phytopathology* 86:940-945.
24. Percival, M. S. 1961. Types of nectar in angiosperms. *New Phytol.* 60:235-281.
25. Pusey, P. L. 1997. Crab apple blossoms as a model for research on bio-

Fig. 5. Effect of synthetic nectar medium composed of 25% total sugar (sucrose/glucose/fructose, 2:1:1) on growth and survival of 28 microorganisms. **A and B,** Population sizes after 24 and 72 h, respectively, for strains indicated by code on the y axis: *Aureobasidium pullulans* E19, *A. pullulans* E6, *Bacillus megaterium* F113, *B. pumilis* F207, *B. thuringiensis* F468, *B. subtilis* E198, *Candida sake* E583, *Erwinia amylovora* Ea153, *E. amylovora* PD494, *E. amylovora* 87-66, *E. amylovora* 87-70, *E. amylovora* 87-80, *E. amylovora* 88-38, *Pantoea agglomerans* C9-1, *Pantoea agglomerans* Eh252, *Pantoea agglomerans* 87-167, *Pantoea agglomerans* C106, *Pantoea agglomerans* E325, *Pantoea agglomerans* E873, *Pantoea agglomerans* F257, *Pseudomonas chlororaphis* F516, *P. chlororaphis* E326, *P. chlororaphis* F224, *P. fluorescens* A506, *P. fluorescens* F395, *P. putida* E300, *P. putida* F143, and *P. syringae* F301. Strains are ranked according to growth, and bar hatching signifies taxonomic group as indicated in legend. Vertical dotted line indicates approximate size of starting population at time of inoculation; horizontal line indicates least significant difference at $P \leq 0.05$.

- logical control of fire blight. *Phytopathology* 87:1096-1102.
26. Raaijmakers, J. M., Weller, D. M., and Thomashow, L. S. 1997. Frequency of antibiotic-producing *Pseudomonas* spp. in natural environments. *Appl. Environ. Microbiol.* 63:881-887.
 27. Roberts, R. G., and Reymond, S. T. 1989. Evaluation of postharvest treatments for eradication of *Erwinia amylovora* from apple fruit. *Crop Prot.* 8:283-288.
 28. Stockwell, V. O., Johnson, K. B., and Loper, J. E. 1996. Compatibility of bacterial antagonists of *Erwinia amylovora* with antibiotics used to control fire blight. *Phytopathology* 86:834-840.
 29. Thomas, H. E., and Ark, P. A. 1934. Nectar and rain in relation to fire blight. *Phytopathology* 24:682-685.
 30. Thomson, S. V. 1986. The role of the stigma in fire blight infections. *Phytopathology* 76:476-482.
 31. van der Zwet, T., and Keil, H. L. 1979. Fire blight: A bacterial disease of rosaceous plants. *Agric. Handb.* 510, U.S. Dep. Agric. Sci. Educ. Admin.
 32. Vanneste, J. L. 1995. *Erwinia amylovora*. Pages 21-46 in: *Pathogenesis and host-parasite specificity in plant diseases: Histopathological, biochemical, genetic and molecular bases*, Vol. 1, Prokaryotes. U. S. Singh, R. P. Singh, and K. Khomoto, eds. Pergamon Press, Oxford.
 33. Vanneste, J. L. 1996. Honey bees and epiphytic bacteria to control fire blight a bacterial disease of apple and pear. *Biocontrol News Inf.* 17:67N-78N.
 34. Vanneste, J. L., and Yu, J. 1996. Biological control of fire blight using *Erwinia herbicola* Eh252 and *Pseudomonas fluorescens* A506 separately or in combination. *Acta Hort.* 411:351-353.
 35. Vanneste, J. L., Yu, J., and Beer, S. V. 1992. Role of antibiotic production by *Erwinia herbicola* Eh252 in biological control of *Erwinia amylovora*. *J. Bacteriol.* 174:2785-2796.
 36. Vansell, G. H. 1942. Factors affecting the usefulness of honey bees in pollination. U.S. Dep. Agric. Circ. No. 650.
 37. Wilson, M., Epton, H. A. S., and Sigee, D. C. 1989. *Erwinia amylovora* infection of hawthorn blossom: II. The stigma. *J. Phytopathol.* 127:15-28.
 38. Wilson, M., Epton, H. A. S., and Sigee, D. C. 1992. Interactions between *Erwinia herbicola* and *E. amylovora* on the stigma of hawthorn blossoms. *Phytopathology* 82:914-918.
 39. Wilson, M., and Lindow, S. E. 1993. Interactions between the biological control agent *Pseudomonas fluorescens* strain A506 and *Erwinia amylovora* in pear blossom. *Phytopathology* 83:117-123.
 40. Wilson, M., Sigee, D. C., and Epton, H. A. S. 1990. *Erwinia amylovora* infection of hawthorn blossom: III. The nectary. *J. Phytopathol.* 128:62-74.
 41. Wykes, G. R. 1952. An investigation of the sugars present in the nectar of flowers of various species. *New Phytol.* 51:210-215.