Comparative Dynamics of Adherent and Nonadherent Bacterial Populations on Maize Leaves

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ABSTRACT

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The dynamics of the adherent and nonadherent populations of three bacterial species on maize leaves were examined to identify the extent to which bacteria adhere to leaves and the importance of this adhesion to leaf colonization. *Pantoea agglomerans* strain BRT98, *Clavibacter michiganensis* subsp. *nebraskensis* strain GH2390, and *Pseudomonas syringae* pv. *syringae* strain HS191R all rapidly adhered to maize leaves following inoculation, but differed in the percentage of cells that adhered to the leaves. Immediately following inoculation, the percentage of adherent cells was highest for the saprophyte *P. agglomerans* (8 to 10%) and was much lower for the pathogens *C. michiganensis* subsp. *nebraskensis* and *P. syringae* pv. *syringae* (2 to 3 and <1%, respectively), although the results for *P. syringae* pv. *syringae* HS191R were based on only one experiment. In the 4 days following inoculation, the percentage of the *P. agglomerans* populations that adhered to the leaves increased to approximately 70%. Similarly, the percentage of *C. michiganensis* subsp.

nebraskensis and *P. syringae* pv. *syringae* cells that resisted removal steadily increased in the days following inoculation, although these increases probably reflected both adherence and localization to endophytic sites. Based on differences in the percentage of cells adhering to several cuticular wax mutants of maize, the rapid adherence of *C. michiganensis* subsp. *nebraskensis* cells to maize leaves was influenced by the cuticular wax properties, while the rapid adherence of *P. agglomerans* was not. Finally, bacterial adherence to leaves was advantageous to *P. agglomerans* survival and growth on leaves based on the finding that the nonadherent populations of the *P. agglomerans* strain decreased significantly more than did the adherent populations in the 24 h following inoculation, and increased much less than did the adherent populations over the next 3 days. Similar results with the *C. michiganensis* subsp. *nebraskensis* and *P. syringae* pv. *syringae* strains indicate that bacterial adherence to leaves, bacterial movement to endophytic sites, or both were advantageous to the survival and growth of these strains on leaves.

Additional keywords: epiphyte, glossy mutants, phyllosphere, *Zea mays*.

Bacterial populations associated with aerial plant leaves are composed of populations that differ in the nature of their association with the leaf surface, as well as in their location relative to the leaf surface. For example, some cells may be adhered to the leaf surface, while others are not, and some may be located in endophytic sites, such as in the substomatal cavities or in the spaces between the mesophyll cells. These distinct populations may differ in their ability to grow and survive on leaves due to differences in their exposure to removal forces, such as wind and rain; to environmental stresses, such as desiccation and solar radiation; and to nutrients, which may differ in endophytic versus surface sites. Knowledge of the size and dynamics of adherent versus nonadherent populations, as well as epiphytic versus endophytic populations, should provide a better understanding of the processes by which bacteria successfully establish and maintain populations on leaves.

Microbial adherence to leaves has been proposed to occur in two stages: a rapid stage that immediately follows leaf contact and a second, slower stage (5,19). Rapid adherence of bacterial cells to leaves has been demonstrated in two previous studies. Haas and Rotem (13) found that, only 1 min after inoculation, gentle washing did not remove 93% of the *Pseudomonas syringae* pv. *lachrymans* cells applied to a cucumber leaf. Similarly, Preece and Wong (18) found that, after 30 min, washing did not remove 11 to

97% of the cells of various bacterial species from leaves. A second, slower stage of bacterial adherence to leaves was suggested in many studies by an increase in the percentage of cells that resisted removal in the days and weeks following inoculation (7,17,27,30,32). In these studies, however, the cells that were not removed may have included cells that were in sites that prevented their removal during the washing treatment. This possibility of endophytic colonization, as well as colonization in "protected" surface sites, thus can hinder the clear detection of bacterial adherence during leaf colonization.

We recently demonstrated that, during colonization of both maize (*Zea mays* L.) and bean (*Phaseolus vulgaris* L.) leaves, a strain of *Pantoea agglomerans* (formerly *Erwinia herbicola*) exclusively colonized leaf surface sites based on its continued sensitivity to a surface sterilant for at least 6 days following inoculation (21). We observed this under both growth chamber and field conditions. Thus, populations of this strain that resist removal are likely to be composed solely of adherent cells. In contrast, we found that the phytopathogenic species *Pseudomonas syringae* pv. *syringae* preferentially colonized endophytic sites of maize and bean leaves (21). This ability to colonize endophytic sites has been demonstrated in previous studies with *P. syringae* pv. *syringae* (1,33), as well as in studies with other gram-negative phytopathogenic bacterial species (9,25,30). The extent to which *P. agglomerans* and *P. syringae* pv. *syringae* are capable of rapid adherence to leaves, and the contribution of surface adherence or endophytic colonization to successful leaf colonization, are currently unknown. Similarly, little is known about the ability of gram-positive bacterial species to adhere to leaf surfaces. In this study, we tested for leaf adherence by the

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gram-positive phytopathogen *Clavibacter michiganensis* subsp. *nebraskensis*, which has been reported to be present on leaf surfaces (23,24).

Although microbial adherence may be mediated by pili (19,20), carbohydrates and glycoproteins (4,14,29), hydrophobic interactions (8), and even a localized positive charge (6), the role of adhesion in bacterial growth and survival on leaves has been critically evaluated only for pilus-mediated adhesion. Using nonpilated *P. syringae* pv. *syringae* mutants, Suoniemi et al. (28) demonstrated that the ability of *P. syringae* pv. *syringae* to adhere to bean leaves via pili contributed to large bacterial population sizes only in experiments in which the leaves were rinsed to remove the bacteria. Similarly, using adhesion-deficient mutants of the yeast *Rhodospidium toruloides*, Buck and Andrews (5) demonstrated that the ability of *R. toruloides* to adhere to barley leaves affected yeast population sizes only under conditions in which the leaves were subjected to washing. Thus, adherence of both of these organisms to leaves contributed to leaf colonization only when removal forces were present. At least for *R. toruloides*, the percentage of cells that were adhered decreased in the days following inoculation. Adherence may have a greater role in colonization by strains that exhibit an increase in adherence over time. To date, the contribution of adherence to leaf colonization by such strains has not been examined.

Bacterial adherence to leaves may be influenced by leaf surface waxes. Removal of leaf waxes with chloroform did not influence the percentage of adhered bacteria for a pilated *P. syringae* pv. *syringae* strain; however, it did influence adherence by a nonpilated mutant (20). Specifically, a smaller percentage of a nonpilated mutant population adhered to a chloroform-treated leaf surface than to a nontreated leaf surface (20), indicating that the leaf surface waxes have a role in bacterial adherence, but that role is secondary when adherence-active pili are present. Buck and Andrews (5) found that the yeast *R. toruloides* adhered better to a cuticular wax mutant of barley than to wild-type barley. We have recently characterized the physicochemical properties of the leaf surfaces of many glossy maize mutants (2); these are mutants that are altered in the biosynthesis of their cuticular waxes (3). The leaf surfaces of these mutants vary in their surface hydrophobicity, in the morphology and density of their epicuticular wax crystals, and in the chemical composition of their waxes (2). Thus, these mutants offer a means to evaluate the influence of leaf surface waxes on bacterial adherence.

The objectives of this study were to test for differences among three bacterial strains in their adherence to maize leaves, to determine whether the adherent and nonadherent populations differed in their survival or growth, and to evaluate whether bacterial adherence to maize leaves was affected by the nature of the leaf surface waxes. The bacterial strains included two maize pathogens, *C. michiganensis* subsp. *nebraskensis*, causal agent of Goss' wilt, and *P. syringae* pv. *syringae*, causal agent of holcus spot, as well as *P. agglomerans*, which is a saprophyte common to leaf surfaces. The model species maize was selected because the high water repellency of its leaves may provide a strong advantage to those bacteria that can resist removal, and because of the availability of maize cuticular mutants whose leaf surface properties have been well characterized. We present evidence that all three bacterial strains, including the gram-positive strain, rapidly adhere to maize leaves, and that the strains differ in the extent of this adherence and in the influence of the leaf surface waxes on adherence. Most importantly, we present evidence that bacterial adherence to leaves can confer a survival advantage upon the bacteria.

MATERIALS AND METHODS

Plant material and plant growth conditions. Seeds of the wild-type *Z. mays* subsp. *mays* L. inbred B73 (genotype *G1*) were

obtained from the North Central Plant Introduction Station (Ames, IA). B73 is susceptible to holcus spot and Goss' wilt. The near-isogenic glossy mutants *gl1*, *gl3*, *gl4*, and *gl5 gl20* were obtained from P. S. Schnable at Iowa State University. These mutants included both spontaneous and chemically induced mutants. Mutants *gl1*, *gl3*, and *gl4* were each homozygous recessive for a single locus involved in cuticular wax biosynthesis, while *gl5 gl20* was homozygous recessive for two glossy loci. The leaf surface properties of these mutants have been described (2).

For each experiment, three to seven pots (five seeds/pot) of each mutant and the wild type were grown in a 1:2:1 peat/perlite/soil potting mixture in a controlled environmental chamber at 28°C, 45% relative humidity (RH) (range: 30 to 60% RH), and a 12-h photoperiod of 350 $\mu\text{Einsteins m}^{-2}\text{s}^{-1}$. Plants were fertilized with a 21:5:20 nitrogen/phosphorous/potassium mixture as the first leaf emerged and 1 week later. Plants were used as seedlings at approximately the time of emergence of leaf 4. The leaf number designations counted the nonleaf coleoptile as 0.

Bacterial strains and culture media. *P. agglomerans* strain BRT98 was a spontaneous rifampicin-resistant mutant of a strain that was isolated from a leaf surface in Brentwood, CA; it was obtained from S. E. Lindow at the University of California-Berkeley. *P. syringae* pv. *syringae* strain HS191R was a spontaneous rifampicin-resistant mutant of HS191 (formerly known as strain 5D430) (10,11), which was obtained from D. C. Gross at Washington State University. *P. syringae* pv. *syringae* strain HS191 was originally isolated from millet in Australia and is known to cause holcus spot on maize (11). Strains BRT98 and HS191R were grown on one-half strength tryptic soy agar (50% TSA) (Microtech Scientific, Orange, CA) containing rifampicin (50 $\mu\text{g/ml}$) for preparation of inocula for plants and for enumeration following recovery from plants. *C. michiganensis* subsp. *nebraskensis* strain GH2390 was obtained from C. Block at the North Central Plant Introduction Station and is known to cause Goss's wilt (23). GH2390 originally was isolated from naturally infected maize seeds from Holt County, NB. GH2390 was grown on nutrient broth yeast agar (NBY) (31) for preparation of inocula for plants. For enumeration following bacterial recovery from plants, GH2390 was grown on sCNS medium (per liter: 8 g of nutrient broth, 2 g of yeast extract, 2 g of K_2HPO_4 , 0.5 g of KH_2PO_4 , 0.001 g of MgSO_4 , 0.02 g of $\text{K}_2\text{Cr}_2\text{O}_7$, 0.04 g of naladixic acid, and 15 g of agar), a semiselective medium for *C. michiganensis* subsp. *nebraskensis* that was modified from CNS (12) by L. Shepherd (26). All media contained cycloheximide (100 $\mu\text{g/ml}$) to inhibit fungal growth. All strains were grown at 28°C. Inocula were prepared with cells grown for 2 days for *P. agglomerans* BRT98, 4 days for *P. syringae* pv. *syringae* HS191R, and 5 days for *C. michiganensis* subsp. *nebraskensis* GH2390.

Inoculum preparation. Bacterial suspensions were prepared by suspending cells grown on solid media in 10 mM potassium phosphate buffer (pH 7) to a density of approximately 10^{10} cells per ml. Cell concentrations were estimated based on optical density measurements at 600 nm (OD_{600}) of cell suspension dilutions and standard curves relating OD_{600} to cell concentration. The maize mutants and wild type varied in their leaf surface hydrophobicity (2), and thus naturally retained distinct volumes of inocula; therefore, the density of the cell suspensions applied to each genotype was adjusted to ensure the retention of similar numbers of cells on each genotype. In the experiments involving *P. agglomerans* BRT98, the inocula contained the following cell densities: 10^8 cells/ml for the wild type, 2×10^7 cells/ml for *gl4*, and 5×10^6 cells/ml for *gl1*, *gl3*, and *gl5 gl20*. In the experiment involving *P. syringae* pv. *syringae* HS191R, as well as in one experiment (designated experiment 4) involving *C. michiganensis* subsp. *nebraskensis* GH2390, the inocula contained the following cell densities: 8×10^8 cells/ml for the wild type, 2×10^8 cells/ml for *gl4*, and 5×10^7 cells/ml for *gl1*, *gl3*, and *gl5 gl20*, while in a second experiment (designated experiment 5), the inocula con-

tained the following densities: 2×10^8 cells/ml for the wild type, 4×10^7 cells/ml for *gl4*, and 1×10^7 cells/ml for *gl1*, *gl3*, and *gl5 gl20*.

Evaluating rapid adherence to leaves and the dynamics of adherent and nonadherent bacterial populations. Each pot of plants was inoculated by spraying approximately 18 ml of inoculum onto the plants using a household sprayer (Trigger sprayer 922H; Continental Manufacturing Co., St. Louis). After inoculation, the plants were incubated in an enclosed plastic tent on the laboratory bench for 8 h. The plants then were transferred to a growth chamber and were incubated at 28°C and 95% RH (range: 75 to 99% RH) with a 12-h photoperiod of 350 $\mu\text{Einsteins m}^{-2}\text{s}^{-1}$ for four additional days. Twelve replicate leaves, including leaf numbers 1, 2, and 3, were harvested immediately following inoculation and at the same time each day for 4 days thereafter. Individual leaves were weighed, placed in test tubes containing 20 ml of washing buffer (0.1 M potassium phosphate buffer, pH 7, containing 0.1% peptone), and sonicated in an ultrasonic cleaning bath (Branson 8210 sonicator; Branson Ultrasonic Corp., Danbury, CT) for 5 min, and then were agitated with a vortex mixer for 10 s. After sonication and agitation, the bacteria in the suspension were enumerated by viable plate counts; this population was designated the nonadherent population. After sonication and agitation of a leaf, the leaf was removed from the sonicate and introduced into sterile washing buffer and the tube containing the leaf was placed at -20°C until processing. At the first two sampling times, 0 and 24 h, the washing buffer contained 10% glycerin to aid bacterial survival during freezing (16). We found that, for leaves sampled at times up to 24 h following inoculation, glycerin amendment improved bacterial cell viability during storage at -20°C, whereas for leaves sampled after 24 h, glycerin amendment did not influence cell viability. To enumerate the bacteria that resisted removal, the frozen leaf was thawed rapidly and homogenized using a Brinkmann's PT 10-35 homogenizer with a 20-mm generator (Brinkmann Instruments, Inc., Westbury, NY) for 40 to 60 s, and the homogenate was plated for viable counts. This population was designated the adherent population. All population sizes were expressed as CFU per gram of leaf (fresh weight).

Six separate experiments were performed. In experiments 1 and 2, *P. agglomerans* BRT98 cells were inoculated onto seedlings of all five maize genotypes, and the nonadherent and adherent populations were examined over a 4-day period. These experiments were replicate experiments except that the RH in the first

8 h following inoculation was lower in experiment 2 than in experiment 1 due to the seasonal use of heated air in the laboratory during experiment 2. In experiment 3, seedlings of the five maize genotypes were inoculated with either *P. agglomerans* BRT98 or *C. michiganensis* subsp. *nebraskensis* GH2390, and the nonadherent and adherent populations of each strain were examined only immediately following inoculation. In experiments 4 and 5, *C. michiganensis* subsp. *nebraskensis* GH2390 cells were inoculated onto seedlings of the five maize genotypes, and the nonadherent and adherent populations were examined over a 4-day period. Like experiments 1 and 2, these experiments were replicate experiments except that the RH in the first 8 h following inoculation was lower in experiment 5 than in experiment 4, due also to the seasonal use of heated air in the laboratory. Lastly, in experiment 6, *P. syringae* pv. *syringae* HS191R cells were inoculated onto seedlings of the five maize genotypes, and the nonadherent and adherent populations were examined over a 4-day period.

Statistical methods. Estimates of bacterial populations on leaves were log-transformed to achieve normality using the transformation $\log(x + 0.5)$, where x is the number of bacteria per gram of leaf (fresh wt). Estimates of the percentage of adherent bacteria were analyzed both as log-transformed data, to achieve normality with the data collected at 0 and 24 h, and untransformed data, which were normally distributed for the data collected at 48, 72, and 96 h; both analyses yielded similar results. Means of the transformed data were compared using one-factor or two-factor analysis of variance (ANOVA) and Fisher's least significant difference (LSD) test. These tests were performed using SAS (Statistical Analysis System; SAS Institute Inc., Cary, NC) or Microsoft Excel. Bacterial population dynamics were evaluated using both log-transformed data and log-transformed data after normalization to the initial population sizes. Comparisons of these normalized values should more accurately reflect true differences among the plant genotypes in the population dynamics of the bacteria. The analyses of the normalized and non-normalized data generally yielded similar results, and when they did not, the differences were clearly attributable to differences in the initial population sizes. Only the analyses for the normalized populations are provided.

RESULTS

Bacterial adherence immediately following inoculation. Rapid adherence to leaves was assessed by inoculating bacterial

TABLE 1. Percentages of the populations of *Pantoea agglomerans* BRT98, *Clavibacter michiganensis* subsp. *nebraskensis* GH2390, and *Pseudomonas syringae* pv. *syringae* HS191R that were adhered to leaves of the wild-type and maize glossy mutants *gl1*, *gl3*, *gl4*, and *gl5 gl20* immediately following inoculation

Bacterial strain	Exp ^w	Percentage of bacteria that were adhered to the leaf surface immediately following inoculation ^x						
		All ^y	Wild type	<i>gl1</i>	<i>gl3</i>	<i>gl4</i>	<i>gl5 gl20</i>	<i>P</i> value ^z
<i>Pantoea agglomerans</i> BRT98	1	8.2	8.4 a	7.8 a	7.8 a	11.2 a	6.4 a	0.25
<i>P. agglomerans</i> BRT98	2	7.7	8.7 a	8.1 a	7.1 a	7.0 a	8.0 a	0.95
<i>P. agglomerans</i> BRT98	3	9.6	8.0 c	7.5 c	8.2 c	11.1 cd	15.3 d	...
<i>C. michiganensis</i> subsp. <i>nebraskensis</i> GH2390	3	2.6	4.5 b	1.8 a	2.0 a	4.0 b	1.7 a	<0.001
<i>C. michiganensis</i> subsp. <i>nebraskensis</i> GH2390	4	2.8	3.1 a	2.8 a	2.7 a	3.5 a	2.0 a	0.17
<i>C. michiganensis</i> subsp. <i>nebraskensis</i> GH2390	5	2.1	2.1 a	1.8 a	1.9 a	3.9 b	2.1 a	0.01
<i>Pseudomonas syringae</i> pv. <i>syringae</i> HS191R	6	0.3	0.9 a	0.2 a	0.4 a	0.5 a	0.3 a	0.06

^w Each experiment (Exp) was performed independently, with the inoculation and sampling occurring under identical conditions. After the samples were collected at 0 h postinoculation, the plants in experiments 1, 2, 4, 5, and 6 were used in studies focused on the dynamics of the populations during colonization.

^x Leaves were collected immediately following spray inoculation with a bacterial suspension. Each leaf was subjected to sonication and washing to enumerate the nonadherent populations, and then to homogenization to enumerate the adherent populations, as described in the materials and methods. Values shown are the percentages of the leaf-associated populations that were adherent, where the leaf-associated population was the sum of the adherent and nonadherent populations. Values represent the mean percentage of 12 leaves per genotype. Within each experiment, values followed by the same letter do not differ significantly by a Fisher's least significant difference (LSD) test of the log-transformed data.

^y Values represent the percentage of bacteria that were adhered to the surface of the leaves of all of the genotypes; thus, these values are the mean percentage of 60 leaves.

^z The *P* value for the Fisher's LSD test is shown. The *P* value for experiment 3, which involved both *Pantoea agglomerans* BRT98 and *C. michiganensis* subsp. *nebraskensis* GH2390, is shown only once.

suspensions onto leaves and immediately subjecting the leaves to sonication and washing to enumerate the nonadherent bacteria in the washate, and then subjecting the leaves to homogenization to enumerate the bacteria that resisted removal. The time between inoculation and sampling was assumed to be too short for significant bacterial movement to sites that offered protection from removal. The percentage of adherent bacteria was relatively consistent among the experiments for each bacterial strain, but differed for the three strains (Table 1). An ANOVA across the experiments, in which the two bacterial strains in experiment 3 were treated as separate experiments, indicated significant differences among the experiments ($P < 0.0001$). Specifically, the percentage of bacteria that were adhered to leaves was significantly larger for *P. agglomerans* BRT98 in experiments 1, 2, and 3 than for *C. michiganensis* subsp. *nebraskensis* GH2390 and *P. syringae* pv. *syringae* HS191R in experiments 3 to 6, and was significantly larger for *C. michiganensis* subsp. *nebraskensis* GH2390 in experiments 3, 4, and 5 than for *P. syringae* pv. *syringae* HS191R in experiment 6, based on a Fisher's LSD test. In experiment 3, which specifically compared the strains *P. agglomerans* BRT98 and *C. michiganensis* subsp. *nebraskensis* GH2390, the percentage of adherent *P. agglomerans* BRT98 cells was significantly larger than the percentage of adherent *C. michiganensis* subsp. *nebraskensis* GH2390 cells on leaves of the wild type and each of the genotypes (Table 1). These results demonstrate that the three strains differ in their ability to rapidly adhere to maize leaf surfaces.

Effect of leaf surface waxes on bacterial adhesion. The leaf surface waxes appeared to have little effect on the ability of *P. agglomerans* BRT98 to rapidly adhere to leaves. With one exception (mutant *gl5 gl20* in experiment 3), there were no significant differences among the mutants and the wild type in the percentage of *P. agglomerans* BRT98 cells that adhered to the leaves within each experiment (Table 1). In contrast, the leaf waxes affected the ability of *C. michiganensis* subsp. *nebraskensis* GH2390 to rapidly adhere to leaves. Specifically, the percentage of adherent *C. michiganensis* subsp. *nebraskensis* GH2390 cells was consistently larger on *gl4* than on the other mutants, and this difference was significant in two of the three experiments (Table 1). The ability of *P. syringae* pv. *syringae* HS191R to rapidly adhere to maize leaves was poor on all of the maize genotypes, and was not significantly influenced by the leaf surface waxes (Table 1).

Survival of the adherent and nonadherent populations in the 24 h following inoculation. In the 24 h following inoculation,

all three bacterial strains exhibited a decrease in the size of the nonadherent and adherent populations. These decreases were observed on all five plant genotypes and in all of the experiments (Table 2). The extent of this decrease was consistently greater for the nonadherent populations than for the adherent populations (Table 2), indicating that the nonadherent cells were more susceptible to death or loss of culturability during this period than were the adherent, or internally located, cells. Based on a two-factor ANOVA in which time and type of population were factors, the decrease in the nonadherent populations between 0 and 24 h postinoculation was significantly larger than that of the adherent populations in the experiments involving the strains *C. michiganensis* subsp. *nebraskensis* GH2390 and *P. syringae* pv. *syringae* HS191R ($P < 0.0001$ in experiments 4, 5, and 6), and was larger, although not significantly, in the experiments involving the saprophyte *Pantoea agglomerans* BRT98 ($P = 0.058$ and 0.13 in experiments 1 and 2, respectively). This difference was significant for *P. agglomerans* BRT98 in experiment 1 if *gl4* was excluded from the analysis ($P = 0.03$). For both populations, the population decreases between 0 and 24 h following inoculation were larger in experiment 2 than in experiment 1, and were larger in experiment 5 than in experiment 4 (Table 2; Fig. 1). The differences between these experiments are reflective of the differences in the RH in the laboratory during the first 6 to 8 h following inoculation in these experiments; specifically, the plants were exposed to a lower RH in experiments 2 and 5 than in experiments 1 and 4 during this period.

The nonadherent populations of *P. syringae* pv. *syringae* HS191R on all five plant genotypes decreased much more in the 24 h following inoculation than did the nonadherent populations of the other two strains (Table 2), suggesting that nonadherent cells of *P. syringae* pv. *syringae* HS191R were particularly poor at surviving under these conditions. The nonadherent populations of *C. michiganensis* subsp. *nebraskensis* GH2390 decreased more than did those of *P. agglomerans* BRT98, at least on the wild type and mutant *gl4*, suggesting that on these genotypes, nonadherent *P. agglomerans* BRT98 cells survived better than did nonadherent *C. michiganensis* subsp. *nebraskensis* GH2390 cells. In contrast to the nonadherent populations, the adherent populations did not exhibit consistent differences among the strains in the relative size of their decrease in the 24 h postinoculation (Table 2).

Effect of leaf surface waxes on bacterial survival in the 24 h following inoculation. The leaf surface waxes affected bacterial survival in the 24 h following inoculation, although the effects were relatively minor. Among the wild type and the glossy mu-

TABLE 2. Proportional decrease in the population sizes of *Pantoea agglomerans* BRT98, *Clavibacter michiganensis* subsp. *nebraskensis* GH2390, and *Pseudomonas syringae* pv. *syringae* HS191R between 0 and 24 h following inoculation

Bacterial strain	Exp ^w	Population ^x	Proportional decrease in population sizes between 0 and 24 h postinoculation ^y						
			All	Wild type	<i>gl1</i>	<i>gl3</i>	<i>gl4</i>	<i>gl5 gl20</i>	<i>P</i> value ^z
<i>Pantoea agglomerans</i> BRT98	1	Nonadherent	5.5	8.9	7.1	5.1	1.4	10.7	0.005
		Adherent	3.2	4.3	4.6	2.8	1.2	5.0	0.09
<i>P. agglomerans</i> BRT98	2	Nonadherent	11.7	11.2	21.7	16.8	5.6	31.7	0.19
		Adherent	6.9	8.6	7.5	8.4	4.1	23.3	0.26
<i>C. michiganensis</i> subsp. <i>nebraskensis</i> GH2390	4	Nonadherent	9.1	16.3	6.7	6.5	9.8	9.0	0.02
		Adherent	1.5	2.8	0.8	1.7	1.2	1.4	0.12
<i>C. michiganensis</i> subsp. <i>nebraskensis</i> GH2390	5	Nonadherent	20.3	32.1	21.0	19.4	20.7	13.2	0.06
		Adherent	5.0	6.7	2.6	5.5	7.7	4.4	0.29
<i>Pseudomonas syringae</i> pv. <i>syringae</i> HS191R	6	Nonadherent	126.0	58.5	116.7	239.5	78.6	217.2	0.62
		Adherent	2.1	1.9	1.5	1.7	2.1	3.9	0.15

^w Experiments (Exp) 1 and 2 were similar except that the relative humidity (RH) in the first 8 h following inoculation was lower in experiment 2 than in experiment 1. Experiments 4 and 5 were similar except that the RH in the first 8 h following inoculation was lower in experiment 5 than in experiment 4.

^x The nonadherent populations were those that were removed from leaves by sonication and washing. The adherent populations were those that remained on the leaves after sonication and washing and were subsequently released by leaf homogenization.

^y Values shown are the mean population size at 0 h postinoculation divided by the mean population size at 24 h postinoculation across all genotypes (All) or on a single genotype within an experiment.

^z A two-factor analysis of variance was performed on the log-transformed population sizes, with time and genotype as factors, to test for an effect of the plant genotype on the population decreases; the resulting *P* values are shown.

tants, mutant *gl4* consistently supported the smallest decreases in both the nonadherent and adherent *P. agglomerans* BRT98 populations, whereas *gl5 gl20* consistently supported the largest decreases. A two-factor ANOVA in which time and genotype were factors, followed by a Fisher's LSD test on the difference in the population sizes at 0 and 24 h postinoculation, indicated that the decrease in the nonadherent population of *P. agglomerans* BRT98 was significantly smaller on *gl4* than on the other genotypes in experiment 1 ($P = 0.005$). However, none of the other differences among the genotypes in experiments 1 and 2 were significant (Table 2). *C. michiganensis* subsp. *nebraskensis* GH2390 also exhibited differences in survival among the genotypes. Specifically, the decrease in the nonadherent populations of *C. michiganensis* subsp. *nebraskensis* GH2390 was smaller on all of the mutants than on the wild type, although this difference was significant at the $P = 0.05$ level only in experiment 4 (Table 2). *P. syringae* pv. *syringae* HS191R did not exhibit significant differences in survival among the genotypes. These results indicate that the leaf surface properties of mutant *gl4* favored the survival of the nonadherent, as well as perhaps the adherent, *P. agglomerans* BRT98 cells, while the leaf surface properties of the wild-type maize disfavored the survival of the non-adherent *C. michiganensis* subsp. *nebraskensis* GH2390 cells.

Dynamics of the adherent and nonadherent populations. In the 4 days following inoculation, the percentage of the *P. agglomerans* BRT98 and *C. michiganensis* subsp. *nebraskensis* GH2390 populations that were adherent increased (Fig. 2). A similar increase occurred for the *P. syringae* pv. *syringae* HS191R populations, although the adherent populations of *P. syringae* pv. *syringae* HS191R decreased to below the limit of detection on the majority of leaves by 72 h. At 48 h postinoculation, the percentage of the leaf-associated populations that were adherent was significantly larger for *C. michiganensis* subsp. *nebraskensis* GH2390 in both experiments (experiments 4 and 5) and for *P. syringae* pv. *syringae* HS191R (experiment 6) than for *P. agglomerans* BRT98 in either experiment (experiments 1 and 2) ($P < 0.0001$). Similarly, at 72 and 96 h postinoculation, the percentage of adherent bacteria was significantly larger for *C. michiganensis* subsp. *nebraskensis* GH2390 ($\geq 78\%$) than for *P. agglomerans* BRT98 (55 to 73%) ($P < 0.0001$). No significant differences were observed among the genotypes in the percentage of adherent populations present on their leaves at any sampling time.

The dynamics of the nonadherent and adherent populations were distinct. The nonadherent populations of *P. agglomerans* BRT98 increased in the period between 24 and 48 h following inoculation in experiments 1 and 2 (Fig. 1A), but then did not increase much more between 48 and 96 h. The nonadherent populations of *C. michiganensis* subsp. *nebraskensis* GH2390 and *P. syringae* pv. *syringae* HS191R decreased in the period between 24 and 48 h (Fig. 1B), and also did not increase much, if at all, between 48 and 96 h. In contrast, the adherent populations of *P. agglomerans* BRT98 and *C. michiganensis* subsp. *nebraskensis* GH2390 exhibited dramatic increases throughout the period between 24 and 72 h following inoculation (Fig. 1A and B), indicating preferential development of these populations during this period. *P. syringae* pv. *syringae* HS191R did not establish populations of any type on the maize leaves under these conditions (Fig. 1C).

Effect of leaf surface waxes on the dynamics of the adherent and nonadherent populations. The nonadherent and adherent populations of *P. agglomerans* BRT98 were consistently larger on mutant *gl4* than on the wild type and other mutants (Fig. 3). Based on a two-factor ANOVA in which time and genotype were factors, the nonadherent and adherent populations of *P. agglomerans* BRT98 were significantly larger on mutant *gl4* than on the wild type in experiment 1 ($P < 0.0001$ and $P = 0.006$, respectively), and the adherent populations were significantly larger in experiment 2 ($P = 0.03$), while the nonadherent populations were not ($P = 0.07$). Similarly, the nonadherent and adherent populations of

C. michiganensis subsp. *nebraskensis* GH2390 were significantly larger on mutant *gl4* than on the wild type in experiment 4 ($P = 0.001$ and $P < 0.0001$, respectively), while the nonadherent populations were significantly larger in experiment 5 ($P < 0.0001$) and the adherent populations were not ($P = 0.3$) (Fig. 4). Thus, the leaf surface waxes of mutant *gl4* favored the development of both of these component populations.

The nonadherent and adherent *P. agglomerans* BRT98 populations were significantly smaller on mutant *gl5 gl20* than on the wild type ($P < 0.03$) (Fig. 3), and the nonadherent and adherent *C. michiganensis* subsp. *nebraskensis* populations were significantly larger on mutant *gl1* than on the wild type (Fig. 4) ($P < 0.03$). Thus, the leaf surface waxes of mutants *gl5 gl20* and *gl1* had a similar effect on both of these component populations.

The leaf surface waxes of maize did not influence either of the component populations of *P. syringae* pv. *syringae* HS191R with one exception. Mutant *gl1* fostered significantly larger adherent *P. syringae* pv. *syringae* HS191R populations than the wild type did ($P = 0.01$) (Fig. 5), indicating that the leaf surface properties of *gl1* may promote *P. syringae* pv. *syringae* HS191R adherence to the surface or localization in protected sites.

Fig. 1. Dynamics of the nonadherent and adherent populations of **A**, *Pantoea agglomerans* BRT98 (experiments 1 and 2), **B**, *Clavibacter michiganensis* subsp. *nebraskensis* GH2390 (experiments 4 and 5), and **C**, *Pseudomonas syringae* pv. *syringae* HS191R (experiment 6) over a 4-day period following inoculation. Experiments 1 and 2 and experiments 4 and 5 differ as described in Table 2. Values shown are the mean log(CFU/g leaf) $\pm$ standard error of the mean of 60 samples. These samples included 12 samples from each of the five maize genotypes tested; differences among the genotypes are shown in Figures 3 to 5.

DISCUSSION

In this article, we evaluated the ability of bacteria to rapidly adhere to leaves by quantifying the percentage of adherent and nonadherent cells immediately following inoculation. We found that up to 15% of the bacteria, depending on the bacterial strain and the maize genotype, adhered to seedling maize leaves immediately after inoculation. We assumed that the bacteria that were not removed immediately after inoculation were adhered to leaves, rather than were located in sites that prevented removal, because the time between inoculation and sampling was sufficiently short to minimize relocation on the leaf. In two previous studies, the percentage of adherent bacterial cells immediately following inoculation was as large as 93 to 97% (13,19). Collectively, these studies and ours provide strong evidence that bacteria are capable of rapidly adhering to leaf surfaces. Although the large difference between our study and the previous two studies in the percentage of adherent cells may be attributable to differences in the bacterial species or plant species, it probably is attributable to differences in technique. Specifically, we used sonication and washing to remove the bacteria, and this has been shown to release more bacteria than simply washing (20), as was done in the previous studies (13,19). For example, we found that 42 to 68% of a *P. agglomerans* population on maize leaves resisted removal by washing the leaves for 5 min, whereas only 12 to 16% of the population on those leaves resisted removal by sonication and washing (A. Struck-Marcell and G. A. Beattie, unpublished data).

We demonstrated that three distinct bacterial strains, representing three distinct species, were capable of rapid adherence. These three strains included *P. syringae* pv. *syringae* HS191R, a gram-negative phytopathogen like *P. marginalis*, *P. syringae* pv. *lachrymans*, and *Xanthomonas campestris* pv. *graminis*, which have all been previously reported to rapidly adhere to leaf surfaces (13,18,20,28). We also demonstrated rapid adherence to leaves by *C. michiganensis* subsp. *nebraskensis* GH2390, a gram-positive phytopathogen; this is, to our knowledge, the first documented report of rapid adherence of a gram-positive phytopathogen to leaves. Lastly, we demonstrated rapid adherence of the saprophyte *P. agglomerans* BRT98 to maize leaves. This result supports three previous reports demonstrating that saprophytic microorganisms can adhere to leaves, two involving *Escherichia coli* (15,18) and one involving the nonphytopathogenic yeast *R. toruloides* (5).

This is the first report to demonstrate significant differences among distinct bacterial strains in their ability to rapidly adhere to the leaves of a single plant species. The percentage of cells that

rapidly adhered to the leaves of the maize genotypes ranged from only 0 to 1% for *P. syringae* pv. *syringae* HS191R cells, to 2 to 5% for *C. michiganensis* subsp. *nebraskensis* GH2390, to 8 to 16% for *P. agglomerans* BRT98, with the ranges for *P. syringae* pv. *syringae* HS191R being based on one experiment and those of *C. michiganensis* subsp. *nebraskensis* GH2390 and *P. agglomerans* BRT98 being based on at least three experiments. The differences in these ranges may reflect distinct survival strategies among the strains. For example, survival by the saprophyte *P. agglomerans* may be more dependent on adherence to the leaf surface than is survival by either pathogen. Specifically, *P. agglomerans* BRT98 appears to be restricted to colonization of leaf surface sites (21); thus, it may depend on adherence for survival more than would an organism that colonizes endophytic sites.

A second stage of bacterial adherence to leaves likely occurs in the hours or days following inoculation. Our recent finding that the *P. agglomerans* strain BRT98 exclusively colonized leaf surface sites of maize leaves (i.e., sites that were exposed to a surface sterilant) (21), indicates that, at all of the sampling times in this study, the populations of *P. agglomerans* BRT98 that resisted removal were composed solely of surface-adhered cells. Thus, the steady increase in the percentage of *P. agglomerans* BRT98 cells that resisted removal from 10% immediately after inoculation to approximately 70% after 4 days provides evidence that *P. agglomerans* BRT98 is capable of a second, slower stage of bacterial adherence. In contrast to the increase with *P. agglomerans*, Buck and Andrews (5) demonstrated that the yeast *R. toruloides* exhibited a consistent decrease in the percentage of cells that were adhered to leaves over a 10-day period. They concluded that *R. toruloides* experienced a first, rapid stage of adherence to leaves, but not a second, slower stage. The extent of adherence of non-phytopathogenic bacteria to leaves during colonization of leaves has not previously been quantified.

The steady increase in the percentage of *C. michiganensis* subsp. *nebraskensis* and *P. syringae* pv. *syringae* cells that resisted removal from maize leaves in the days following inoculation indicates that phytopathogenic bacteria may also adhere to leaves via a two-stage process. Previous studies with several foliar phytopathogens (9,25,30), including *C. michiganensis* subsp. *nebraskensis* (23,24) and *P. syringae* pv. *syringae* (1,21,33), have found that these phytopathogens were in endophytic and epiphytic sites. Based on this observation, the populations of *C. michiganensis* subsp. *nebraskensis* and *P. syringae* pv. *syringae* that resisted removal may have included both surface-adhered cells and cells located in sites that prevented removal; thus, the ability of these strains to employ a second, slower stage of adherence remains unclear.

Fig. 2. Percentage of the leaf-associated bacterial populations that was adhered to maize leaves over a 4-day period following inoculation. The experiments are as described in Table 2. The percentage of adherent bacteria could not be accurately estimated beyond 48 h in experiment 6 due to the adherent populations decreasing below the detection limit on the majority of leaves. Values shown are the mean percentage $\pm$ standard error of 60 samples. These samples included 12 samples from each of the five maize genotypes tested; no significant differences among the genotypes were observed.

The absence of detectable differences among the wild-type and cuticular maize mutants in the percentage of *P. agglomerans* BRT98 cells that were adhered to leaves immediately after spray application demonstrated that the leaf surface waxes did not influence the rapid adherence of *P. agglomerans* BRT98 to maize leaves. Similarly, the absence of detectable differences in the days following inoculation demonstrated that, at least under the conditions tested, the leaf surface waxes also did not influence long-term adherence of *P. agglomerans* BRT98 to maize leaves. In contrast, the leaf surface waxes appeared to have a role, albeit a small role, in the ability of *C. michiganensis* subsp. *nebraskensis* GH2390 to rapidly adhere to maize leaves. This conclusion is based on the consistent finding that approximately twice as many *C. michiganensis* subsp. *nebraskensis* GH2390 cells, on a percentage basis, adhered to *gl4* leaves as adhered to the leaves of the other mutants immediately after inoculation. Furthermore, in the 72 h following inoculation, mutant *gl4* generally supported a larger percentage of adherent or endophytic *C. michiganensis* subsp. *nebraskensis* GH2390 cells than did the other genotypes, although the differences often were not statistically significant. The leaf surface properties of the wild type and the mutants have been characterized (2). Among the mutants, *gl4* is the most similar to the wild type in the types of epicuticular crystalline waxes it produces (2), suggesting that it produces cuticular waxes that are most similar in composition to those of the wild type. Like the other mutants, *gl4* produced much less cuticular wax than the wild type and a reduced density of epicuticular crystalline waxes. Unfortunately, no single leaf surface property or combination of properties explains the favorable influence of *gl4* on bacterial adhesion.

The adherent and nonadherent populations exhibited distinct dynamics during the 4-day colonization period. The larger de-

Fig. 3. Dynamics of the nonadherent and adherent populations of *Pantoea agglomerans* BRT98 on wild-type maize (WT) and on the four maize glossy mutants *gl1*, *gl3*, *gl4*, and *gl5 gl20* over a 4-day period following inoculation in two experiments. The experiments differ as described in Table 2. Values were normalized to the mean log(CFU/g leaf) at 0 h after inoculation. Each value represents the mean normalized log(CFU/g leaf) $\pm$ standard error of 12 samples.

Fig. 4. Dynamics of the nonadherent and adherent populations of *Clavibacter michiganensis* subsp. *nebraskensis* GH2390 on wild-type maize (WT) and on the two maize glossy mutants *gl1* and *gl4* over a 4-day period following inoculation in two experiments. The experiments differ as described in Table 2. The population dynamics on mutants *gl3* and *gl5 gl20* were similar to those on the wild type and are not shown. Values were normalized to the mean log(CFU/g leaf) at 0 h after inoculation. Each value represents the mean normalized log(CFU/g leaf) $\pm$ standard error of 12 samples.

Fig. 5. Dynamics of the nonadherent and adherent populations of *Pseudomonas syringae* pv. *syringae* HS191R on wild-type maize (WT) and on the four maize glossy mutants *gl1*, *gl3*, *gl4*, and *gl5 gl20* over a 4-day period following inoculation. Values were normalized to the mean log(CFU/g leaf) at 0 h after inoculation. Each value represents the mean normalized log(CFU/g leaf) $\pm$ standard error of 12 samples at 0 h, of 6 to 12 samples with detectable populations at 24 and 48 h, and of 3 to 12 samples with detectable populations at 72 and 96 h. Mean values representing fewer than three samples with detectable populations are not shown.

crease in the size of the nonadherent populations than in the adherent populations in the 24 h after inoculation provides evidence that the nonadherent cells were more susceptible to death or loss of culturability on leaves. A previous study demonstrated that cells in sites that are protected from surface sterilants survived better than cells in exposed sites when the plants were incubated under low-RH conditions (33). The fact that the adherent, and possibly endophytic, populations increased within 24 h after inoculation while the nonadherent populations did not provides evidence that surface adherence (*P. agglomerans*), as well as possibly internal colonization (*C. michiganensis* subsp. *nebraskensis*, *P. syringae* pv. *syringae*), favors bacterial growth and survival following inoculation. Several reports have demonstrated that surface populations decrease rapidly following inoculation (1,13,22,33), but to our knowledge only two (7,30) have shown a simultaneous increase in the adherent populations. The mechanistic basis for the poorer survival of the nonadherent cells is unlikely to be that the nonadherent cells were removed from the leaves more rapidly than were the adhered cells, because there were no obvious removal forces, such as rain or wind, in these experiments. Instead, the mechanistic basis is probably that the nonadherent cells were more exposed to environmental stresses, such as desiccation or limited nutritional resources, either of which may negatively impact survival or culturability. This increased exposure could be due either to localization of the nonadherent cells in sites that provided limited water or nutrients, such as in sites that promoted water evaporation, or to the absence of large amounts of exopolysaccharides that could function in roles such as hydration, nutrient accumulation, and adhesion.

In summary, we demonstrated differences among three strains in the relative sizes of the adherent populations present on a leaf. The strains represented three distinct species; therefore, these results suggest species specificity in the extent of bacterial adherence to leaves, as well as possibly in the extent of bacterial localization to endophytic leaf sites. We also demonstrated that the cuticular wax-dependent leaf surface properties did not detectably influence *P. agglomerans* adherence to leaves, but did influence *C. michiganensis* subsp. *nebraskensis* adherence, suggesting *C. michiganensis* subsp. *nebraskensis* recognition of these waxes. Lastly, we demonstrated that the adherent and nonadherent populations of the three strains differed in their dynamics during leaf colonization, indicating that they may be differentially influenced by the environment. In particular, the superior survival of the adherent populations suggested that adhesion, and possibly endophytic colonization, conferred a survival advantage upon the bacteria.

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