

# The Facultative Symbiont *Rickettsia* Protects an Invasive Whitefly against Entomopathogenic *Pseudomonas syringae* Strains

Tory A. Hendry,<sup>a\*</sup> Martha S. Hunter,<sup>b</sup> David A. Baltrus<sup>a</sup>

School of Plant Sciences, University of Arizona, Tucson, Arizona, USA<sup>a</sup>; Department of Entomology, University of Arizona, Tucson, Arizona, USA<sup>b</sup>

Facultative endosymbionts can benefit insect hosts in a variety of ways, including context-dependent roles, such as providing defense against pathogens. The role of some symbionts in defense may be overlooked, however, when pathogen infection is transient, sporadic, or asymptomatic. The facultative endosymbiont *Rickettsia* increases the fitness of the sweet potato whitefly (*Bemisia tabaci*) in some populations through mechanisms that are not yet understood. In this study, we investigated the role of *Rickettsia* in mediating the interaction between the sweet potato whitefly and *Pseudomonas syringae*, a common environmental bacterium, some strains of which are pathogenic to aphids. Our results show that *P. syringae* multiplies within whiteflies, leading to host death, and that whiteflies infected with *Rickettsia* show a decreased rate of death due to *P. syringae*. Experiments using plants coated with *P. syringae* confirmed that whiteflies can acquire the bacteria at a low rate while feeding, leading to increased mortality, particularly when the whiteflies are not infected with *Rickettsia*. These results suggest that *P. syringae* may affect whitefly populations in nature and that *Rickettsia* can ameliorate this effect. This study highlights the possible importance of interactions among opportunistic environmental pathogens and endosymbionts of insects.

Bacterial symbionts can affect the fitness of insects in multiple ways. Perhaps the most important of these are the primary endosymbiotic nutritional mutualists required by some insects for growth (1–3). However, many insects also harbor facultative, or secondary, symbiotic bacteria that manipulate reproduction or provide fitness benefits in particular ecological contexts (4–10). For example, the secondary symbiont *Hamiltonella defensa* provides protection against a parasitoid wasp to pea aphids and may be common in pea aphid populations that experience high levels of parasitism (4, 6). Laboratory studies have shown that symbionts can also play a major role in protecting their insect hosts from microbial pathogens (11, 12). Insects regularly interact with numerous environmental bacteria, some of which are likely to be specialists or opportunistic pathogens and thus possibly influence insect fitness and the dynamics of defensive symbionts. However, given the transience of infections and difficulty with the sampling of microbial pathogens from naturally infected insects, the evolutionary effects of such pathogens and symbionts remain poorly understood (12, 13).

Sweet potato whiteflies (*Bemisia tabaci*, Hemiptera: Aleyrodidae) are serious agricultural pests and invasive in many warm temperate and tropical parts of the world (14). The alphaproteobacterium *Rickettsia* species near *bellii* (*Rickettsia* sp. nr. *bellii*), a secondary symbiont of the sweet potato whitefly, confers fitness benefits to the host (15, 16). The increased fitness observed for *Rickettsia*-infected ( $R^+$ ) whiteflies in the lab appears to be due to both increased fecundity and better survival to adulthood. However, the mechanistic basis for these effects, as well as how environmental context influences their magnitude, remains unknown. Whitefly populations vary in the degree of benefit that they receive due to *Rickettsia* infection. For instance, whitefly populations in the southwestern United States receive an increased fitness benefit from *Rickettsia* infection and have high infection frequencies, whereas whitefly populations in Israel receive less of a fitness benefit and have lower infection frequencies (15–17). Himpler and colleagues suggested that, among other possible mechanisms, a role in defense would contribute to *Rickettsia* spread in

the field (16). This idea is supported by work demonstrating that a closely related *Rickettsia* sp. nr. *bellii* strain protects pea aphids (*Acyrtosiphon pisum*) against a fungal pathogen (11, 18). Laboratory assays that consistently show the better performance of  $R^+$  whiteflies in the absence of any known pathogens may make defense against pathogens unlikely to be the only role for the symbiont in whiteflies (16), but defense could contribute to the success of infected whiteflies in areas where pathogens are common.

Phloem-feeding insects continually encounter bacteria on plants while feeding, and while the effect of most of these bacteria on the insects is unknown, a growing number of studies suggest that phyllosphere bacteria may represent a source of pathogens of plant-associated insects (19–21). Such pathogens may be cryptic, in that they do not produce visible symptoms, and furthermore, they may infect at a low rate, making them difficult to observe. It was recently discovered that some strains of the plant pathogen *Pseudomonas syringae* cause high rates of mortality after ingestion by pea aphids (22). *Pseudomonas syringae* is a common plant epiphyte (23, 24) and is therefore likely to be encountered by whiteflies on plants. Here we investigate the potential for *Rickettsia* to protect sweet potato whiteflies against *P. syringae*. Our objective was to determine whether whiteflies, as well as aphids, are affected by exposure to this bacterium. To address this, we endeavored to confirm previous patterns of *P. syringae* pathogenicity in pea aphids and to compare these results to the patterns in whiteflies. Secondarily, we sought to determine if the symbiont *Rickettsia*

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Address correspondence to Tory A. Hendry, toryahendry@gmail.com.

\* Present address: Tory A. Hendry, Department of Environmental Science, Policy, and Management, University of California, Berkeley, Berkeley, California, USA.

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influences the interaction between whiteflies and this possible pathogen.

## MATERIALS AND METHODS

**Whitefly and aphid maintenance.** The whitefly *Bemisia tabaci* is a cryptic species complex with more than 28 species worldwide (25), among which the invasive Middle East-Asia Minor 1 (MEAM1) species (= B biotype) is established in the field in the United States and is the focus of this study. Whiteflies were originally collected in Maricopa, AZ, in 2006 and kept on cowpea (*Vigna unguiculata*) plants at 27°C. *Rickettsia*-positive (R<sup>+</sup>) and *Rickettsia*-negative (R<sup>-</sup>) whitefly lines were introgressed as described by Himler et al. and were used in all experiments (16). These lines differ in *Rickettsia* infection status but share >98% of their nuclear alleles and were grown under the same environmental conditions. Cowpea seedlings in adjacent cages were infested with adult R<sup>+</sup> or R<sup>-</sup> whiteflies at the same time, and the insects were allowed to multiply to a high density. Whiteflies of each line were then collected at the same time and immediately placed under the experimental conditions. Care was taken to prevent contamination between R<sup>+</sup> and R<sup>-</sup> whiteflies during the experiments, and whitefly cultures were routinely checked for *Rickettsia* infection status using diagnostic PCR assays.

Pea aphids (*A. pisum* clone 5A, collected by N. Moran in 1999 in Madison, WI) were kept on fava bean plants at 24°C with 16 h of light and 8 h of darkness. This clone bears the obligate nutritional symbiont *Buchnera* but no secondary symbionts (26). For aphid assays, 5 to 10 adults were placed on each of 10 2-week-old plants and allowed to multiply to a high density for 5 to 7 days. At the start of an assay, second- and third-instar aphids were collected from all healthy plants. To control for maternal effects or health differences between plants, these aphids were then divided into treatments so that each treatment received approximately the same number of second- and third-instar aphids as well as individuals from each of the collection plants.

**In vitro pathogenicity assays.** *In vitro* feeding assays were used to test for the pathogenicity of two *P. syringae* strains, *P. syringae* pv. *syringae* B728a and *P. syringae* pv. *tomato* DC3000, to whiteflies after oral exposure. Both *P. syringae* strains display resistance to rifampin and show no genomic sequence difference from the original isolates obtained from Steven Lindow (*P. syringae* pv. *syringae* B728a) and Allan Collmer (*P. syringae* pv. *tomato* DC3000). For each assay, adult whiteflies were simultaneously collected from one cage and placed in 35-mm petri dishes covered in Parafilm. Each dish contained 5 to 20 whiteflies, with a total of 50 to 100 whiteflies being used per treatment. The Parafilm on each dish was covered with 1 ml of 5% sucrose in 10 mM sodium phosphate buffer (pH 7.0) and then sealed with another layer of Parafilm to create a feeding sachet. To make the bacterial treatments, 1 ml of an overnight culture was pelleted, washed twice in 10 mM MgCl<sub>2</sub> solution, and then resuspended in 5 ml of sucrose solution. For each solution, serial dilutions were then plated to confirm that bacterial concentrations were within a range from 3 × 10<sup>8</sup> to 5 × 10<sup>8</sup> CFU/ml during inoculation. Assay dishes were kept at 24°C for 4 to 6 days. All dishes were checked for dead whiteflies 1 to 2 h after the dishes were set up to identify any insects that died due to transfer. During the course of the experiment, dishes were checked for dead whiteflies twice daily at times 5 to 18 h apart. Insects were counted as dead if they turned brown or if they remained without movement at the bottom of the dish for over 1 min of observation. Each assay included a negative-control treatment (sucrose in buffer only) and a bacterial treatment with each *P. syringae* strain. Both *P. syringae* strains have previously been shown to cause death in aphids and are widely used laboratory strains. For each *P. syringae* strain, some bacterial sucrose solutions were checked after 2 days to confirm that the bacterial numbers were not increasing during the course of the experiment, and no significant growth was observed. All assays were independently replicated twice, with different batches of plants and whiteflies being used in each replicate of the experiment.

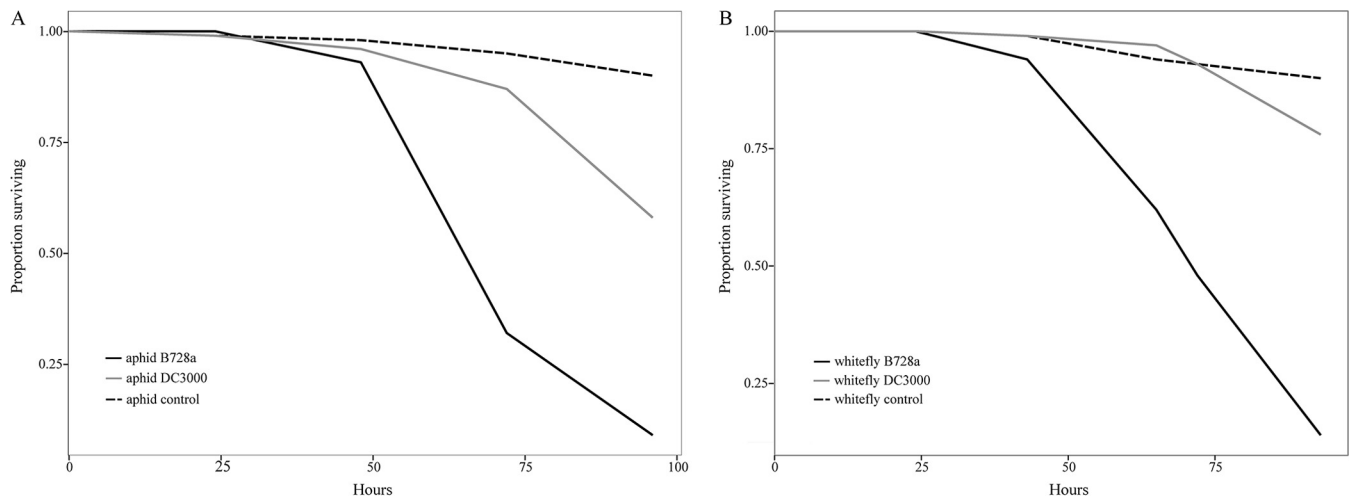
To compare the effects of *P. syringae* on whiteflies to the effects on aphids, we performed pathogenicity assays on aphids, modeled after those

done by Stavrinides et al. (22). Negative-control and bacterial treatments were prepared as described above for whiteflies, except that artificial aphid diet (27) instead of sucrose solution was used. Each well of a 96-well plate was filled with 200 μl of diet, and the plate was covered in Parafilm to make a feeding sachet. As described above, second- and third-instar aphids were placed individually in wells of a second 96-well plate, and the feeding sachet plate was inverted above them. This placement allowed the insects to feed through the Parafilm but kept them within the plate wells. The plates were kept under the same environmental conditions described above for whiteflies. After the aphids were allowed to feed for 24 h, the feeding sachet was replaced with a new one containing sterile artificial diet. This was repeated every 24 h for 4 days. At the time that the sachet was replaced, the aphids were also checked for mortality. An aphid was counted as dead if it had turned brown or was at the bottom of the well and did not move during the observation, even after gentle tapping of the plate on a benchtop. If an aphid was on the Parafilm of the feeding sachet but not moving, it was assumed to be feeding and alive. Each aphid assay included a negative-control treatment, a *P. syringae* pv. *syringae* B728a treatment, and a *P. syringae* pv. *tomato* DC3000 treatment, with the same number of aphids being used in each treatment. Aphid assays were replicated twice with 24 and 96 aphids per treatment in the respective assays.

**Bacterial growth estimates.** To track changes in bacterial titers in whiteflies over time after an initial exposure to bacteria, whiteflies were fed for 48 h on bacteria in sucrose solution, as described above, and then transferred to sterile sucrose solution. For each treatment with each bacterial strain or sucrose only, approximately 150 whiteflies were fed in the same 50-ml tube topped with a feeding sachet. After 48 h, living whiteflies were collected and placed in groups of 20 to 30 in 35-mm petri dishes and given a feeding sachet of sterile sucrose solution.

Bacterial sampling dishes were observed daily, and any dead whiteflies were marked with a dot on the bottom of the dish. Each bacterial sampling dish contained 20 to 30 whiteflies, and all whiteflies from a single dish were sampled at each time point. Living whiteflies were cooled by briefly placing the dish on ice and transferred with sterile forceps to a microcentrifuge tube containing silica beads and 10 mM MgCl<sub>2</sub> solution. The tubes were shaken for 20 s in an MP Biomedicals FastPrep-24 tissue homogenizer to release internal bacteria. Previous tests (data not shown) found no difference in bacterial numbers from whiteflies that had been surface sterilized by washing in 70% ethanol and those that had not, suggesting that bacteria on whitefly surfaces do not contribute significantly to the bacterial numbers observed in assays. For each time point, three groups of whiteflies were collected by combining 5 to 10 whiteflies per tube. In a second bacterial titer assay, three groups of both living and dead whiteflies from each treatment were separately sampled after 142 h on sterile medium. Dead whiteflies had been marked each day prior to sample collection, so any dead whiteflies sampled had died in the previous 24 h. For both assays, each homogenate was serially diluted in 10 mM MgCl<sub>2</sub> and the serial dilutions were plated on King's B agar plates containing rifampin. Bacterial titers per whitefly were determined for each group by averaging the colony counts across countable dilutions and replicates and then dividing by the number of whiteflies included in the group.

**Exposure on plants.** In order to test whether whiteflies can acquire bacteria from feeding on plants with epiphytic *P. syringae*, we exposed whiteflies to plants that had been painted with bacteria. Whole-leaf surfaces of 10-day-old cowpea seedlings (3 per pot) were painted, using a sterile cotton-tipped swab, with either *P. syringae* pv. *syringae* B728a cells in solution or sterile solution alone. For the bacterial solution, 4 ml of an overnight *P. syringae* pv. *syringae* B728a culture was pelleted, washed, and resuspended in 2 ml of 10 mM MgCl<sub>2</sub>. This solution was divided between seedlings in two pots, and 2 ml of sterile 10 mM MgCl<sub>2</sub> was painted on the seedlings in two other pots. Based on the optical density of the bacterial culture and the leaf surface area, we estimate that approximately 10<sup>6</sup> CFU/cm<sup>2</sup> was placed on the leaves, though only a portion of these cells likely persisted during the experiment, making the bacterial numbers on leaves within naturally occurring ranges (28). The plants were allowed to air dry,



**FIG 1** Proportion of surviving individuals over time for pea aphids and sweet potato whiteflies exposed to *P. syringae* strains *P. syringae* pv. *syringae* B728a and *P. syringae* pv. *tomato* DC3000 or no bacteria (control) in oral infection assays. (A) Proportion of surviving individuals for aphids averaged between two independent assays for each treatment (total  $n = 120$  aphids). Time zero represents the time when aphids were placed on artificial medium with bacteria. (B) Proportion of surviving individuals for whiteflies averaged between two independent assays for each treatment (total  $n = 147$  for control whiteflies, 156 for whiteflies exposed to B728a, and 160 for whiteflies exposed to DC3000). *Rickettsia*-infected whiteflies were used, and time zero represents the time when insects were placed in feeding assays.

and then each pot was covered and infested with approximately 200 whiteflies, with one pot of each treatment receiving  $R^+$  whiteflies and the other receiving  $R^-$  whiteflies. The pots were kept at 24°C for 48 h. After they had been on the plants for 48 h, 60 to 120 whiteflies from each treatment were collected and transferred to feeding dishes containing a sterile sucrose solution. In order to track the survival of whiteflies exposed to epiphytic bacteria, whiteflies were placed in feeding dishes in groups of 5 to 20 per dish and monitored for 118 or 139 h in two replicate assays. Additionally, 60 whiteflies from each treatment were sampled for the presence of *P. syringae* to determine if the whiteflies had been infected while feeding on the plants. For bacterial sampling, 20 groups of 3 whiteflies each (60 whiteflies in total) were homogenized as described above and the homogenate was placed in 10- $\mu$ l spots on plates. Spots with bacteria had either very few colonies or large numbers of colonies (uncountable), and samples with less than 10 colonies in a spot were considered not infected.

**Statistical analysis.** In each survival assay, survival was modeled in R (29) using Kaplan-Meier survival curves, which are frequently used in ecological studies of survival since they allow the inclusion of data on all individuals, including those that do not die during the observation time (30). Differences in survival between treatments were tested for significance using log rank tests. Data from all replicates of each assay were combined for analysis. Unless otherwise stated in the Results, the effect of replicates on the assay results was not significant, suggesting that the results of replicates of the assays were highly similar. For ease of interpretation, the results presented in the figures show the proportion of surviving individuals over time, while the reported statistical values are the result of survival curves and log rank tests.

## RESULTS

***P. syringae* is similarly pathogenic to whiteflies and aphids.** Sweet potato whiteflies orally exposed to *P. syringae* show highly elevated mortality compared to the negative controls (Fig. 1; Table 1). These patterns are similar to the previously documented pathogenic effects of *P. syringae* on pea aphids (22), which we have replicated here (Fig. 1A). The strain *P. syringae* pv. *syringae* B728a was highly virulent to both whiteflies and pea aphids, causing 100% mortality for the duration of the assays, while a second

strain, *P. syringae* pv. *tomato* DC3000, was less virulent but still caused insect death (Fig. 1) (22). We note that the methods used for assays with each species were slightly different, with whiteflies being exposed to bacteria continuously and aphids being exposed for 24 h. It is possible that aphids continuously exposed to bacteria could die more quickly. However, the trends in the effects of each strain on each insect compared to those for the controls would likely remain the same.

### *Rickettsia* infection provides protection against *P. syringae*.

Whereas  $R^+$  and  $R^-$  whiteflies survived equally well when fed on sterile artificial medium for 141 h (log rank test,  $\chi^2 = 0.1$ ,  $P = 0.735$ ), *Rickettsia* infection caused a significant decrease in the death rate related to exposure to the two *P. syringae* strains, *P. syringae* pv. *syringae* B728a (log rank test,  $\chi^2 = 35.6$ ,  $P = 2.76 \times 10^{-9}$ ) and *P. syringae* pv. *tomato* DC3000 (log rank test,  $\chi^2 = 13.8$ ,  $P = 2.04 \times 10^{-4}$ ) (Fig. 2). As with pea aphid death (22), whitefly death appeared to correspond to the growth of bacteria within the insect (Fig. 3). Both *P. syringae* strains grew within  $R^+$  and  $R^-$  whiteflies after oral exposure. Living whiteflies that were exposed

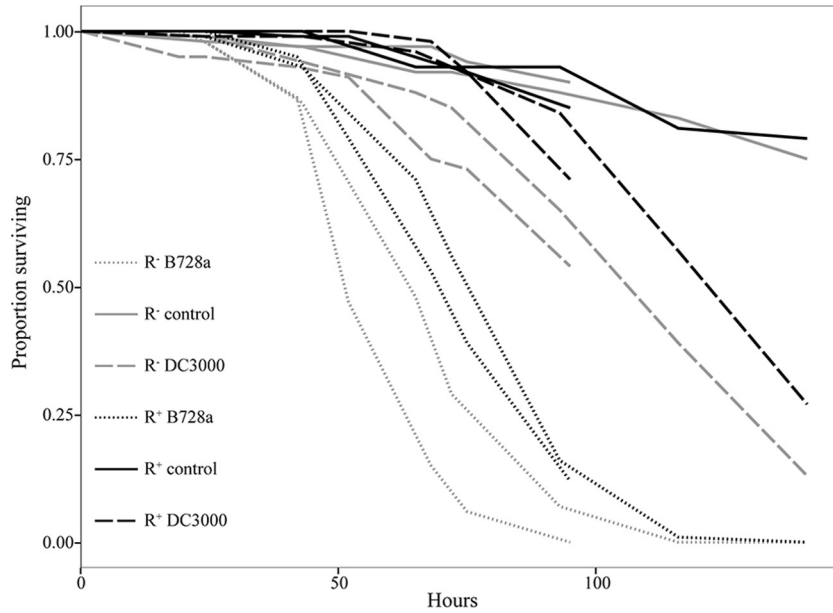
**TABLE 1** Results of log rank tests comparing Kaplan-Meier survival curves in aphids and whitefly treatments<sup>a</sup>

Insect and comparison group	df	$\chi^2$
Pea aphids		
B728a vs NC	1	228 <sup>b</sup>
DC3000 vs NC	1	36.3 <sup>c</sup>
B728a vs DC3000	1	122 <sup>b</sup>
Whiteflies		
B728a vs NC	1	269 <sup>b</sup>
DC3000 vs NC	1	48.9 <sup>c</sup>
B728a vs DC3000	1	154 <sup>b</sup>

<sup>a</sup> df, number of degrees of freedom; NC, negative-control treatment.

<sup>b</sup>  $P = 0$ .

<sup>c</sup>  $P = 0.00000001$ .

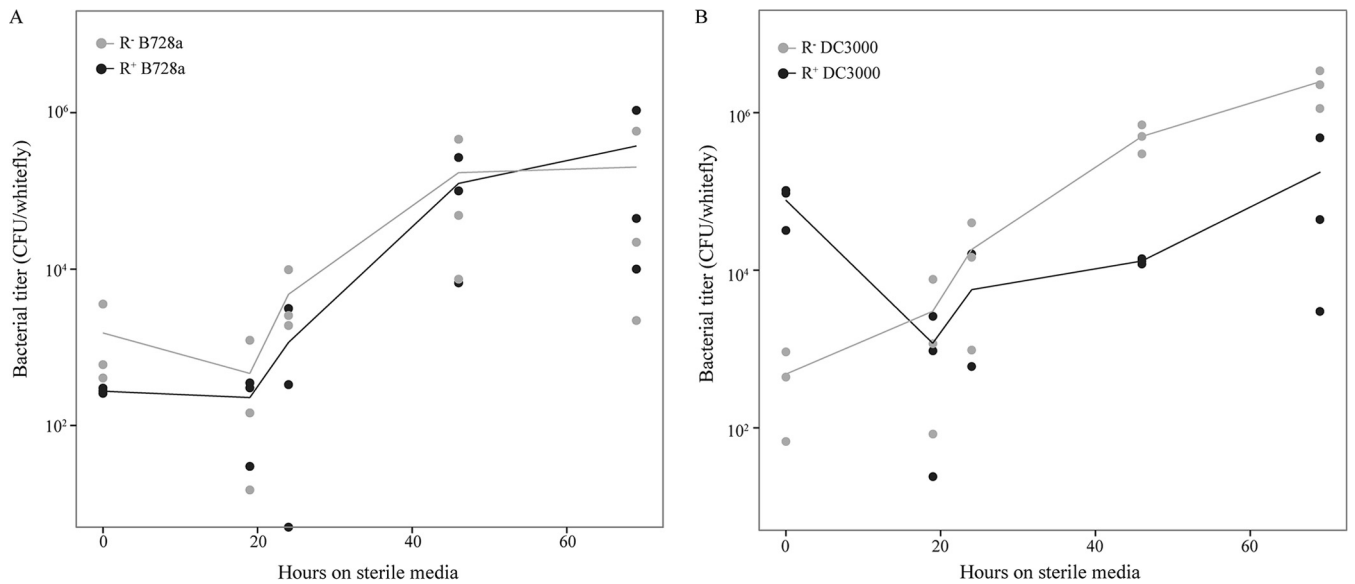


**FIG 2** Proportion of surviving individuals for whiteflies differing in *Rickettsia* infection exposed to *P. syringae* strains. Whiteflies were exposed to strains *P. syringae* pv. *syringae* B728a and *P. syringae* pv. *tomato* DC3000 or no bacteria (control) in oral infection assays. The results of two replicate assays are shown. For each assay combined, the total numbers of whiteflies were 147 for the R<sup>-</sup> whitefly controls, 156 for R<sup>+</sup> whiteflies exposed to B728a, 160 for R<sup>-</sup> whiteflies exposed to DC3000, 129 for the R<sup>+</sup> whitefly control, 142 for R<sup>+</sup> whiteflies exposed to B728a, and 129 for R<sup>+</sup> whiteflies exposed to DC3000. Whiteflies were exposed to bacteria throughout the assay, and time zero is the time of initial assay setup. One assay was followed for 91 h, and the second was followed for 141 h.

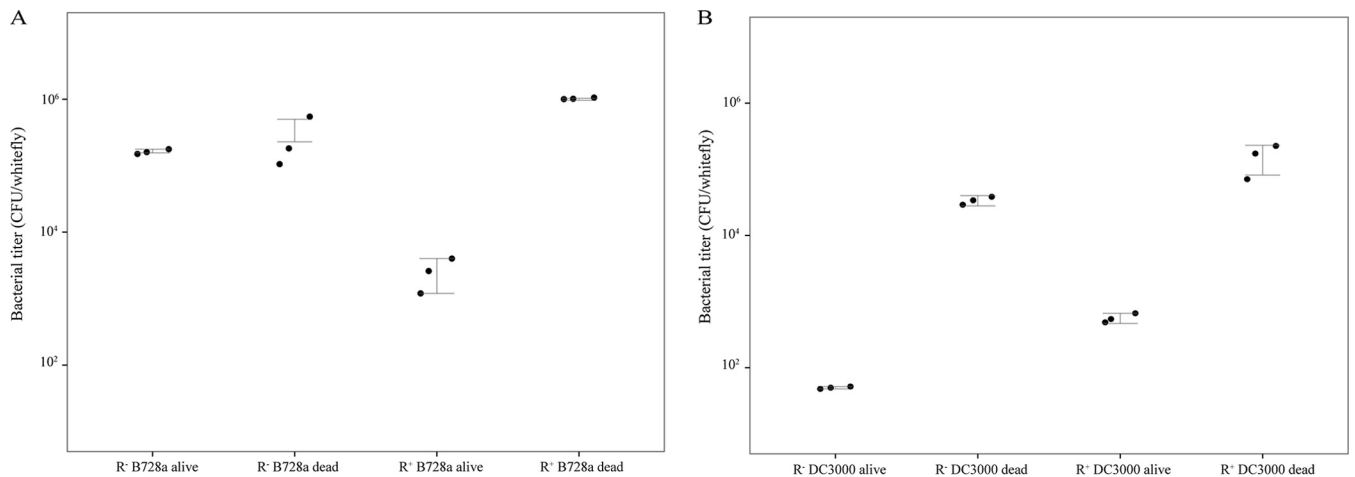
to bacteria for 48 h and then kept on sterile medium first showed a slight decrease or no change in bacterial titer for 19 h and then a steady increase in titer, presumably due to bacterial growth in the insects (Fig. 3). Both R<sup>+</sup> and R<sup>-</sup> whiteflies that died after 142 h on sterile medium after oral exposure had higher bacterial titers for both *P. syringae* strains than whiteflies that were still alive (10- to 1,000-fold differences, depending on the treatment; Fig. 4). Some

differences in *P. syringae* titers between the two types of whiteflies are suggested by our data. For both *P. syringae* strains, bacterial titers at most time points tended to be higher for living R<sup>-</sup> whiteflies (Fig. 3). Whiteflies that were not infected with *Rickettsia* and that died after 48 h of exposure were found to have approximately 10-fold lower levels of bacteria than R<sup>+</sup> whiteflies as well.

**Whiteflies and *P. syringae* on plants.** Our results show that *P.*



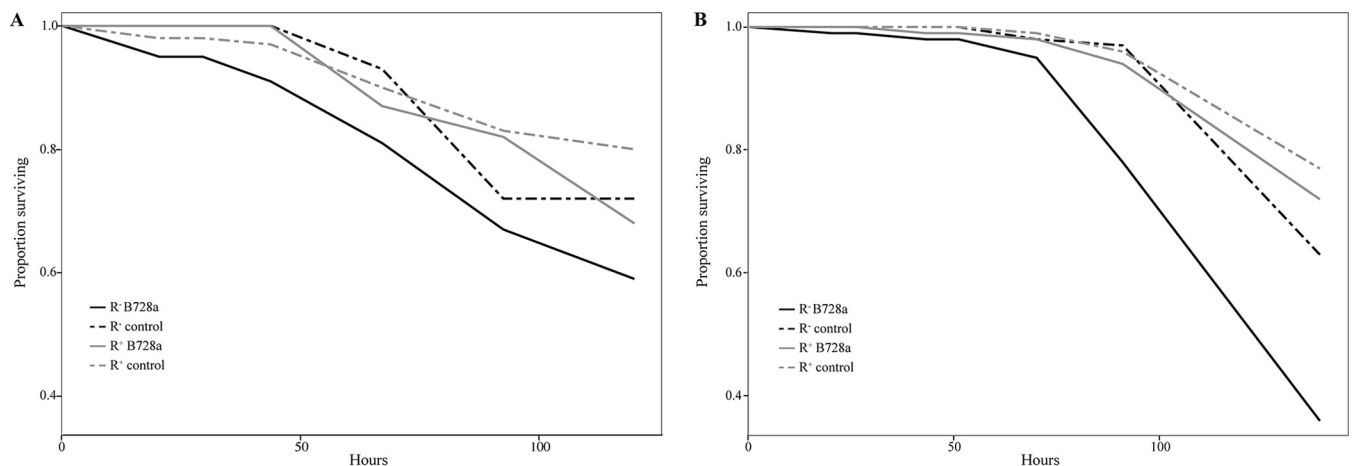
**FIG 3** Growth of *P. syringae* strains in whiteflies exposed to bacteria for 48 h and then fed on sterile medium. The time on the x axis begins after transfer to sterile medium, and three groups of 5 to 10 living whiteflies were taken for each time point. Each point represents the titer per whitefly for a group, and lines are drawn through mean values for each treatment at each time point. (A) R<sup>+</sup> and R<sup>-</sup> whiteflies infected with *P. syringae* pv. *syringae* B728a; (B) *Rickettsia*-positive and -negative whiteflies infected with *P. syringae* pv. *tomato* DC3000.



**FIG 4** *Pseudomonas syringae* titers in dead or living whiteflies after 48 h of bacterial exposure followed by 142 h on sterile medium. Three groups of 5 to 10 living or dead whiteflies were taken at each time point. Each point represents the titer per whitefly for a group, and error bars show the mean  $\pm$  standard error. (A) Whiteflies exposed to *P. syringae* pv. *syringae* B728a in sucrose solution for 48 h; (B) whiteflies exposed to *P. syringae* pv. *tomato* DC3000 in sucrose solution for 48 h.

*syringae* can be acquired at a low rate by insects feeding on plants. Whiteflies were allowed to feed for 48 h on plants that had been painted with *P. syringae* pv. *syringae* B728a. Bacteria were recoverable from these insects at a rate of 1.67 to 5.00% infected whiteflies, out of 240 individuals from two replicate assays, for both  $R^+$  and  $R^-$  whiteflies at the end of 48 h. Given the lag in bacterial growth within the whiteflies shown in Fig. 3, it is likely that more individuals were infected with low numbers of bacteria that were not detectable with our methods. To test if this apparently low infection rate could influence whitefly survival, we removed whiteflies from bacterially coated plants, placed them on sterile medium, and observed their survival. In two replicate assays,  $R^-$  whiteflies exposed to *P. syringae* on plants both began dying more quickly than whiteflies receiving the other treatments and showed the highest death rate overall (Fig. 5). *Rickettsia*-negative whiteflies exposed to *P. syringae* on plants showed a death rate significantly increased compared to that of the  $R^-$  controls at 66 and 70

h on sterile medium for assays 1 and 2, respectively (for assay 1, log rank test,  $\chi^2 = 11.1$ ,  $P = 8.44 \times 10^{-4}$ ; for assay 2, log rank test,  $\chi^2 = 16.33$ ,  $P = 5.31 \times 10^{-5}$ ). With the data from both assays combined, the rates of survival in all treatments were significantly different (Table 2), with all  $R^-$  whiteflies having a lower survival rate than  $R^+$  whiteflies and both  $R^+$  and  $R^-$  whiteflies exposed to *P. syringae* having a lower survival rate than controls with the same *Rickettsia* infection status, as expected (Fig. 5). The group with the highest mortality in both assays,  $R^-$  whiteflies exposed to bacteria, had a considerably lower rate of survival than the group with the second-highest mortality, the  $R^-$  control whiteflies (36% versus 63% for assay 2), and in assay 2,  $R^-$  whiteflies exposed to bacteria had half as many surviving individuals as  $R^+$  whiteflies exposed to bacteria (36% versus 72%). We note that the two replicate assays had different overall death rates, as well as small differences in the relative death rate, between treatments at some time points (Fig. 5). Reflective of this, the effect of the assay was significant in the



**FIG 5** Proportion of surviving individuals of whiteflies that were exposed to *P. syringae* pv. *syringae* B728a on plants for 48 h and then fed on sterile medium. Time zero represents the time of transfer to sterile medium. (A) Assay 1 was followed for 118 h on sterile medium with 60 ( $R^+$  whitefly controls), 72 ( $R^-$  whitefly controls), 60 ( $R^+$  whiteflies exposed to B728a), and 85 ( $R^-$  whiteflies exposed to B728a) whiteflies; (B) assay 2 was followed for 139 h on sterile medium with 112 ( $R^+$  whitefly controls), 110 ( $R^-$  whitefly controls), 104 ( $R^+$  whiteflies exposed to B728a), and 129 ( $R^-$  whiteflies exposed to B728a) whiteflies.

**TABLE 2** Results of log rank tests comparing Kaplan-Meier survival curves in whiteflies exposed to bacteria or control treatments on plants, including interaction terms for different treatments<sup>a</sup>

Comparison group	df	$\chi^2$
R <sup>-</sup> B728a vs R <sup>-</sup> NC	1	23.34 <sup>b</sup>
R <sup>+</sup> B728a vs R <sup>+</sup> NC	1	7.72 <sup>c</sup>
R <sup>-</sup> B728a vs R <sup>+</sup> B728a	1	28.13 <sup>b</sup>
R <sup>-</sup> NC vs R <sup>+</sup> NC	1	5.26 <sup>d</sup>
Assay replicate	1	56.46 <sup>b</sup>
(B728a vs NC) × (R <sup>+</sup> vs R <sup>-</sup> )	3	41.45 <sup>b</sup>
(B728a vs NC) × (R <sup>+</sup> vs R <sup>-</sup> ) × (assay replicate)	7	107.7 <sup>b</sup>

<sup>a</sup> df, number of degrees of freedom; NC, negative-control treatment.

<sup>b</sup>  $P < 0.000001$ .

<sup>c</sup>  $P < 0.001$ .

<sup>d</sup>  $P < 0.05$ .

analysis of the combined data (Table 2). Nevertheless, the relative trends between treatments were similar in the two assays. Taken together, these data suggest that whiteflies are able to acquire *P. syringae* from plants and that exposure to these bacteria in this manner leads to increased mortality. Furthermore, infection with *Rickettsia* appears to greatly improve survivorship after exposure to *P. syringae* on plants.

## DISCUSSION

This study provides evidence that oral exposure to strains of the common plant epiphyte *P. syringae* can increase mortality in the sweet potato whitefly, and as with pea aphids, the effect is consistent across multiple *P. syringae* strains. Furthermore, our results demonstrate that the facultative symbiont *Rickettsia* protects against pathogenic infection by *P. syringae*. *Pseudomonas syringae* is well-known as a plant pathogen and epiphyte but has only recently been documented to be an entomopathogen (22). This study provides evidence that the pathogenicity of *P. syringae* is not restricted to aphids but extends to another phloem feeder in the Sternorrhyncha Hemiptera, the whitefly *B. tabaci*. The results of this study provide further support for the suggestion that *P. syringae* can grow within and could possibly be vectored by whiteflies. These results are consistent with work suggesting that whitefly stylets are wide enough to allow the uptake of small bacterial cells (31, 32) but run counter to suggestions that phloem-feeding whiteflies may not commonly encounter or be influenced by bacteria on plant surfaces (33). These findings further suggest that bacterial epiphytes, some of which are of interest for use in the biological control of plant pathogens, could be explored as well for their potential use in insect pest management (21, 34). We found that the rate of death caused by *P. syringae* is similar in both pea aphids and whiteflies, even though the insects differ in body size and the size limitations of their digestive tracts, and that the relative effects of the two *P. syringae* strains tested were the same in both insect species (22, 32, 35). It appears that in both insect species, the death rate may correspond to the bacterial titer inside the insect. Stavrinides and colleagues suggested that mortality in aphids was linked to *P. syringae* growth reaching a lethal dose (22). Here, dead whiteflies had higher bacterial titers than living whiteflies, supporting the idea that whiteflies with higher bacterial concentrations are more likely to die. However, dead whiteflies were not sampled at their time of death but were sampled only within 24 h of death, so it is possible that the bacteria were more numer-

ous because they grew more quickly in dead whiteflies than living whiteflies.

*Rickettsia* has been shown to impact whiteflies in multiple ways, including influencing reproduction and survivorship. For instance, a rapid spread of *Rickettsia* through whitefly populations in the southwestern United States was recently documented and linked to the maternally transmitted bacterium's ability both to create female-biased sex ratios in offspring and to increase the reproductive output of infected females (16). Though *Rickettsia* in whiteflies has not previously been shown to influence responses to pathogens, closely related rickettsiae have been shown to be protective against a fungal pathogen in two aphid species (18).

How *Rickettsia* might slow pathogen-induced death is not known, though our results suggest two possibilities for further study. The finding that R<sup>+</sup> whiteflies tended to contain lower numbers of bacteria than R<sup>-</sup> whiteflies suggests that *P. syringae* either grows more slowly in R<sup>+</sup> whiteflies or is less able to infect them. A similar relationship has been found with the endosymbiont *Wolbachia* in flies challenged with viral and protist pathogens, in which pathogen density is reduced in the presence of the symbiont (9, 36). It is also possible that an interaction between *P. syringae* and *Rickettsia* could benefit infected whiteflies, for example, if the symbiont produces a toxin that kills or slows the growth of the pathogenic bacteria (37). Alternatively, higher bacterial doses may be required to kill R<sup>+</sup> whiteflies, as suggested by the fact that dead R<sup>+</sup> whiteflies had higher bacterial titers than dead R<sup>-</sup> whiteflies. It is possible that R<sup>+</sup> whiteflies are more robust and can therefore support higher numbers of pathogenic bacteria for longer time periods. *Rickettsia*-infected whiteflies have been found to perform better overall in laboratory cultures, and so they may be better able to survive the negative impacts of a parasite or they could be better able to mount an immune response to slow pathogen growth and prevent infection (16, 38).

*Rickettsia* infection lowered the rate of death due to both *P. syringae* strains, though it did not prevent eventual death. It should be noted that to ensure infection and death in a short time period, whiteflies in these experiments were exposed to high levels of bacteria during our *in vitro* experiments. It is possible that *Rickettsia* infection could be more effective at preventing death when insects are exposed to lower numbers of pathogenic bacteria, as is more likely in nature. In support of this, whiteflies that encountered *P. syringae* on experimental plant surfaces had higher rates of survival (survival that was twice as high in one assay) if they were infected with *Rickettsia*. The increased survival of R<sup>+</sup> whiteflies after *P. syringae* exposure under more natural conditions suggests that the presence of *Rickettsia* could significantly benefit whitefly populations that encounter epiphytic bacterial pathogens in nature.

*Bemisia tabaci* is susceptible to a number of fungal pathogens (39), but known bacterial pathogens are rare. To our knowledge, the most likely candidate for a bacterial pathogen of whiteflies is *Enterobacter cloacae*, which can be ingested and grow to high densities in *B. tabaci* adults, causing mortality (31), though it is not known how commonly whiteflies encounter the bacterium. Other bacterial species have been implicated in causing low to moderate rates of mortality in whiteflies, though in many cases death appears to be caused by a toxic, bacterially produced compound rather than ingestion of the bacteria themselves (40, 41). However, plant-associated insects, such as aphids and whiteflies, can encounter a variety of bacterial species while feeding on plants, and

as this study has shown, these bacteria may have negative effects on the insects. *P. syringae* is a widespread plant epiphyte that insects could regularly encounter on plants (23, 24). *Pseudomonas* species and other plant-associated bacteria are often recovered from surface-sterilized sweet potato whiteflies and other whitefly species using both culture-dependent and culture-independent methods (42–45). The results of this study suggest that whiteflies may acquire *P. syringae* while feeding on plants, though at a low rate. However, given that strains of the bacterium cause very high mortality, our results indicate that even a low initial infection rate can be detrimental to insects. The negative effects of *P. syringae* on insects may be particularly strong if a strain with high virulence, such as *P. syringae* pv. *syringae* B728a, was encountered, though the apparent variation in virulence between strains creates the possibility for context-dependent effects in nature. The protective effect of *Rickettsia* might increase the spread of the facultative symbiont in populations where the pathogenic bacterial strains are commonly encountered. We note that the frequencies of *Rickettsia* in this invasive whitefly are high in many countries around the world (46, 47), and while we expect that defense against opportunistic bacterial pathogens is unlikely to be important for spread in some geographic areas, particularly arid regions where *P. syringae* is uncommon, it may influence the frequency of *Rickettsia* in others.

These results demonstrate that the environmentally common bacterium *P. syringae* can be acquired by plant-associated sweet potato whiteflies and cause increased mortality for the insects. Furthermore, the facultative symbiont *Rickettsia* can ameliorate the effects of the pathogenic bacteria on the insects, possibly having significant effects in natural settings. This scenario represents an example of how a pathogen might help explain the prevalence of a facultative symbiont. This study highlights the possible importance of both environmental bacteria and symbionts, as well as the interactions of the two, on insect health.

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