Interactions Between *Frankliniella fusca* and *Pantoea ananatis* in the Center Rot Epidemic of Onion (*Allium cepa*)

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ABSTRACT

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An *Enterobacteriaceae* bacterium, *Pantoea ananatis* (Serrano) Mergaert, is the causal agent of an economically important disease of onion, center rot. *P. ananatis* is transmitted by an onion-infesting thrips, *Frankliniella fusca* (Hinds). However, interactions between *F. fusca* and *P. ananatis* as well as transmission mechanisms largely remain uncharacterized. This study investigated *P. ananatis* acquisition by thrips and transstadial persistence. Furthermore, the effects of bacterial acquisition on thrips fitness were also evaluated. When thrips larvae and adults were provided with acquisition access periods (AAP) on peanut leaflets contaminated with the

bacterium, an exponentially positive relationship was observed between AAP and *P. ananatis* acquisition ($R^2 \geq 0.77$, $P = 0.01$). *P. ananatis* persisted in thrips through several life stages (larvae, pupae, and adult). Despite the bacterial persistence, no significant effects on thrips fitness parameters such as fecundity and development were observed. Immunofluorescence microscopy of adult thrips with *P. ananatis*-specific antibody after 48 h AAP on contaminated food revealed that the bacterium was localized only in the gut. These results suggested that the pathogen is not circulative and could be transmitted through feces. Mechanical inoculation of onion seedlings with fecal rinsates produced center rot symptoms, whereas inoculation with rinsates potentially containing salivary secretions did not. These results provide evidence for stercorarian transmission (transmission through feces) of *P. ananatis* by *F. fusca*.

A number of *Enterobacteriaceae* members are phytopathogenic, and infections of these bacteria cause diseases in agricultural crops of economic importance (Bell et al. 2007; Bruton et al. 2003; Gitaitis and Gay 1997; Medrano and Bell 2007; Mitchell and Hanks 2009; Schwartz and Otto 2000). Examples include center rot of onion, bacterial wilt in cucumber, and yellow vine of cucurbits caused by *Pantoea ananatis* (Serrano) Mergaert, *Erwinia tracheiphila* (Smith), and *Serratia marcescens* Bizio, respectively (Bruton et al. 2003; Gitaitis and Gay 1997; Mitchell and Hanks 2009). Insects serve as vectors of several phytopathogenic *Enterobacteriaceae* members (Bell et al. 2007; Bruton et al. 2003; Gitaitis and Gay 1997; Medrano and Bell 2007; Mitchell and Hanks 2009). However, details on effects of enterobacteria on vector preference and fitness are limited when compared with transmission of other bacterial pathogens such as *Xylella fastidiosa* (Wells et al.), '*Candidatus Liberibacter asiaticus*' (Jagoueix et al.), and viruses (Almeida et al. 2005; Daugherty and Almeida 2009; Hill and Purcell 1995; Nachappa et al. 2012). In the majority of such interactions, the intricacy of vector-pathogen interactions seems to largely influence vector preference and fitness (Mauck et al. 2012). The effects of phytopathogenic enterobacteria on vectors' preference and fitness remain uninvestigated.

In this study, an attempt was made to further characterize interactions of enterobacteria with their arthropod vectors using the onion center rot pathosystem as a model. Center rot is one of the important bacterial diseases of onion, which affects onion production in the United States (Gitaitis and Gay 1997; Gitaitis et al. 2002, 2003). The symptoms of center rot include white streaks with water soaked margins running the length of the leaf and eventual soft rotting

of bulbs. Under favorable conditions, this disease has the potential to cause 100% yield loss (Gitaitis et al. 2002, 2003). Though *P. ananatis* could be seedborne (Walcott et al. 2002), it is widely believed that the primary mode of *P. ananatis* transmission to onion in the southern United States is by an insect vector tobacco thrips (*Frankliniella fusca* (Hinds)) (Gitaitis et al. 2003; Wells et al. 2002). *F. fusca* is also the most prevalent thrips species on onion plants in the southern United States (Diffe et al. 2008; Sparks et al. 2011).

Gitaitis et al. (2003) indicated that onion-colonizing thrips such as *F. fusca* could transmit *P. ananatis* but did not elucidate the interactions between thrips and *P. ananatis*. In general, details on interactions between phytopathogenic *Enterobacteriaceae* members and their vectors from an anatomical and physiological standpoint are, as yet, unclear. Particularly, details on pathogen localization or infection of insect tissues, persistence within the insect, and effects on vector fitness are not known. It is also not clear whether bacteria are transmitted to a susceptible host through feeding by salivary secretions or through other means such as regurgitation following biofilm formation and proventriculus blockage, as in the case of an animal-infecting *Enterobacteriaceae* member, *Yersinia pestis* (Jarrett et al. 2004). Very few studies have examined plant-infecting enterobacteria localization in arthropod vectors. Dutta et al. (2014) demonstrated that *P. ananatis* was localized only in the gut of its thrips vector, *Thrips tabaci* (Lindeman). Nevertheless, it is unknown whether the localization pattern of *P. ananatis* is conserved among thrips vectors.

The goals of this study were to gather basic information pertaining to acquisition of *P. ananatis* by its thrips vector (*F. fusca*) and examine persistence in thrips. An additional goal was to assess whether persistence of *P. ananatis* has any effect on the fitness of its vector. Experiments to assess the acquisition efficiency of *P. ananatis* by *F. fusca* adults and larvae and persistence of *P. ananatis* in *F. fusca* were conducted. To evaluate the effects of *P. ananatis* infection on *F. fusca* fitness, microcosms (Plexiglas cages) were used to monitor

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the development of *F. fusca* for an entire generation (adult to adult). Immunofluorescent labeling with *P. ananatis*-specific antibodies was used to localize *P. ananatis* in thrips. Subsequently, membrane feeding assays and inoculation assays were conducted to investigate the mechanism of *P. ananatis* transmission by *F. fusca*. A previously optimized peanut leaf assay was used to study the acquisition and persistence of *P. ananatis* by *F. fusca* (Gitaitis et al. 2003; Wells et al. 2002).

MATERIALS AND METHODS

Bacterial strains and inoculum preparation. *P. ananatis* strain PNA 97-1 isolated from symptomatic onion foliage showing center rot symptoms was used in this study (Gitaitis and Gay 1997). Inoculum preparation was done by inoculating 3 ml of nutrient broth with single colonies of bacterial strain from a 48-h-old culture on nutrient agar (NA). The inoculated nutrient broth was incubated overnight at 28°C on a rotary shaker (Innova; New Brunswick Scientific) at 150 rpm. After incubation, 3 ml of bacterial suspension was centrifuged at $6,000 \times g$ (Allegra 25R; Beckman Coulter) for 5 min. The supernatant was discarded and the pellet was resuspended in 1 ml of 0.1 M phosphate-buffered saline solution (PBS) (0.01 M KH_2PO_4 - K_2HPO_4 , pH 7.0, and 0.75% NaCl). The bacterial concentration was adjusted to an optical density of 0.3 at 600 nm (approximately 1×10^8 CFU/ml) using a spectrophotometer (Spectronic 20; Bausch and Lomb), and 10-fold serial dilutions were prepared in 0.9 ml of PBS to obtain desired concentrations of 1×10^6 CFU/ml. Confirmation of bacterial populations was checked by dilution plating of a 0.1-ml aliquot on NA.

***F. fusca* maintenance.** A colony of *F. fusca* was established in 2009 on noninfected 'Georgia Green' peanut leaflets with thrips collected from peanut blooms from the Belflower Farm, University of Georgia, Tifton. Since then, thrips were maintained in Munger cages (Munger 1942) on peanut (Georgia Green) leaflets. Munger cages were maintained in a growth chamber (Thermo Scientific) at 25 to 30°C with a photoperiod of 14 h of light and 10 h of darkness per 24 h. In order to confirm whether the peanut plants were negative for *P. ananatis*, leaflets ($n = 5$) without surface sterilization were rinsed in 2 ml of 0.1 M PBS for 2 min. The aliquots of 0.1 ml were then spread-plated on PA-20 semiselective medium (Goszczynska et al. 2006) and incubated. Thrips in the colony were also crushed and spread-plated on PA-20 medium to check for *P. ananatis* contamination. The majority (>90%) of individuals in the colonies were females. Adult females were subsequently used for all experiments.

Surface sterilization of peanut leaflets and *F. fusca* larvae and adults. Peanut leaflets ($n = 15$) were collected from thrips-free plants maintained in the greenhouse, which were surface sterilized using 10% sodium hypochlorite for 30 s and transferred to 70% ethanol for an additional 30 s. After rinsing twice in 2 ml of 0.1 M PBS, leaflets were placed in individual petri plates with partially opened lids and air dried in a laminar airflow chamber for 15 to 20 min. After drying, individual leaflets were placed in separate 2-ml microcentrifuge tubes. The efficiency of surface sterilization was assessed by spread plating 0.1-ml rinsates of five individual leaflets from the last rinsing step in replicates ($n = 3$) on PA-20 plates and incubating for 6 days at 28°C.

For surface sterilization of thrips, individuals were randomly collected from the laboratory-maintained colony and surface sterilized using the same procedure as described above. Rinsates from three replicates (from the last rinsing step with PBS) of 10 thrips were assessed separately by spread plating 0.1-ml aliquots on PA-20 plates followed by incubation for 6 days at 28°C. Additionally, the confirmation of laboratory-reared thrips (adult and larvae) being devoid of *P. ananatis* populations in their gut was achieved by macerating three replicates of 10 surface-sterilized thrips (adult and larvae) separately in 1.5-ml microcentrifuge tubes. The macerates were then suspended in 1 ml of 0.1 M PBS followed by spread plating of 0.1-ml aliquots on PA-20 agar plates. Upon incubation at

28°C for 6 days, plates were assessed for *P. ananatis* bacterial colonies.

Confirmatory tests for *P. ananatis*. Bacterial colonies were characterized by the presence of yellow colonies (3 to 4 mm in diameter) with dark inclusion body on PA-20 (Goszczynska et al. 2006), gram reaction, utilization of glucose in oxidative and fermentative reaction, indole reaction, and nitrate reductase reaction. In addition, real-time polymerase chain reaction (PCR) specific for *P. ananatis* was also conducted on three random colonies per agar plate. For PCR, a single bacterial colony was suspended in 25 μl of sterile deionized water and heated (Modular Dry Block Heaters) for 3 min at 95°C. DNA extract (5 μl) was amplified using a commercial real-time PCR mastermix (Bio-Rad iQ Supermix; Bio-Rad) in a 25- μl reaction with 25 μM each primer PanITS1 (5'-GTCTGAT AGAAAGATAAAGAC-3') and EC5 (5'-CGGTGGATG CCCTG GCA-3') (Walcott et al. 2002) and 10 μM TaqMan probe 6-FAM AGCGGTTAGGACTCCGCCCTTTCA-BHQ. The real-time PCR was carried out in a Cepheid Smart Cycler using the following thermal profile: denaturation at 95°C for 180 s, and 35 cycles each of denaturation at 95°C for 15 s and annealing at 60°C for 40 s. Samples with cycle threshold values less than 35 were considered positive for *P. ananatis*.

Acquisition of *P. ananatis* by *F. fusca* adults and larvae. After air drying, peanut leaflets ($n = 15$) were placed in a 2-ml microcentrifuge tube containing 1 ml of *P. ananatis* at 1×10^6 CFU/ml. Leaflets were incubated for 5 min and air dried for 2 to 3 min. Two to three surface-sterilized peanut leaflets (dipped in a bacterial suspension of 1×10^6 CFU/ml) were placed inside Munger cells (11.5 by 9 cm) lined with a moistened filter paper (Whatman number 2). Peanut leaflets incubated in PBS and air dried in Munger cells served as negative controls. Five cells were used for each treatment and PBS control. Fifty adults and larvae of *F. fusca* were released per Munger cell, and the percentage of thrips acquiring bacteria was assessed in a time-course study. At each acquisition access period (AAP) (0, 1, 6, 24, and 48 h), four individual adults and larvae of *F. fusca* were randomly sampled from each Munger cell and placed into separate 1.5-ml microcentrifuge tubes. After surface sterilization, the collected thrips (adult and larvae) were assayed individually for the presence of bacteria using dilution plating on PA-20 medium. Surface sterilization rinsate (from the last rinsing step with PBS) was also tested for the presence of *P. ananatis* by plating. Thrips fed on PBS-inoculated leaves served as a negative control. Percentages of *P. ananatis*-positive thrips (adult and larvae) at different AAP were recorded. This experiment was repeated five more times, with four replicates per treatment per time point. Regression analysis was used to determine the relationship between AAP and percentage of thrips (adult and larvae) acquiring *P. ananatis*. Regression analysis was conducted using PROC REG in SAS (version 9.1; SAS Institute) after assessing for differences among experiments using PROC GENMOD in SAS.

Transstadial persistence of *P. ananatis* in *F. fusca*. For this experiment, second-instar *F. fusca* larvae that developed from a cohort of eggs were exposed to *P. ananatis*-contaminated peanut leaflets. Surface-disinfested peanut leaflets ($n = 15$) were inoculated (leaflets dipped in bacterial suspension of 1×10^6 CFU/ml) with *P. ananatis* and placed in Munger cells. Fifty noninfected larvae of *F. fusca*, reared on *P. ananatis*-free peanut leaflets, were released per Munger cell and were given an AAP of 48 h. After incubation, larvae were removed and transferred to a separate Munger cell with surface-disinfested peanut leaflets, which served as a clean source of food. The surface-disinfested leaflets were replaced every alternate day. Samples of four individuals were randomly selected and surface sterilized from each Munger cell at 6 h postexposure at the pupal stage (96 h postexposure) and the adult stage (144 h postexposure). Samples collected were placed into separate 1.5-ml microcentrifuge tubes. Surface sterilization rinsates (from the last rinsing step with PBS) were also plated and evaluated to ensure no *P. ananatis* surface contamination in thrips. Presence of

P. ananatis from individual thrips was assayed separately by macerating and plating on PA-20 medium, as described above. After a period of incubation, percentages of *P. ananatis*-positive thrips at each sampling period were recorded. This experiment was repeated five more times, with four replicates per treatment per time point.

Effect of *P. ananatis* acquisition on *F. fusca* fitness. Time taken to complete an entire generation (adult to adult) and adult development were monitored on surface-sterilized peanut leaflets with and without epiphytic populations of *P. ananatis*. Peanut leaflets were placed in separate Munger cages and 10 thrips were released in each cage. Ten cages were set up (five for thrips with *P. ananatis*-contaminated leaflets and five for thrips with peanut leaflets without *P. ananatis*), and the experiment was repeated two more times ($n = 300$ thrips on leaflets with and without *P. ananatis* populations). Munger cages were kept in a growth chamber (Thermo Scientific) at 25 to 30°C with a 14-h photoperiod. Leaflets with or without *P. ananatis* in Munger cages were replaced every alternate day. The released adult thrips were removed from cages 5 days later, the cages were monitored daily under a compound microscope (Meiji Techno), and newly hatched larvae were recorded. The number of adults emerging from each cage was recorded at 24-h intervals, and the adults were removed. Observations were recorded until there were no more larvae or adults in each cage. Differences in the number of adults produced were estimated by subjecting the data to linear mixed models using PROC GLIMMIX. Treatments were considered as fixed effects and replications were considered as random effects. The median development time required for the first adult to develop was estimated for thrips on leaflets with and without *P. ananatis* populations. Statistical significance of differences in development time on leaflets with and without *P. ananatis* was estimated by a Wilcoxon Rank Sum test with a continuity correction factor of 0.5 and a Kruskal-Wallis test.

Immunolocalization of *P. ananatis* in adult *F. fusca*. Adult *F. fusca* ($n = 10$) were allowed to feed on peanut leaflets dipped in a suspension containing *P. ananatis* at 1×10^6 CFU/ml for 48 h and sampled for immunofluorescence microscopy. Thrips ($n = 10$) fed on PBS-treated peanut leaflets served as negative controls. Thrips samples were fixed, washed (buffer and sterile water), and dehydrated using an ascending ethanol series (Dutta et al. 2014). The dehydrated tissues were gradually infiltrated with LR White embedding resin (Ted Pella Inc.) and transferred to gelatin capsules containing fresh 100% LR White resin (Electron Microscopy Sciences) for polymerization. Longitudinal sections (250 nm) of thrips were made using a Leica EM UC6 ultra-microtome (Leica Microsystems) and mounted on glass slides. Two sections per sample were stained with 0.05% Toluidine blue to visualize the basic cellular arrangement. The remaining sections were immunolabeled with polyclonal rabbit anti-PNA (primary) (Walcott et al. 2002) and Alexa Fluor 488 conjugated goat antirabbit (secondary). Sections were observed using a fluorescence microscope (Nikon Eclipse 80i) with filter (wavelength 450 to 650 nm) attached to a Nikon DS-Ri1 camera, and bacterial localization in thrips was documented.

Transmission of *P. ananatis* to onion seedlings through *F. fusca* salivary and fecal rinsates obtained through membrane feeding. Laboratory-maintained thrips (negative for *P. ananatis*) in three subsets ($n = 15$ /subset) were allowed to feed on peanut leaflets dipped in a suspension containing *P. ananatis* at 1×10^6 CFU/ml for 72 h. Later, thrips were surface sterilized and transferred to sterile 1.5-ml microcentrifuge tubes. The opening of each tube was sealed with stretched paraffin film (Parafilm; American Can Company) to facilitate thrips probing and feeding. A drop (50 μ l) of sucrose solution (7% wt/vol) was placed on top of the paraffin film. A second layer of paraffin film, stretched likewise, was placed at the top of the first layer. Two to three holes were made on the paraffin film (on both layers) for aeration using a fine entomological pin (Indigo Instruments). The paraffin layers were removed after 3 days of incubation and thrips were extracted. Thrips feces were collected

by thoroughly rinsing the tubes with sterile PBS (500 μ l). Adult thrips fed on PBS-inoculated peanut leaflets and processed similarly for feces collection served as a negative control. Salivary secretions in sucrose solution were collected by rinsing the lower Parafilm layer with sterile PBS (200 μ l). For the pathogenicity test on onion, 0.1 ml of the rinsed buffer from each subset was placed at the cut end of an onion leaf. Onion seedlings were scored and center rot incidence (percent) was recorded at 6 to 12 days postinoculation (DPI). Onion leaves mechanically inoculated with *P. ananatis* at 1×10^6 CFU/ml as described above served as a positive control. Additionally, the presence of *P. ananatis* in fecal rinsates was confirmed by spread plating 0.1 ml of rinsate from each thrips subset on PA-20 agar plates. The presence of bacterial colonies was determined after 6 days of incubation at 28°C. In all, 10 replicates were used in a single experiment and the experiment was repeated one more time (20 replicates in total). Furthermore, to ascertain the presence of *P. ananatis* in thrips saliva, sucrose solution containing salivary secretions trapped between the two paraffin layers was collected and processed as described above. This experiment was also repeated one more time (20 replicates in total).

Confirmation of center rot of onion symptomatic seedlings. Bacterial isolations were performed to confirm that the symptoms observed on seedlings were caused by *P. ananatis*. Small pieces (2 mm²) of tissue bordering the symptomatic and asymptomatic areas from 10 seedlings per experiment were macerated using a sterile scalpel with a number 3 blade (DR Instruments) in 20 μ l of 0.1 M PBS. Tissue macerates were streaked onto both PA-20 and tryptic soy broth agar plates and incubated at 28°C. After incubation, bacterial colonies were characterized based on morphology, gram reaction, oxidative and fermentative utilization of glucose, indole reaction, and nitrate reductase test. Furthermore, *P. ananatis* colonies were confirmed by real-time PCR assays with species-specific primers (Walcott et al. 2002), as described above.

Confirmation of *P. ananatis* strain PNA 97-1 reisolated from *F. fusca* or seedlings with center rot symptoms by repetitive extragenic palindrome PCR. Bacterial colonies (isolated from *F. fusca* or seedlings with center rot symptoms) confirmed as *P. ananatis* by growth on PA-20 semiselective medium, physiological tests, and real-time PCR assay were reconfirmed as the same strain used for inoculation by repetitive extragenic palindrome (rep)-PCR. Bacterial strains were inoculated into 3 ml of nutrient broth and incubated on a rotary shaker (Innova; New Brunswick Scientific Co.) at 250 rpm for 18 h. After incubation, cells were harvested by centrifugation at $6,000 \times g$ (Allegra 25R; Beckman Coulter) for 5 min and DNA was extracted using the UltraClean Microbial DNA Kit (Mo Bio), according to the manufacturer's instructions. For rep-PCR, 2 μ l of bacterial DNA was amplified using 10 μ M BOXA1R primer (5'-CTA CGG CAA GGC GAC GCT GAC G-3') according to PCR conditions, as described previously (Versalovic et al. 1994). PCR products (10 μ l) were separated by electrophoresis at 125 V for 4 h on a 1.5% agarose gel in 1 $\times$ Tris-borate EDTA buffer. For comparison, rep-PCR was also conducted on DNA extracted from *P. ananatis* strain PNA 97-1, which was used as a reference bacterial strain. The DNA fingerprinting pattern of a reference strain was compared with the patterns obtained for the isolated strains (thrips and symptomatic seedlings).

RESULTS

Surface sterilization of peanut leaflets and *F. fusca* larvae and adults. *P. ananatis* colonies were not isolated from the rinsates of surface-disinfested and noninfected peanut leaflets on PA-20 agar plates. Additionally, bacterial colonies were also not recovered from *F. fusca* rinsates (from the last rinsing step with PBS) postsurface sterilization.

Acquisition of *P. ananatis* by *F. fusca* adults and larvae. *F. fusca* larvae and adults did not acquire *P. ananatis* after a 48-h AAP from PBS-treated peanut leaflets. In contrast, *F. fusca* adults

and larvae acquired *P. ananatis* following acquisition access on treated leaflets. *P. ananatis* acquisition percentages in *F. fusca* adults after 1- and 6-h AAP were 7.1 and 28.5%, respectively (Fig. 1A). The mean percentages of *P. ananatis* acquisition by larvae after 1- and 6-h AAP were 4.2 and 25%, respectively (Fig. 1B). At a 24-h AAP, 47.6 and 45.8% of the adults and larvae, respectively, were positive for *P. ananatis*. Acquisition percentages did not vary with experiments in adults ($df = 4,115$, $\chi^2 = 2.55$, $P = 0.77$) and larvae ($df = 4,115$, $\chi^2 = 2.23$, $P = 0.82$). Data from experiments were combined and subjected to regression analysis. Results showed an exponentially positive relationship between AAP and percentages of adult *F. fusca* acquiring *P. ananatis* ($R^2 = 0.79$, $P < 0.01$), with 73.8% of adults acquiring the bacterium after a 48-h AAP (Fig. 1A). An exponentially positive relationship also was observed between AAP and percentages of *F. fusca* larvae acquiring *P. ananatis* ($R^2 = 0.77$, $P < 0.01$), with 70.8% of larvae acquiring the bacterium (Fig. 1B) after a 48-h AAP.

Transstadial persistence of *P. ananatis* in *F. fusca*. *P. ananatis* colonies were not recovered from *F. fusca* samples exposed to PBS-treated leaflets. The mean percentages of larvae (6 h postexposure) and pupae (96 h postexposure) that tested positive for *P. ananatis* were 51.2 and 50%, respectively. In contrast, at the adult stage (144 h postexposure), the percentage of positive samples was approximately six times less (8.3%) than in the other two stages. In this experiment, following AAP, thrips were reared on surface-disinfested leaflets changed on alternate days. Also, *F. fusca* pupae are not mobile and do not feed. Therefore, presence of *P. ananatis* in the adults following molting from the pupal stages confirmed that *P. ananatis* persisted in thrips transstadially.

Effect of *P. ananatis* acquisition on *F. fusca* fitness. Thrips were monitored for an entire generation (adult to adult) in Munger cages on peanut leaflets with and without epiphytic populations of *P. ananatis*. The number of adults developed per adult released did not vary with the repeats of the experiment ($df = 2, 27$, $F = 1.74$, $P = 0.19$). Also, no differences between treatments were noticed ($df = 1, 28$, $F = 1.74$, $P = 0.74$). These results indicated that the presence of epiphytic populations of *P. ananatis* had no effect on thrips development. The median developmental time to complete one generation (adult to adult) on leaves with and without *P. ananatis* populations were 15 days (12 to 19) and 15 days (14 to 18), respectively. Numbers in parentheses represent ranges in first

adult development time in individual Munger cells. Developmental time of *F. fusca* was not influenced by the presence of *P. ananatis* ($df = 1, 22$, $Z = 1.39$, $Pr > |Z| = 0.16$).

Immunolocalization of *P. ananatis* in *F. fusca* adults. The toluidine-blue-stained section showed anatomical features of *F. fusca* (Fig. 2A and C). Sections of negative controls (*F. fusca* on PBS-treated peanut leaflets) that were labeled with antirabbit immunoglobulin G conjugated with Alexa-fluor 488 alone did not produce fluorescent signals (Fig. 2B). This indicated the absence of nonspecific binding of the secondary antibody to thrips tissues (Fig. 2B). When the sections of *F. fusca* were immunolabeled with primary and secondary antibodies and observed under a fluorescent microscope, immunolabeling of *P. ananatis* was visible in the esophagus, midgut 1, midgut 2, midgut 3, and hindgut (Fig. 2D). *P. ananatis* was not observed in any other tissues within thrips or on thrips cuticle. Immunolabeled sections from all 10 thrips indicated that *P. ananatis* was exclusively localized in the gut.

Transmission of *P. ananatis* to onion seedlings through *F. fusca* salivary and fecal rinsates obtained through membrane feeding. *P. ananatis* colonies were not recovered from sucrose solution trapped between paraffin film layers by plating on PA-20 medium, suggesting that the bacterium was absent in salivary secretions. In contrast, *P. ananatis* colonies were recovered from 100% of the fecal rinsates that were exposed to the bacterium. In two independent experiments, 45% (average) of the onion seedlings that were inoculated with thrips fecal rinsates developed center rot symptoms by 12 DPI. Of the seedlings that were mechanically inoculated with *P. ananatis* as a positive control, 100% displayed typical center rot symptoms at 6 DPI.

Leaf tissues from seedlings that displayed center rot symptoms were used for bacterial isolation. Bacterial isolates were further tested for *P. ananatis*. Target bacterial colonies were isolated from 100% of the symptomatic seedlings. The isolated colonies were observed as small (3 to 4 mm in diameter), yellow-colored, shiny colonies with dark yellow granules at the center, on PA-20 semiselective medium. Further testing of colonies revealed that they were gram negative, could utilize glucose as a facultative anaerobe, and were positive for indole and negative for nitrate reductase tests. Amplification of DNA from three random colonies per plate by *P. ananatis*-specific real-time PCR primers confirmed the identity of the bacterium. Seedlings inoculated with sucrose solution remained asymptomatic.

Fig. 1. Mean percentage of **A**, adults and **B**, larvae of *Frankliniella fusca* acquiring *Pantoea ananatis* after acquisition access periods (0, 1, 6, 24, and 48 h) on peanut leaflets supporting epiphytic bacterial populations. Individual adult thrips were randomly sampled and assayed separately for the presence of bacteria using dilution plating on respective semiselective medium. Four individuals were used at each acquisition access period, and the experiment was repeated six times. Each data point represents the mean of six experiments and the error bars represent standard error for the mean.

Confirmation of *P. ananatis* strain PNA 97-1 reisolated from *F. fusca* or seedlings with center rot symptoms by repetitive extragenic palindrome (rep)-PCR. DNA fingerprinting patterns obtained by rep-PCR of bacterial strains isolated from thrips or center rot infected seedlings were identical to known pattern of a *P. ananatis* reference strain (PNA 97-1) (Fig. 3).

DISCUSSION

Prior to this study, it was not clear how acquisition of a phytopathogenic bacterium varied with life stages of its thrips vector, and how long the acquired bacterium persisted in thrips. Results from this study revealed that both adults and larvae of *F. fusca* could efficiently acquire *P. ananatis* from a contaminated food source. Once acquired,

the bacterium persisted transstadially in *F. fusca*. Despite the acquisition and persistence in the insect, there seems to be no noticeable effects on thrips fecundity (adults recovered per adult released) and developmental time. Additionally, immunolabeled microscopy revealed that *P. ananatis* was only localized in the gut of *F. fusca* and nowhere else in the insect body. These results suggest that the bacterium is not circulative in the insect. Absence of *P. ananatis* infection in onion seedlings following mechanical inoculation of sucrose solution potentially containing salivary secretions indicated that the bacterium was not transmitted through saliva. Furthermore, infection of *P. ananatis* on onion seedlings following mechanical inoculation of fecal rinsates suggested that the bacterium was likely transmitted to onion seedlings via feces.

Fig. 2. Immunolocalization of *Pantoea ananatis* in adult *Frankliniella fusca*. **A**, Micrograph of negative control *F. fusca* stained with toluidine blue dye; **B**, immunolabeled micrographs of negative control *F. fusca*; **C**, *F. fusca* micrograph stained with toluidine blue dye that fed on epiphytic populations of *P. ananatis*; **D**, immunolabeled micrograph of *F. fusca* fed on epiphytic populations of *P. ananatis*. TH = thorax; Oe = esophagus; MG = midgut 1, 2, and 3; and HG = hindgut. Fluorescing green areas show the localization of *P. ananatis* cells in the gut of *F. fusca*. Bars correspond to 100 μ m.

P. ananatis was found in surface-sterilized larvae and adults of *F. fusca* following acquisition access on contaminated peanut leaflets. Both life stages acquired *P. ananatis* within 1 h of AAP and, by 48 h of AAP, approximately 70% of the thrips tested acquired the bacterium. In addition, there was a strong linear relationship between AAP and *P. ananatis* acquisition from a contaminated food source. Longer acquisition periods exponentially increased the probability of *P. ananatis* acquisition by *F. fusca* larvae and adults. Similar observations were made in the case of another phytopathogenic bacterium (*X. fastidiosa*) and the blue green sharpshooter (*Graphocephala atropunctata* Signoret), where a minimum AAP of 2 h was required for the vectors to become infective (Purcell and Finlay 1979). Other experiments demonstrated that an increase in AAP up to 12 h led to increase in *X. fastidiosa* transmission by 68% of the adults (Hill and Purcell 1995). Daugherty and Almeida (2009) observed that the acquisition efficiency of *X. fastidiosa* by glassy-winged sharpshooter (*Homalodisca vitripennis* Germar) was dependent on pathogen populations in infected plants and lengths of AAP. It is not clear whether *P. ananatis* acquisition is dependent upon epiphytic pathogen densities because the experiments were conducted using high densities of *P. ananatis* (1×10^6 CFU/ml); however, it is likely that the acquisition of *P. ananatis* by *F. fusca* would also be density dependent.

Even though *P. ananatis* persisted through the different life stages of *F. fusca*, the infection percentages decreased with time. The infection percentages in surface-sterilized larvae and pupae were more or less similar (approximately 50%) but were substantially (six times) less in adults. The loss of bacteria in adults could be due, in part, to the fact that thrips is a hemimetabolous insect, and anatomical reorganization from pupae to adults is more extensive than transitioning from larvae to pupae (Johnson and Triplehorn 2004; Tipping 2008). Persistence of *Enterobacteriaceae* members through vector life stages is also documented in other pathosystems. For instance, *P. stewartii* (Smith) persisted in the flea beetle *Chaetocnema pulicaria* (F. E. Melsheimer) for its life, upon

acquisition at the larval stage (Esker and Nutter 2003; Menelas et al. 2006).

Fitness experiments indicated that the persistence of the bacterium had no effect on the fecundity (adults recovered per adult released) of thrips as well as on thrips developmental time. These results indicated that persistence of the bacterium had no beneficial or deleterious fitness effects on thrips themselves. These findings were contrary to observations made by de Vries et al. (2004). Their study identified positive fitness effects in the case of the western flower thrips, *F. occidentalis* (Pergande), with the gut bacterium *Erwinia* sp. when compared with thrips without the gut bacterium. Bacteria-laden thrips had a shorter larval development time and increased fecundity when compared with thrips without the enterobacterium (de Vries et al. 2001). Even though *P. ananatis* and *Erwinia* sp. found on *F. occidentalis* are taxonomically similar (members of *Enterobacteriaceae*), it is not clear why such fitness benefits were not observed with *F. fusca*.

Immunofluorescence microscopy further shed some light on bacteria localization in thrips and allowed us to identify the mechanism of transmission. Immunofluorescence micrographs of adult *F. fusca* with a 48-h AAP on *P. ananatis*-infected leaflets indicated that the bacterium was localized only in the gut. The absence of immunofluorescence on the outer cuticle further reiterated that there was no contamination and that surface sterilization was effective. The absence of *P. ananatis* in other insect tissues such as salivary glands suggested that the pathogen is not circulative. These results are similar to what was observed with onion thrips, *T. tabaci* and *P. ananatis* (Dutta et al. 2014). This observation was not surprising because *P. ananatis* is a member of *Enterobacteriaceae*, which includes several gut-residing bacteria such as *Erwinia* and *Pectobacterium* spp. in thrips and other insects (de Vries et al. 2001).

P. ananatis populations were not associated with mouth parts of *F. fusca*, thereby suggesting that the bacterium might not be transmitted through thrips feeding. Membrane-based feeding assays showed that *P. ananatis* might be transmitted via feces and not by salivary secretions. Together, our findings suggest that the pathogen could infect onion through the feeding injuries and simultaneous fecal contamination. These results were in line with our earlier findings, which documented *P. ananatis* transmission by *T. tabaci* through feces (Dutta et al. 2014). The transmission of *P. ananatis* by *T. tabaci* and *F. fusca* through feces suggests that mode of transmission is similar among thrips vectors.

Though our previous research also indicated that *T. tabaci* could transmit *P. ananatis* via feces (Dutta et al. 2014), the findings in this study include information on acquisition and persistence of *P. ananatis* in various life stages of thrips, and also on thrips fecundity and development. Prior to this study, such information pertaining to transmission of plant-pathogenic *Enterobacteriaceae* members by thrips was lacking. In addition, the findings in this manuscript validate our previous findings on the mode of *P. ananatis* transmission by thrips (Dutta et al. 2014), and suggest that the mode of *P. ananatis* transmission via feces (setcorarion transmission) is conserved among vector thrips species.

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LITERATURE CITED

- Almeida, R. P. P., Winstrom, C., Hill, B. L., Hashim, J., and Purcell, A. H. 2005. Vector transmission of *Xylella fastidiosa* to dormant grape. *Plant Dis.* 89:419-424.
- Bell, A. A., Medrano, E. G., Lopez, J. D., and Luff, R. K. 2007. Transmission and importance of *Pantoea ananatis* during feeding on cotton buds (*Gossypium hirsutum* L.) by cotton fleahoppers (*Pseudatomoscelis seriatus* Reuter). In: *Proc. World Cotton Res. Conf.* Lubbock, TX.

Fig. 3. DNA fingerprinting pattern of *Pantoea ananatis* strains reisolated from symptomatic onion seedlings and thrips (after acquisition and persistence experiments) using repetitive extragenic palindrome polymerase chain reaction. Lanes in the gel image denote the following: lane L = 100-bp ladder (New England Biolabs), lane 1 = PNA 97-1 (reference strain), lanes 2 to 4 = *P. ananatis* reisolated from symptomatic onion seedlings, and lanes 5 and 6 = *P. ananatis* reisolated after acquisition and persistence experiments.

- Bruton, B. D., Mitchell, F., Fletcher, J., Pair, S. D., Wayadande, A., Melcher, U., Brady, J., Bextine, B., and Popham, T. W. 2003. *Serratia marcescens*, a phloem-colonizing, squash bug transmitted bacterium: Causal agent of cucurbit yellow vine disease. *Plant Dis.* 87:937-944.
- Daugherty, M. P., and Almeida, R. P. P. 2009. Estimating *Xylella fastidiosa* transmission parameters: Decoupling sharpshooter number and feeding period. *Entomol. Exp. Appl.* 132:84-92.
- de Vries, E. J., Breeuwer, J. A. J., Jacobs, G., and Mollema, C. 2001. The association of western flower thrips, *Frankliniella occidentalis*, with a near *Erwinia* species gut bacteria: Transient or permanent? *J. Invertebr. Pathol.* 77:120-128.
- de Vries, E. J., Jacobs, G., Breeuwer, J. A. J., Sabelis, M. W., and Menken, S. B. J. 2004. Diet dependent fitness effects of gut bacteria on their insect host: The symbiosis of *Erwinia* sp. and western flower thrips. *Proc. R. Soc. Lond. B* 271:2171-2178.
- Diffé, S., Edwards, G. B., and Mound, L. A. 2008. Thysanoptera of south-eastern U.S.A.: A checklist for Florida and Georgia. *Zootaxa* 1787:45-62.
- Dutta, B., Barman, A. K., Srinivasan, R., Avcı, U., Ullman, D. E., Langston, D. B., and Gitaitis, R. D. 2014. Transmission of *Pantoea ananatis* and *Pantoea agglomerans*, causal agents of center rot of onion (*Allium cepa* L.), by onion thrips (*Thrips tabaci* Lindeman) through feces. *Phytopathology* 104:812-819.
- Esker, P. D., and Nutter, F. W. 2003. Temporal dynamics of corn flea beetle populations infested with *Pantoea stewartii*, causal agent of Stewart's disease of corn. *Phytopathology* 93:210-218.
- Gitaitis, R., Walcott, R., Culpepper, S., Sanders, H., Zolobowska, L., and Langston, D. 2002. Recovery of *Pantoea ananatis*, causal agent of center rot of onion, from weeds and crops in Georgia, USA. *Crop Prot.* 21:983-989.
- Gitaitis, R. D., and Gay, J. D. 1997. First report of a leaf blight, seed stalk rot, and bulb decay of onion by *Pantoea ananas* in Georgia. *Plant Dis.* 81:1096.
- Gitaitis, R. D., Walcott, R. R., Wells, M. L., Diaz Perez, J. C., and Sanders, F. H. 2003. Transmission of *Pantoea ananatis*, causal agent of center rot of onion, by tobacco thrips, *Frankliniella fusca*. *Plant Dis.* 87:675-678.
- Goszczyńska, T., Venter, S. N., and Coutinho, T. A. 2006. PA-20, a semi-selective medium for isolation and enumeration of *Pantoea ananatis*. *J. Microbiol. Methods* 64:225-231.
- Hill, B. L., and Purcell, A. H. 1995. Acquisition and retention of *Xylella fastidiosa* by an efficient vector, *Graphocephala atropunctata*. *Phytopathology* 85:209-212.
- Jarrett, C. O., Deak, E., Isherwood, K. E., Oyston, P. C., Fischer, E. R., Whitney, A. R., Kobayashi, S. D., DeLeo, F. R., and Hinnebusch, B. J. 2004. Transmission of *Yersinia pestis* from an infectious biofilm in the flea vector. *J. Infect. Dis.* 190:782-792.
- Johnson, N. F., and Triplehorn, C. A. 2004. Order Thysanoptera: Thrips. Pages 327-342 in: *Introduction to the Study of Insects*. D. J. Borrer, C. A. Triplehorn, and N. F. Johnson, eds. Cengage Learning, Inc., Independence, KY.
- Mauck, K., Bosque-Pérez, N. A., Eigenbrode, S. D., Moraes, C. M., and Mescher, M. C. 2012. Transmission mechanisms shape pathogen effects on host-vector interactions: Evidence from plant viruses. *Funct. Ecol.* 26:1162-1175.
- Medrano, E. G., and Bell, A. A. 2007. Role of *Pantoea agglomerans* in opportunistic bacterial seed and boll rot of cotton (*Gossypium hirsutum*) grown in the field. *J. Appl. Microbiol.* 102:134-143.
- Menelas, B., Block, C. C., Esker, P. D., and Nutter, F. W. 2006. Quantifying the feeding periods required by corn flea beetles to acquire and transmit *Pantoea stewartii*. *Plant Dis.* 90:319-324.
- Mitchell, R. F., and Hanks, L. M. 2009. Insect frass as a pathway for transmission of bacterial wilt of cucurbits. *Environ. Entomol.* 38:395-403.
- Munger, F. 1942. A method of rearing citrus thrips in the laboratory. *J. Econ. Entomol.* 35:373-375.
- Nachappa, P., Shapiro, A. A., and Tamborindéguy, C. 2012. Effect of '*Candidatus Liberibacter solanacearum*' on fitness of its insect vector, *Bactericera cockerelli* (Hemiptera: Trioziidae), on tomato. *Phytopathology* 102:41-46.
- Purcell, A. H., and Finlay, A. H. 1979. Evidence of non-circulative transmission of Pierce's disease bacterium by sharpshooter leafhoppers. *Phytopathology* 69:393-395.
- Schwartz, H. F., and Otto, K. 2000. First report of a leaf blight and bulb decay of onion by *Pantoea ananatis* in Colorado. *Plant Dis.* 84:808.
- Sparks, A. N., Diffé, S., and Riley, D. G. 2011. Thrips species composition shift on onions in the Vidalia region of Georgia. *J. Environ. Sci. (China)* 46:40-45.
- Tipping, C. 2008. Thrips (Thysanoptera). Pages 3769-3771 in: *Encyclopedia of Entomology*. J. L. Capinera, ed. Springer, New York.
- Versalovic, J., Schneider, M., de Bruijn, F. J., and Lupski, J. R. 1994. Genomic fingerprinting of bacteria using repetitive sequence-based polymerase chain reaction. *Methods Mol. Cell. Biol.* 5:25-40.
- Walcott, R. R., Gitaitis, R. D., Castro, A. C., Sanders, F. H., Jr., and Diaz-Perez, J. C. 2002. Natural infestation of onion seed by *Pantoea ananatis*, causal agent of center rot. *Plant Dis.* 86:106-111.
- Wells, M. L., Gitaitis, R. D., and Sanders, F. H. 2002. The Association of tobacco thrips, *Frankliniella fusca*, (Thysanoptera: Thripidae) with two species of bacteria of the genus *Pantoea*. *Ann. Entomol. Soc. Am.* 95:719-723.