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Inoculation of Stigma-Colonizing Microbes to Apple Stigmas Alters Microbiome Structure and Reduces the Occurrence of Fire Blight Disease

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ABSTRACT

Flowers secrete nutrient-rich exudates that support the growth of an assemblage of microorganisms, including both beneficial and pathogenic members, most of which belong to the phylum *Proteobacteria*. Given the potential role of the microbiome in plant health, manipulating the microbiome to promote growth of beneficial members holds promise in controlling plant diseases. In this study, we inoculated four different bacterial strains that were originally isolated from apple stigmas, alone or in mixtures of increasing complexity, onto apple flowers during bloom. We tested whether such treatments would influence fire blight occurrence, a disease caused by *Erwinia amylovora*, and whether we could detect a shift in the structure of the microbiome due to the treatments. We show that various inoculations did influence the occurrence of fire blight, although the level of disease suppression was dependent upon specific bacterial strains. Furthermore,

treatments using different strains or strain mixtures predominantly resulted in increased representation of the inoculated strains, suggesting that disease suppression was due to an alteration of the stigma microbiome structure. Compared with treatments using single strains, a *Pantoea*–*Pseudomonas* strain mixture produced a homogeneous microbiome structure with less interflower variability. Findings from this study suggest that the microbiome on the flower stigma can be manipulated through microbial inoculation. Due to flowers' short life span yet important role in plant disease infection, even a short-term influence on microbiome composition may result in significant decreases in disease susceptibility.

Keywords: bacteriology, fire blight, flower, microbiome, microbiome manipulation, rhizosphere and phyllosphere, stigma

Flowers play a critical role in lifecycles of angiosperm plants by providing a platform for diverse pollination possibilities resulting in the union of sperm with ovules. During pollination, flowers produce nutrient-rich exudates, including stigma exudates, pollen exudates, and nectar, to promote germination of pollen grains or to attract pollinators (Crawford and Yanofsky 2008). Such exudates also support the growth of many microorganisms, including those that

are either beneficial or pathogenic to the host (Alekkett et al. 2014; Pusey et al. 2009; Spinelli et al. 2005).

Our recent studies characterized the composition and assembly of the microbiome on apple flowers (Steven et al. 2018), with a focus on the stigma (Cui et al. in press). In general, the apple flower microbiome is dominated by bacteria in the phylum *Proteobacteria* (>90%), with small contributions by species of *Cyanobacteria*, *Actinobacteria*, *Firmicutes*, and *Bacteroidetes* (Steven et al. 2018). Temporal patterns in the stigma microbiome development revealed a diverse bacterial community at the early stage of bloom evolving into a community dominated by two families within the phylum *Proteobacteria*, the *Pseudomonadaceae* and *Enterobacteriaceae* (Cui et al. in press). This suggests that members within the *Pseudomonadaceae* and *Enterobacteriaceae* are best adapted for the stigma environment, and form the core members of the apple stigma microbiome (Cui et al. in press; Pusey et al. 2009; Stockwell et al. 2010).

Fire blight, caused by a bacterial pathogen *Erwinia amylovora*, is an important disease of Rosaceous plants such as apple and pear (van der Zwet et al. 2012). One of the major entry points of *E. amylovora* to host plants is flowers, and successful infection of flowers is highly dependent on establishing an epiphytic pathogen

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population on the stigma portion of flowers during bloom (Thomson 1986). Stigmatic colonization by *E. amylovora* is influenced by various biotic and abiotic factors such as temperature, humidity, flower age, and morphology (Pusey 2000; Steiner 1990). To date, no sustainable and completely effective control methods have been developed for this disease, and control mainly relies on the use of copper formulations and the antibiotic streptomycin (Sundin et al. 2016; Vanneste et al. 1995). However, such practices are becoming increasingly limited due to concerns about the phytotoxicity caused by copper and the recent development and spread of streptomycin resistance in *E. amylovora* populations (McGhee et al. 2011; Sundin et al. 2016). An alternative tool for plant disease management is biological control (biocontrol), the use of a beneficial microorganism to suppress plant disease (Ait Bahadou et al. 2018; Bonaterra et al. 2007; Johnson and Stockwell 1998; Pusey et al. 2009; Sundin et al. 2009; Wilson et al. 1992). In order for these treatments to be effective, the inoculated microbe needs to colonize the host, in essence becoming a member of the microbiome. However, there exists a significant knowledge gap concerning how plant microbiomes are acquired and develop, and how ecological processes such as invasion occur.

There is evidence that microbiome composition can influence plant disease development (Berendsen et al. 2018; Morella et al. 2019; Purahong et al. 2018). A large number of plant pathogens such as *Pseudomonas syringae*, *E. amylovora*, and *Xanthomonas citri* grow epiphytically on plant surfaces prior to causing infection in the plants (Brunings and Gabriel 2003; Pusey 2000; Xin and He 2013). By secreting carbon to the plant surface, plants recruit an assemblage of microbes to compete with the pathogens for nutrients and space, thereby acting as a first line of defense (Leben 1965; Weller et al. 2002). With regards to fire blight control, previous studies have investigated inoculating beneficial bacterial strains to open flowers as biological control (Stockwell et al. 2010, 2011; Sundin et al. 2009). Selection of such strains is often initiated from an in vitro test for antibiosis, or on detached flowers, followed by field testing (Johnson and Stockwell 2000; Pusey 1997). Strains tested for disease control efficacy include *P. fluorescens* A506, *Pantoea vagans* C9-1, and *Bacillus amyloliquefaciens* QST 713 (Ait Bahadou et al. 2018; Chen et al. 2009; Mikiciński et al. 2020; Pusey et al. 2011; Stockwell et al. 2010). Although these treatments have shown promising results in suppressing fire blight disease occurrence and reducing pathogen populations, the downstream effects on the composition of the microbiome have not been assessed.

In this study, we used the apple flower as model to test the hypothesis that manipulating the structure of the microbiome would be associated with changes in disease incidence. Furthermore, we predicted that more complex inoculations consisting of multiple bacterial strains would potentially outperform single-species inoculations by forming a more diverse microbiome structure. Our results demonstrate that inoculating natural stigma-inhabiting bacteria during bloom altered the structure of the stigma microbiome and reduced fire blight disease incidence. However, contrary to our hypothesis, more complex inoculations were not associated with better performance as a biocontrol. Instead, the presence of a specific bacterium, with no observed antagonistic activity, was responsible for decreasing fire blight. Taken together, these data indicate the utility of microbiome manipulation as a tool to control fire blight while also shedding light on factors that control the assembly and composition of the flower microbiome.

MATERIALS AND METHODS

Sampling site. To exclude the effects of host and environmental conditions, studies were conducted with flowers from 27 trees of the

same apple cultivar (*Malus domestica* 'Golden smoothie') on MM104 rootstock planted in two adjacent blocks at Lockwood Farm, Hamden, CT (41.406 N, 72.906 W) in a 16-foot-by-21-foot spacing. All trees were the same age (planted in 1970) and were maintained under the same management program (<https://ag.umass.edu/fruit/publications/2020-new-england-tree-fruit-management-guide>). The trees were used in fire blight research in previous years but were subject to extensive pruning to remove the overwinter inoculum prior to the flowering season. During bloom, approximately 300 flowers were produced on each tree. Weather data (temperature and humidity) prior to and during bloom (from 1 to 30 May 2019) is summarized in Supplementary Table S2.

Isolation of stigma colonizing bacteria. Stigma-colonizing bacteria were isolated from the stigma portion of Early Macoun apple flowers at full bloom on 8 May 2018. The stigma portion of a single apple flower was dissected and placed in a sterile 1.5-ml microcentrifuge tube containing 200 μ l of 0.5 $\times$ phosphate-buffered saline (PBS). In total, 100 single flowers from six trees were sampled. The microcentrifuge tubes containing stigma were placed in a water bath sonicator for 5 min followed by 30 s of vortexing. PBS (5 μ l) from each tube was spread on Lysogen Broth (LB) agar plates containing cycloheximide (50 μ g ml⁻¹) to inhibit fungal growth. Single colonies of different morphologies from each plate were restreaked onto a fresh LB agar plate to obtain pure cultures. More than 1,000 bacterial isolates were obtained. The 16S ribosomal RNA (rRNA) of the isolates was PCR amplified using 63f (5'-CAG GCC TAA CAC ATG CAA GTC-3') and 1387r (5'-GGG CGG WGT GTA CAA GGC-3') and sequenced by Sanger sequencing. The 88 strains described in this study make up a representative subset based on the strains' colony morphology.

Bacterial treatments of open flowers. Bacterial strains of *Pseudomonas* sp. strain CT-1059, *Pantoea* sp. strain CT-1039, *Enterobacter* sp. strain CT-1341, and *Curtobacterium* sp. strain CT-1342 were stored at -80°C in 20% glycerol. All strains were cultured in LB medium at 28°C overnight and adjusted to an optical density at 600 nm (OD₆₀₀) = 1.0 (approximately 10⁹ CFU/ml). The 27 apple trees were randomly labeled into nine treatment groups, with 3 replicate trees in each treatment group. Each treatment group received with one of nine treatments: (i) *Pseudomonas*, (ii) *Pantoea*, (iii) *Enterobacter*, (iv) *Curtobacterium*, (v) *Pseudomonas*-*Pantoea*, (vi) *Pseudomonas*-*Pantoea*-*Enterobacter*, (vii) *Pseudomonas*-*Pantoea*-*Enterobacter*-*Curtobacterium*, (viii) water, and (ix) streptomycin. Microbial treatments, regardless of single strain or strain mixtures, contained equal amount of the total bacteria (4 $\times$ 10⁷ CFU/ml). Overnight bacterial culture (600 ml, adjusting to OD₆₀₀ = 1.0) was directly diluted into 14.2 liters of water and used for spray. Equal amounts of ingredient bacteria were mixed together at the site of application. Water was used as a negative control.

Microbial and water treatments were applied twice, once at 50% bloom (8 May 2019) and again at 70% bloom (9 May). Approximately 3.8 liters of bacterial suspension at the final concentration of 4 $\times$ 10⁷ CFU/ml was applied to each tree. *E. amylovora* was inoculated onto the flowers by spraying a bacterial suspension (1 $\times$ 10⁶ CFU/ml) on 12 May (100% bloom), immediately after sampling of stigma microbiome. The antibiotic streptomycin (100 ppm) was applied on the same day (12 May), 2 h after *E. amylovora* inoculation, and again at 24 h after the first inoculation.

Sampling for the stigma microbiome and quantification of disease incidence. Sampling of stigma microbiomes was performed on 12 May 2019 at 100% bloom, 3 days after the second microbial treatments. Ten flowers were collected from a labeled branch of each treated tree, from which the unopened flowers were removed prior to microbial treatment to ensure that all flowers received the inoculum. The stigma portion of each individual flower was

harvested with a sterile scissors and placed in a sterile 1.5-ml microcentrifuge tube. Collected samples were kept on ice during transportation and were stored at -80°C until DNA isolation.

Three weeks after pathogen inoculation (30 May), inoculated flowers were rated for the development of blossom blight symptoms (black withering, dying of the apple flowers, and emergence of ooze droplets). Percentage of diseased flower clusters in the total number of flower clusters was determined for each tree (Johnson and Temple 2013). For each individual tree, disease symptoms were rated on more than 200 flower clusters by three individual people.

DNA extraction and 16S rRNA gene sequencing. DNA was extracted from the surface of stigmas collected from each individual flower. Stigmas were submerged in 200 μl of 0.5 $\times$ PBS buffer in a microcentrifuge tube. Epiphytic microbes were removed from stigma surface by water bath sonication for 5 min, followed by vortex for 30 s. Stigmas were removed from the tube and the DNA was extracted from the solution using the DNeasy PowerSoil Pro Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The isolated DNA in 100 μl of water was further concentrated by precipitating in 100% ethanol. Isolated DNA was used as template in a PCR to amplify the V4 region of the bacterial 16S rRNA gene using the 515f/806r primer set, with both primers containing a 6-bp barcode unique to each sample (Sinclair et al. 2015). The amount of template DNA added in the PCR (25 μl) ranged from 10.0 to 20.0 ng, as determined by Nanodrop2000 (Thermo Fisher Scientific, Waltham, MA, U.S.A.). Peptide nucleic acid clamps to block the PCR amplification of apple plastid and mitochondrial sequences were included in the reaction, as previously described by Steven et al. (2018). Successful PCR amplifications at the correct amplicon size were confirmed by gel electrophoresis. The PCR products were purified and normalized using the SeqalPrep normalization plate kit (Invitrogen, Carlsbad, CA, U.S.A.). Pyrosequencing was conducted on an Illumina MiSeq platform through services provided by the Yale Center for Genome Analysis.

Bioinformatics and statistical analysis. Illumina sequencing reads were assembled into contigs and quality screened using mothur v1.39.5 (Schloss et al. 2009). Sequences that were at least 253 bp in length, contained no ambiguous bases, and no homopolymers of more than 8 bp were used in the analysis. Chimeric sequences were identified by using the VSEARCH (Rognes et al. 2016) as implemented in mothur, and all potentially chimeric sequences were removed. To maintain a similar sampling effort between samples, samples with <10,000 sequences per sample were also removed. The resulting sequence counts per sample are presented in Supplementary Table S1. Negative control (PCR using sterile H_2O as a template) was also included in sequence datasets. The sequence data are deposited at the Sequence Read Archive under accession number PRJNA628946.

Sampling effort was normalized to the depth of the smallest sample and operational taxonomic units (OTUs) were defined at 100% sequence identity, employing the OptiClust algorithm in mothur (Westcott and Schloss 2017). Taxonomic classification of sequences was performed with the Ribosomal Database Project classifier against the SILVA v132 reference alignment in mothur (Quast et al. 2012; Wang et al. 2007). Nonmetric multidimensional scaling (NMDS) was used to visualize the pairwise distances among samples with Bray-Curtis distances in the Vegan package in R (Dixon 2003). Descriptive diversity statistics were calculated in mothur. The nonparametric Shannon's diversity index and disease incidence in each treatment were generated with the ggplot2.0 package for R (Wickham 2016). Statistically significant differences in diversity statistics were identified with a one-way analysis of variance and the Tukey-Kramer post hoc test in the agricolae package in R.

In vitro coinoculation assay and antagonistic assay. Overnight LB cultures of *Pseudomonas* sp. strain CT-1059, *Pantoea* sp. strain CT-1039, *Enterobacter* sp. strain CT-1341, and *Curtobacterium* sp. strain CT-1342 were adjusted to $\text{OD}_{600} = 1.0$ (approximately 10^8 CFU/ml). A narrow line of each strain inoculum was streaked on both horizontal and perpendicular orientations on the LB agar plate using an inoculum loop. For the antagonistic assay, a testing strain was inoculated at the center of an LB agar plate. After 48 h postincubation, *E. amylovora* strain 110 was stamped onto the plates using a prong dipped in bacterial suspension (approximately 10^2 CFU/ml). Each treatment had three biological replicates and the experiment was repeated twice.

RESULTS

Selection of strains for microbiome manipulation. A culture collection of 88 bacterial strains was obtained from the stigmas of apple flowers of cultivar Early Macoun collected from Lockwood Farm, CT (41.406 N, 72.906 W). 16S rRNA gene sequencing placed the isolates in three phyla and five families (Supplementary Fig. S1). We chose four strains for testing their efficacy in suppressing fire blight disease (Table 1; Supplementary Fig. S1B). The rationale for choosing these four strains was as follows. *Pseudomonas* and *Pantoea* were chosen because there is a history of using related strains in fire blight control (Stockwell et al. 2010, 2011). Genus *Enterobacter* was chosen because it was closely related to a highly abundant OTU found in our previous work (Cui et al. in press). We anticipated that it might inhibit pathogen colonization by niche exclusion, by effectively monopolizing the stigma environment. Finally, genus *Curtobacterium* was selected as an out-group. Our previous data showed that bacteria within the phylum *Proteobacteria* generally accounted for >90% of the microbial diversity of the stigma (Cui et al. in press). We wanted to test whether a bacterium outside this phylum could colonize the stigma and observe the interaction with disease development.

We also wanted to design more complex strain mixtures in order to test whether increasing the complexity of the inoculated strains would affect disease suppression. A coinoculation assay demonstrated that the strains tested did not antagonize each other when cultured under an in vitro condition (Supplementary Fig. S2). We employed *Pseudomonas* and *Pantoea* spp. as the base strains for the mixtures based upon the previous use of related strains in fire blight biocontrol (Stockwell et al. 2010). The strain mixtures increased in complexity from two to four strains, with equal representation of each component strain (mix 1, *Pseudomonas* and *Pantoea*; mix 2, *Pseudomonas*, *Pantoea*, and *Enterobacter*; mix 3, *Pseudomonas*, *Pantoea*, *Enterobacter*, and *Curtobacterium*).

Impact of microbiome manipulation on fire blight disease occurrence. The microbial inoculum consisted of overnight cultures diluted to a final density of 4×10^7 CFU ml^{-1} . Water was used as a negative control. The seven inoculations, including four single-strain treatments and three strain mixtures, were spray inoculated onto open flowers of apple trees twice during the period of bloom at the point at which approximately 50 and 70% of flowers were observed to be open. To evaluate whether the microbial treatments had any impact on the occurrence of fire blight disease, we inoculated the pathogen *E. amylovora* to flowers 4 days after the initial microbial treatments. The antibiotic streptomycin was used as a positive control treatment, and was sprayed to flowers twice, at 2 and 24 h post pathogen inoculation. The percentage of flowers that developed disease symptoms was assessed 3 weeks later. In total, 33.2% of water-treated and 11.7% of streptomycin-treated flowers developed fire blight symptoms (Fig. 1). Compared with the water treatment, flowers that received a treatment of any single or multiple

strains showed a trend toward lower disease incidence, although the difference was only significant for the *Pantoea*-alone treatment and the various strain mixtures (Fig. 1). None of the treatments achieved the same disease reduction as the streptomycin treatment, yet the *Pseudomonas*-*Pantoea* and the four-strain mixture produced statistically similar results (Fig. 1). Among the single-microbe treatments, flowers treated with *Pseudomonas* or *Pantoea* showed lower disease incidence than those treated with *Enterobacter* and *Curtobacterium*, though the *Pseudomonas* treatment was not significantly different than the water control (Fig. 1). Compared with the single-strain treatments, the strain mixtures did reduce disease incidence; however, they were not significantly different than *Pantoea* alone (Fig. 1). These data indicate that *Pantoea* may be the core contributor to the control of fire blight on apple flowers, although its activity may be partially enhanced with the addition of other flower microbiome members.

Microbiome composition after manipulation. To determine whether the inoculated microbes influenced the stigma microbiome, we performed 16S rRNA gene sequencing to evaluate the microbiome composition of each individual flower sample 3 days after the second microbial treatment (right before pathogen inoculation). In total, 11,610,049 high-quality filtered sequences were obtained from 78 samples, with the number of sequences ranging from 68,767 to 190,579 (Supplementary Table S1). These sequences were clustered into 147,102 OTUs (mean 2,823 per sample) at 100% sequence similarity.

The Bray-Curtis distances among samples of each microbial treatment were visualized using NMDS, which showed that samples of the same microbial treatment clustered together and separated from samples from other treatments (permutation analysis of variance, $P = 0.001$) (Fig. 2A). This result was confirmed by β -dispersion test ($P = 0.002$) and suggests that the inoculated microbes did indeed affect the microbiome structure. Samples of the control dataset (water-treated) showed a large dispersion, suggesting a high variation of microbiome structure among individual flowers under natural conditions (Fig. 2A). Among different single-strain treatments, a higher level of variation in the microbiome structure among individual flowers was observed in *Pseudomonas* (Fig. 2A, red) and *Curtobacterium* (Fig. 2A, yellow) treatments than the *Pantoea* (Fig. 2A, green) and *Enterobacter* (Fig. 2A, light blue) treatments, suggesting that the impact of microbial manipulation on the microbiome structure is largely strain dependent. The structures of microbiome manipulated by mixtures of three (Fig. 2A, dark blue) and four (Fig. 2A, purple) microbes largely overlapped, suggesting that addition of multiple strains drove the structure of the

microbiome to a similar composition. Notably, a consistent observation was that the treatments that included the *Pantoea* strain showed tighter clustering between samples, suggesting a smaller variability in microbiome structure between individual stigmas (Fig. 2A).

Diversity of the stigma communities was assessed by calculating the Shannon's diversity index. Samples of different single-strain treatments displayed various levels of diversity, with the diversity in *Enterobacter*-treated samples being the lowest in comparison with others. In samples treated with double-, triple-, and quadruple-strain mixtures, we observed an increase in microbial diversity as the number of strains included in the treatment increased (Fig. 2B). Differences in diversity were further supported by the similar patterns in community richness and evenness (Fig. 2B, lower panels). These findings suggest that inoculation of different strains

Fig. 1. Percentage of apple flower clusters that developed fire blight symptoms. Bacterial treatments were applied at 50 and 70% bloom, with three individual apple trees included in each treatment. *Erwinia amylovora* strain 110 was applied to all trees at 100% bloom 3 days after bacterial inoculations. Change in disease incidence compared with the water-treated control are indicated in parentheses. Different letters denote statistical significance ($P < 0.05$, identified by analysis of variance comparisons of means, employing a posthoc Tukey-Cramer test for multiple comparisons). Control: flowers sprayed with H_2O , Streptomycin: flowers sprayed with antibiotic streptomycin (100 ppm), Pseu: *Pseudomonas* spp., Panto: *Pantoea* spp., Entero: *Enterobacter* spp., and Curto: *Curtobacterium* spp.

TABLE 1
Taxonomic assignments of stigma-isolated bacteria used for this study^a

Isolates	Blastn by NCBI		Relationship to representative OTU sequences		Accession ^d
	Closest BLAST match	SI (%) ^b	Match ^c	SI (%) ^b	
<i>Pseudomonas</i> spp. CT-1059	<i>Pseudomonas graminis</i>	100	OTU2	100	MT396635
<i>Pantoea</i> spp. CT-1039	<i>Pantoea agglomerans</i>	100	OTU3	100	MT396714
<i>Enterobacter</i> spp. CT-1341	<i>Enterobacter</i> spp.	100	OTU1	100	MT396725
<i>Curtobacterium</i> spp. CT-1342	<i>Curtobacterium flaccumfaciens</i>	100	OTU4	100	MT396712

^a Operational taxonomic unit (OTU) numbers: OTU1 = *Enterobacter* sp. strain CT-1341, OTU2 = *Pseudomonas* sp. strain CT-1059, OTU3 = *Pantoea* sp. strain CT-1039, and OTU4 = *Curtobacterium* sp. strain CT-1342.
^b Sequence identity.
^c Closest match.
^d Accession number.

and strain mixtures to flowers affected the flower microbiome, resulting in different community structures and diversity.

Phylum level taxonomic bins and the *Enterobacteriaceae* and *Pseudomonadaceae* ratio. To investigate how the microbial inoculations affected the composition of the stigma microbiome, 16S rRNA gene sequences were classified to the phylum level. In total, 24 phyla were detected across the different treatments. Excluding the *Curtobacterium* treatment, samples were composed predominantly of *Proteobacteria* (94.1 to 99.9%), with a small percentage of *Actinobacteria* (0.1 to 5.9%). This included the water control, with no bacterial treatment (Fig. 3A). In the samples manipulated with genus *Curtobacterium*, a member of the *Actinobacteria*, the percentage of *Proteobacteria* was reduced to a mean of 53.8% (Fig. 3A). Looking at the ratio of *Actinobacteria* sequences in each of the flowers treated with *Curtobacterium* spp., a wide variation between flowers was observed, with *Proteobacteria* sequences still being dominant in several of the individual samples (Fig. 3B). Moreover, samples manipulated with the four-microbe mixture also exhibited a slight decrease in percentage of *Proteobacteria*, which could be explained by the colonization of *Curtobacterium* (Fig. 3A). These findings indicated that, although *Curtobacterium* is not typically an abundant core member of the stigma microbiome, it is still able to

predominate on stigmas when given a competitive advantage by artificial inoculation.

We have previously shown that two families of bacteria tend to predominate on the stigma, the *Enterobacteriaceae* and the *Pseudomonadaceae* (Cui et al. in press; Steven et al. 2018). We determined the ratio of *Enterobacteriaceae* to *Pseudomonadaceae* ($R_{E/P}$) of individual flowers across the treatments. The $R_{E/P}$ was reshaped by the various microbiome manipulations. The $R_{E/P}$ of the water-treated dataset varied significantly (243%) among the samples (Fig. 3C). Some microbial treatments still displayed high variations in the $R_{E/P}$ (such as the single-strain treatments of *Pseudomonas* and *Curtobacterium*), yet many other treatments tended to stabilize the $R_{E/P}$ between individuals, as observed in single-strain treatments of *Pantoea* and *Enterobacter* and all strain mixture treatments (Fig. 3C). The effect was most pronounced in the single-strain treatments of *Pantoea* and *Enterobacter*, which became almost exclusively dominated by the family *Enterobacteriaceae* to the exclusion of the family *Pseudomonadaceae* (Fig. 3C). Interestingly, the *Pseudomonas* treatment did not result in a similar exclusion of *Enterobacteriaceae*, suggesting that *Pseudomonas* is not as adept at monopolizing the stigma environment. Similarly, the multiple-strain mixtures that included *Pseudomonas*

Fig. 2. A, Nonmetric multidimensional scaling (NMDS) plot displaying relationships of stigma microbial community composition in samples from water-treated control (black X) and different bacterial treatments. Distances were calculated using the Bray-Curtis metric and the stress value of the ordination is indicated. Statistically significant differences in clustering were evaluated via both permutation analysis of variance and the β -disper test, and P values (95% confidence) are indicated. **B,** Comparative analysis of community diversity (Shannon index), richness (number of recovered operational taxonomic units), and evenness (Shannon's evenness) among stigma samples. Different letters denote statistically significant differences (P value < 0.05) identified by analysis of variance comparisons of means, employing a posthoc Tukey-Cramer test for multiple comparisons. Control datasets are included for reference only and were not included in the statistical comparisons. Control: flowers sprayed with sterile H_2O , *Pseu*: *Pseudomonas* spp., *Panto*: *Pantoea* spp., *Entero*: *Enterobacter* spp., and *Curto*: *Curtobacterium* spp.

consistently showed a higher ratio of *Enterobacteriaceae*, suggesting that *Enterobacteriaceae* consistently outcompetes *Pseudomonadaceae* and achieves higher relative abundances.

Identification of the inoculated strains in the microbiome. The sequences in each dataset were classified to the OTU level (100% sequence identity) and representative sequences for each OTU were interrogated against the isolate sequences. Four OTUs were identified that shared 100% sequence identity to the isolates: OTU1-*Enterobacter*, OTU2-*Pseudomonas*, OTU3-*Pantoea*, and OTU4-*Curtobacterium*. The relative abundance of each OTU was employed as a metric to determine the isolate's abundance in the stigma microbiome. OTU1 was the most abundant and conserved in the dataset (relative abundance ranging from 0.6 to 94.2% of recovered sequences), followed by OTU2 (0 to 94.9%), OTU3 (0 to 72.2%), and OTU4 (0 to 92.1%). In the water-treated control samples, the majority of OTUs were accounted for by OTU1 (mean 62.2%) and OTU2 (mean 6.7%), with a large abundance of other OTUs (rare = 31.1%) (Fig. 4). OTU abundance was generally predictable from the treatments, in that the inoculated OTUs were enriched in the treatment datasets (Fig. 4). There were two notable exceptions. OTU1-*Enterobacter* was highly abundant whether or

not it was included in the inoculation. OTU1 showed the lowest abundance when it was coinoculated with *Pseudomonas*. However, including other bacteria in the inoculation once again allowed OTU1 to dominate the stigma microbial community. The second exception to an inoculated strain gaining an advantage in the microbiome was OTU3-*Pantoea*. When inoculated alone, it achieved a mean relative abundance of 42.0% yet, when coinoculated with *Pseudomonas*, it only accounted for 0.04% of sequences. However, the mean relative abundance of *Pseudomonas* in the *Pseudomonas*–*Pantoea* mixture (9.6%) was significantly lower than when *Pseudomonas* was sprayed alone (75.4%). Moreover, the coinoculation of *Pseudomonas* and *Pantoea* together resulted in a relative abundance of OTU1-*Enterobacter* similar to when *Enterobacter* was inoculated alone. This apparent paradox of coinoculating *Pseudomonas* and *Pantoea* benefitting the non-inoculated *Enterobacter* may point to a competition between two organisms opening a niche for a third. Taken together, these observations point to complex interactions in the plant microbiome that underpin our need to characterize the functional ecology of this system, and documenting the cooperative and antagonistic relationships between community members.

Fig. 3. **A**, Mean relative abundance (RA) of the predominant bacterial phylum *Proteobacteria*. **B**, Relative abundance of the phyla *Actinobacteria* and *Proteobacteria* on individual flowers manipulated with *Curtobacterium*. The category "rare" represents the sum of the remaining taxa. **C**, Relative abundance of the two predominant bacterial families upon water and microbial treatments. Each dot represents a single flower sample. The ratio of *Enterobacteriaceae* to *Pseudomonadaceae* is presented on the top of each treatment with standard deviation. Statistical significance was determined by analysis of variance comparisons of means, employing a posthoc Tukey-Cramer test for multiple comparisons. Control: flowers sprayed with sterile H₂O, Pseu: *Pseudomonas* spp., Panto: *Pantoea* spp., Entero: *Enterobacter* spp., and Curto: *Curtobacterium* spp.

In vitro antagonistic test of selected strains against *E. amylovora*. The selection criteria of the strains tested in this study were not the biocontrol activities but, rather, their representation of different members of the stigma microbiome. Our final goal was to determine the potential mechanisms of disease suppression by the inoculated strains in an in vitro assay. Of all four strains tested, only *Pseudomonas* showed inhibition against the growth of *E. amylovora* on the LB agar medium (Fig. 5). Interestingly, despite the antagonistic activity toward *E. amylovora*, the *Pseudomonas* strain did not show the highest disease inhibition (Fig. 5). Instead, the *Pantoea* strain, alone or in a strain mixture, showed the greatest disease suppression (Fig. 5). One potential explanation for the reduced disease incidence could be attributable to niche exclusion of *E. amylovora* on apple stigmas, which is consistent with previous observations (Giddens et al. 2003). However, when the *Pantoea* was mixed with *Pseudomonas*, disease suppression was similar to *Pantoea* alone or in the other strain mixtures (Fig. 5) but the relative abundance of *Pantoea* dropped to >1% of recovered 16S rRNA sequences (Fig. 4). This suggests that the presence of *Pantoea* is still potentially inhibitory to fire blight development, even when present in low relative abundance. Thus, these data suggest that *Pantoea* antagonistic activities were not apparent on LB media and would not have been predicted from the relative abundance of *Pantoea*-related sequences (particularly in the *Pseudomonas*–*Pantoea* mixture). These data highlight the importance of monitoring the microbiome in situ to assess the complex interactions that occur between members and how they may interact with outcomes such as disease suppression.

DISCUSSION

In this study, we demonstrated that the microbiome structure can be altered by artificial inoculation, which resulted in attenuated disease occurrence. Many trials in manipulation of the microbiome with the intention to influence host–microbe interactions, suppress diseases, or promote host-beneficial traits have been attempted (Brugman et al. 2018). The outcomes of such efforts vary

significantly, and are highly influenced by the host environment, nutrient stage, and manipulation approaches (Berg and Koskella 2018; Brugman et al. 2018). Unlike many host environments, flowers are nutrient rich but also directly exposed to the natural environment, providing an easy access for manipulation. Compared with some environments such as the root microbiome that require a long-term, steadfast microbiome intervention to produce beneficial effects (Bakker et al. 2013), flowers have a short window of bloom; thus, the microbiome manipulation could be short-term yet the influence on disease control and plant health could still be significant.

Taxonomic diversity is an important measure of microbiome structure and function (Hu et al. 2016; Trivedi et al. 2012). Our data suggest that the diversity of the microbial community on the apple stigma is not directly correlated with the occurrence of fire blight. For example, similar taxonomic diversity was observed on flowers treated with strains of *Enterobacter* and a mixture of *Pseudomonas* and *Pantoea*, although the disease occurrence of such treatments differed (Figs. 1 and 2B). Furthermore, among treatments of double-, triple-, and quadruple-strain mixtures, although the diversity gradually increased in accordance with the increasing number of strains, no significant difference in their disease protection was observed (Figs. 1 and 2B; Supplementary Fig. S3). In other plant hosts such as tomato, the level of disease protection ability of a constructed bacterial community was also found to be independent of overall bacterial diversity (Berg and Koskella 2018). Thus, the hypothesis that a more complex microbial inoculation would result in better disease suppression was not supported. Here, our data point to a trend that treatments with *Pantoea* generally lead to better disease suppression than treatments that didn't include *Pantoea*.

Natural flowers harbored varied microbiome communities, resulting in unique structures. The largest effect of the microbial treatments on the microbiome was a reduction in the interindividual variability, with the exception of treatments with *Pseudomonas* and *Curtobacterium*. In particular, microbial treatments using *Pseudomonas*–*Pantoea*-based double-, triple-, and quadruple-strain

Fig. 4. Identification of inoculated strains in the microbiome. Each column represents the microbial composition of a single flower. Four operational taxonomic units (OTUs) with 100% sequence identity to the inoculated strains are displayed, and the category “other” represents the sum of the remaining taxa. OTU1: *Enterobacter* sp. strain CT-1341, OTU2: *Pseudomonas* sp. strain CT-1059, OTU3: *Pantoea* sp. strain CT-1039, and OTU4: *Curtobacterium* sp. strain CT-1342.

mixtures resulted in the smaller distances between samples in the NMDS clustering (Fig. 2A) and the similarity in the R_{EP} between replicate flowers (Fig. 3C). This is important because it suggests that there should also be more uniformity in any phenotypic effects induced by the microbiome. A common observation is that only a proportion of flowers sprayed with *E. amylovora* in the field will later go on to develop fire blight (Cui et al. in press; Sundin et al. 2009). Similarly, many biocontrol strains have been described with excellent antagonistic potential under in vitro or in planta conditions; however, they present inconsistent effectiveness and year-to-

year variation under field conditions (Johnson and Stockwell 1998; Sundin et al. 2009). One potential reason for the variability observed at the individual flower level and for interannual irregularity could be the large interindividual disparity in microbiome structure between flowers. Thus, by shifting the microbiome to a state that is predictable, repeatable, and stable and also induces disease resistance, we may be able to manage such variations. Although none of the manipulations employed here attained the same disease reduction as the streptomycin treatment, they did produce a highly conserved microbiome structure (Fig. 4) and up to an approximately

Fig. 5. In vitro antagonism of strain **A**, *Pseudomonas* spp.; **B**, *Pantoea* spp.; **C**, *Enterobacter* spp.; and **D**, *Curtobacterium* spp. against *Erwinia amylovora* strain 110. Tested strains were precultured on Lysogen Broth agar plates for 48 h. *E. amylovora* 110 was inoculated onto the plates using a prong dipped in bacterial suspension.

45% decrease fire blight (Fig. 1). This suggests that a microbiome-informed treatment strategy may be a viable path to controlling plant diseases.

Recent work trying to predict community assembly from first principals found that complex communities seeded on a glucose minimal medium predictably adopted a state dominated by members of the families *Enterobacteriaceae* and *Pseudomonadaceae* in a phenomenon termed “emergent simplicity” (Goldford et al. 2018). The predictability of this assembly points to certain metabolic rules, based on cross-feeding, determining the final state of the microbiome (Estrela et al. 2020; Treves et al. 1998; Turner et al. 1996). Here, we observed a similar phenomenon in a natural environment. The ratio of families and the particular members of the microbiome can be seeded early in the development of the stigma microbiome, leading to a predictable community structure. Untangling the metabolic interactions between community members will better inform mechanisms by which the microbiome can be shifted to potentially beneficial states.

One limitation of this study is that some LB ingredients were sprayed to flowers during the microbial treatments, because the overnight culture of the bacterial inoculum was directly diluted in water prior to application on flowers. This limitation is caused by the need for large volumes of inoculum for field application and the unavailability of a large centrifuge that can process liters of bacterial culture to remove the media ingredients. The dilution factor for the bacterial cultures was approximately 25-fold and the media content was largely depleted after bacterial growth; thus, the amount of nutrients remaining in the bacterial inoculum was likely minimal. However, the leftover nutrients and potential secondary metabolites were not present in the water controls, which could account for some of the observed differences. Thus, during the analysis of the microbiome data, we only directly compared the different microbial treatments. The microbiome structure, composition, and diversity in water controls were provided as a reference for the development of the microbiome in the absence of manipulation.

In summary, our data point to a common state of the stigma microbiome being dominated by bacteria in two families, *Enterobacteriaceae* and *Pseudomonadaceae*. However, a probiotic spray in the field can determine the particular members that make up this state. Treating flowers with certain microbial members, particularly a strain of *Pantoea*, was associated with disease suppression. Multispecies inoculations showed that there are complex interactions between the treatment strains but there is some evidence that a more complex inoculation can enhance disease resistance. These data will inform both treatments for fire blight as well as the ecological principals and interactions that determine the structure and function of the flower microbiome.

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