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Influence of *prgH* on the Persistence of Ingested *Salmonella enterica* in the Leafhopper *Macrosteles quadrilineatus*

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Phytophagous insects can encounter *Salmonella enterica* on contaminated plant surfaces and transmit externally adhered and internalized bacteria on and among leaves. Excretion of ingested *S. enterica* by the leafhopper *Macrosteles quadrilineatus* has been previously reported; however, the sites of persistence of ingested bacteria remain undetermined. Fluorescence microscopy revealed the presence and persistence of *S. enterica* in various organs of *M. quadrilineatus* fed an inoculated diet for 12 h and then moved to two consecutive noninoculated diets for a total of 48 h. Ingested *S. enterica* was predominantly observed in the filter chamber, midgut, and Malpighian tubules of *M. quadrilineatus* dissected immediately after acquisition and at 24- and 48-h post-acquisition access periods (post-AAPs). Additionally, we examined the potential roles of the *Salmonella* pathogenicity island 1 (SPI-1) and SPI-2 type III secretion systems (T3SSs) in the persistence and excretion of ingested *S. enterica*. In competition assays, a *prgH* mutant lacking a functional SPI-1 T3SS was recovered at significantly lower levels than the WT in insect homogenates at 24 h post-AAP, and complementation with *prgH* restored *S. enterica* persistence in *M. quadrilineatus*. Moreover, expression of *prgH* inside *M. quadrilineatus* was observed up to 48 post-AAP. No differences were observed between the WT and an *ssaK* mutant lacking a functional SPI-2 T3SS in insect homogenates or between the WT and either mutant in insect excretions. This study provides novel insight into the presence and persistence of *S. enterica* inside *M. quadrilineatus* and demonstrates that the SPI-1 T3SS influences the persistence of the pathogen in the gut of a potential vector.

Salmonella enterica is the leading cause of bacterial foodborne illness in the United States (1). In the last few decades, salmonellosis outbreaks attributed to *S. enterica*-contaminated fresh produce have increased, and produce is now considered the most likely vehicle of disease transmission (2). Plant contamination with *S. enterica* is thought to primarily occur in the field before harvest, and the pathogen has the ability to adhere, colonize, and persist in and on plant surfaces, in some cases for extended periods (3, 4). Domestic and wild animals represent one of the main entry routes for *S. enterica* to produce fields, where they can directly introduce the bacterial pathogens to plants or agricultural water through excretion of contaminated waste (3). Additionally, synanthropic and coprophagic insects can introduce human bacterial pathogens to produce fields by dispersing bacteria associated with their exoskeletons onto plant surfaces (5). These events increase the chances that phytophagous insects could encounter a human enteric pathogen as a result of feeding or wandering on contaminated plant surfaces. The abundance and distribution of phytophagous insects within agricultural fields suggest that their contamination could influence the dispersal of enteric pathogens in the field (6).

The aster leafhopper, *Macrosteles quadrilineatus* Forbes (Hemiptera: Cicadellidae), is an important agricultural pest of several fresh vegetable crops that have been linked to foodborne illness outbreaks (7). Previous studies demonstrated that *M. quadrilineatus* can become contaminated with *S. enterica* after feeding on contaminated plant material, and plant infestation with these insects enhanced the persistence of the pathogen on lettuce (8). *M. quadrilineatus* can also transmit externally attached and ingested bacteria to noninoculated leaves or artificial diets, thereby serving as vectors of *S. enterica* (6). One route of transmission of ingested bacteria was excretion of ingested *S. enterica* in honeydew, a sugar-rich secretion deposited in large volumes on plant surfaces by phloem sap-feeding insects. However, the status and sites of persistence of ingested *S. enterica* within *M. quadrilineatus*, and the molecular and biochemical underpinnings of these interactions, remain undetermined.

Members of the family *Enterobacteriaceae*, which contains *S. enterica*, have an intimate association with insects, exploiting them as alternate hosts or vectors or acting as pathogens or symbionts (9, 10). Many of these bacteria employ type III secretion systems (T3SSs), similar to those used by mammalian and plant pathogens during pathogenesis, to establish associations with insects. These secretion systems act as a molecular syringe that allows bacteria to deliver effector proteins necessary for multiple purposes, including host invasion, defense suppression, and pathogen multiplication within host cells (11). Phylogenetic analyses have identified seven distinct families of T3SSs, including the SPI-1 (also known as Inv-Mxi-Spa) and SPI-2 (also known as Ssa-Esc) families, associated with animal pathogens, and the *Rhizobiales*, Hrp1, and Hrp2 families, associated with plant pathogens (11). Some bacterial pathogens have multiple T3SSs from different families, and each of them is required at different points during interactions with their host. *S. enterica* has two T3SSs, *Salmonella* pathogenicity island 1 (SPI-1), required for host cell penetration, and SPI-2, important for subsequent intracellular

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TABLE 1 *S. enterica* strains and plasmids used in this study

Strain or plasmid	Genotype or relevant characteristics	Source or reference
Strains		
14028s	Wild type	ATCC
JDB1034	Wild type spontaneously resistant to Nal	This study
JDB1253	1034 wild-type-pKT-kan	This study
JDB1254	1034 *prgH*-pProbeNT	This study
JDB1256	14028s Δ*prgH*::Kan^r	This study
JDB1257	14028s Δ*prgH*::Cm^r	This study
JDB1258	14028s Δ*ssaK*::Kan^r	This study
JDB1261	14028s Δ*prgH*::Cm^r *prgH*-pEVS (complement)	This study
JDB1262	14028s Δ*prgH*::Cm^r-pKT-kan	This study
JDB1279	14028s Δ*prgH*::Cm^r-pEVS143 empty	This study
Plasmids		
pKT-kan	*gfp* expression and Kan resistance	17
pProbe-NT	*gfp* reporter	18
pEVS143	Broad-host-range cloning vector; Kan resistance	50

proliferation (12). Similarly, the endosymbiont *Sodalis glossinidius* of the tsetse fly (*Glossina palpalis*) contains two distinct SPI-1 T3SSs, one required for invasion of host cells and the second required for bacterial survival after entry (13). Interestingly, the plant-pathogenic bacterium *Pantoea stewartii*, the causal agent of Stewart's wilt of corn (*Zea mays* L.), also possesses two T3SSs, an Hrp1 required for plant pathogenesis and an SPI-1 required for bacterial persistence in the corn flea beetle (*Chaetocnema pulicaria*) vector and for subsequent transmission to host plants (14). Thus, although the SPI-1 of *S. enterica* is best known for its role in mammalian pathogenesis, it is possible that it is important in interactions with insect vectors. The role of T3SSs in *S. enterica* interactions with mammalian and plant hosts has been previously described (15, 16). However, the role of T3SSs in *S. enterica*-insect interactions remains unknown.

The main objective of this study was to investigate the acquisition of *S. enterica* and to determine the sites of persistence within *M. quadrilineatus*. We hypothesize that *S. enterica* establishes a temporary association with phytophagous insects that enhances the bacterium's epiphytic survival on plants and movement between plants. To test this hypothesis, we examined *S. enterica* persistence within *M. quadrilineatus* following acquisition using fluorescence microscopy. Furthermore, we investigated a possible role for the two T3SSs, SPI-1 and SPI-2, in the presence, persistence, and excretion of ingested *S. enterica*. The results of this study suggest that a functional SPI-1 T3SS needle complex is required in bacterium-hemipteran interactions, allowing the pathogen to persist within phytophagous insects, which to this point had conventionally been considered nonhosts.

MATERIALS AND METHODS

Bacterial strains, media, and culture conditions. The bacterial strains and plasmids used in this study are listed in Table 1. All strains were grown in, or plated on, Luria-Bertani (LB) medium. Media were amended with the following antibiotics as necessary: kanamycin (Kan) (50 μg/ml), chloramphenicol (Cm) (20 μg/ml), or nalidixic acid (Nal) (50 μg/ml).

Generation of fluorescent *S. enterica* strains. Plasmid pKT-kan was transformed into wild-type (WT) *S. enterica* JDB1034 (WT-pKT-kan) and Δ*prgH* JDB1257 (Δ*prgH*::Cm^r-pKT-kan); this plasmid confers Kan resistance and green fluorescent protein (GFP) gene (*gfp*) expression and has been shown to have no effect on the survival and growth of *S. enterica* (17, 18).

Generation of the *S. enterica prgH* pProbeNT strain. Additionally, the putative promoter region of the *prgH* gene was PCR amplified from *S. enterica* 14028s using primers prgHForXbal (NNNTCTAGATAAGTTAT CTGCGGCAGG) and prgHRevEcoRI (NNNGAATTCCCTGGGCTTGT TATCGTC), digested with EcoRI/XbaI, and cloned into pProbeNT (18). All constructs were confirmed and the promoter orientations were determined by sequencing (Quintara Biosciences, Berkeley, CA), and the *prgH* pProbeNT construct was electroporated into *S. enterica* JDB1034. Plasmid uptake by *S. enterica* JDB1034 was confirmed by plating the transformed strains (WT pKT-kan and *prgH* pProbeNT) on Kan-amended media, and these strains were used in the insect-feeding and confocal-imaging assays described below. The constitutive expression of *gfp* was seen in all colonies of the WT pKT-kan strain grown on LB plates by visualizing bright-green fluorescence through microscopy or by naked eye under UV light in the dark. On the other hand, green fluorescence was not observed in colonies of the *prgH* pProbeNT strain grown on LB plates. To confirm that *gfp* was constitutively expressed only in the WT pKT-kan strain and not in the *prgH* pProbeNT strain, fluorescence for each strain was measured. Briefly, JDB1034, JDB1253, and JDB1254 were grown overnight in LB medium at 37°C with agitation. Overnight cultures of each strain were diluted 1:10 and grown to an optical density (OD) of 1, and 200-μl aliquots were loaded into the wells of a 96-well plate. Additionally, serial dilutions of the WT pKT-kan strain (10^8, 10^6, 10^4, 10^2, and 10^1) were made and loaded into separate wells. Three replicate wells were included for each strain and dilution, as well as for the water control. Samples were incubated for 5 min, and endpoint fluorescence from each strain was measured at an excitation wavelength of 485 nm and an emission wavelength of 520 nm, using a plate reader (Synergy HT; BioTek, Winooski, VT). Averages of the fluorescence values for the three replicates were calculated and used to compare fluorescence between the *prgH* pProbeNT, WT, and WT pKT-kan strains. The fluorescence values of the undiluted *prgH* pProbeNT suspension (79 fluorescence units) were similar to those of the undiluted WT (24 fluorescence units) and the most diluted (10^2 and 10^1) WT-pKT-kan suspensions (95 and 54 fluorescence units, respectively) but significantly lower than those of the undiluted WT-pKT-kan suspensions (582 fluorescence units). Therefore, it was considered that *gfp* is not constitutively expressed in the *prgH* pProbeNT strain and exhibits fluorescence only when the *prgH* gene is expressed.

Generation of deletion mutants and complementing plasmids. The *prgH* and *ssaK* mutants used in this study were taken from a deletion library generated by the lambda Red recombinase method in *S. enterica* serotype Typhimurium ATCC 14028s (19). To obtain isogenic strains and reduce the possibility of secondary mutations, each mutation was transduced with phage P22 into a clean 14028s background (20). The complemented strain was constructed by amplifying the *prgH* gene from *S. enterica* 14028s using primers prgHForAvr (NNNCCTAGGGAGAACGAC AGACATCGC) and prgHRevBam (NNNGGATCCCCTGACCAAGGTG TTGCC). The amplicon was then digested with AvrII/BamHI, excised and purified from a 1% agarose gel (QIAquick gel extraction kit; Qiagen Inc., Valencia, CA), and cloned into pEVS143, which is a medium-copy-number plasmid that confers Kan resistance. The presence of the correct insert was confirmed by sequencing (Quintara Biosciences, Berkeley, CA), and the corresponding plasmid was electroporated into the Δ*prgH*::Cm^r mutant strain. Uptake of the plasmid by the Δ*prgH*::Cm^r strain was confirmed by PCR using primers to amplify the *prgH* gene and by plating the transformed strains (Δ*prgH*::Cm^r *prgH*-pEVS) on Kan-amended media. Additionally, an empty plasmid, pEVS143, was electroporated into the Δ*prgH*::Cm^r mutant strain, and uptake of the plasmid by the Δ*prgH*::Cm^r strain was confirmed by plating the transformed strain (Δ*prgH*::Cm^r-pEVS empty) on Kan-Cm-amended media. These strains (the *prgH* mutant, the complement, and the mutant transformed with the empty vec-

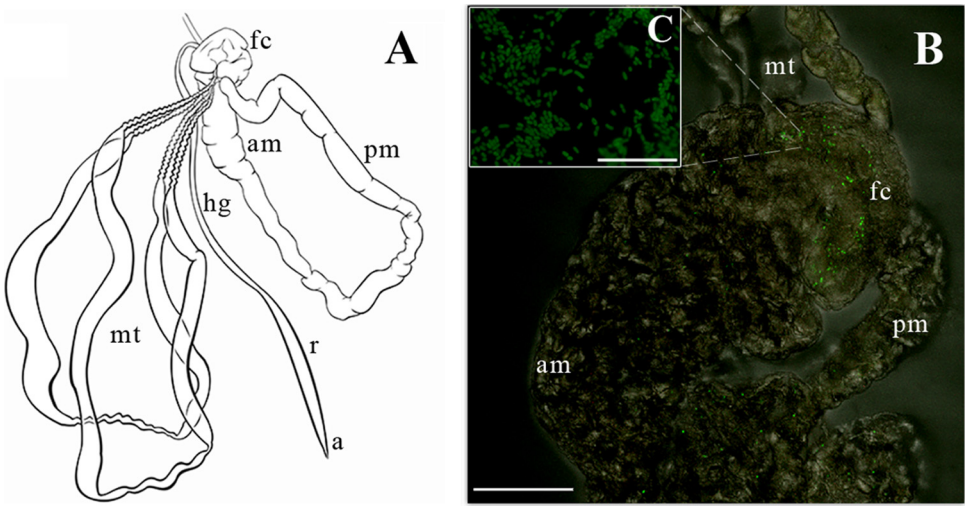

FIG 1 Presence of ingested *S. enterica* in the alimentary canal of *M. quadrilineatus*. (A) Diagram depicting the relative positions of the *M. quadrilineatus* filter chamber (fc), anterior midgut (am), posterior midgut (pm), Malpighian tubules (mt), hindgut (hg), rectum (r), and anus (a). (B) Confocal micrograph of dissected guts of an insect fed an *S. enterica*-inoculated liquid diet confined in Parafilm sachets following a 12-h AAP. (C) Representative close-up image of fluorescent *S. enterica* (green) in a dissected insect observed at higher magnification with an epifluorescence microscope. Fluorescent *S. enterica* bacteria are mainly present in the fc and throughout the am. The experiment was repeated 7 times, and a total of 54 insects were dissected and examined after the 12-h AAP. Scale bars, 100 μm.

tor) were used for complementation studies. To rule out the possibility that the mutant strains had general growth defects compared to the WT, growth kinetics in LB medium and minimal medium (M9) were determined for each strain. Bacteria were inoculated in LB medium with appropriate antibiotics for overnight growth at 37°C with agitation. Overnight cultures were used to prepare bacterial suspensions at an OD of 0.2, which were then diluted 1:1,000, and 2-μl aliquots of the diluted culture of each strain were transferred into the wells of a 96-well plate containing 200 μl of LB medium. Similarly, undiluted cultures were transferred into wells containing 200 μl of M9 medium. Three replicate wells were included for each strain, each medium, and the corresponding controls. Kinetic bacterial growth was recorded by reading the optical density at 600 nm (OD_{600}) every 30 min using a plate reader (Synergy HT; BioTek, Winooski, VT) at 37°C with continuous shaking for 48 h. Averages of the OD_{600} values for the three replicates were calculated and used to compare growth phenotypes between the WT and the mutant strains. Additionally, after the 48-h incubation period, each culture was sampled, diluted 1:10,000, plated on LB medium and LB medium with antibiotics, and incubated overnight at 37°C. Bacterial populations were enumerated and compared between media with and without antibiotics to make sure that the lack of antibiotic did not affect bacterial growth. There were no significant differences in growth kinetics for any of the strains (data not shown), suggesting that the cultures would grow at the same rate in the competition assays.

Insect feeding of fluorescent *S. enterica*. Adult *M. quadrilineatus* insects were collected from a colony maintained on oat (*Avena sativa* L.) seedlings as previously described (6). To visualize the presence and persistence of ingested *S. enterica* in the alimentary canal of *M. quadrilineatus*, individual insects were contained inside 1.5-ml microcentrifuge tubes and fed a synthetic liquid diet through Parafilm sachets as previously described (6). Parafilm sachets were used to limit physical contact of the insect body with the liquid diet but allow oral ingestion. The liquid diets consisted of either GFP-fluorescent *S. enterica* (WT pKT-kan; 10^6 CFU/ml) or sterile water mixed at a 1:1 ratio with a 10% sucrose solution; insects were allowed to feed on these diets for a 12-h acquisition access period (AAP). After the 12-h AAP, the insects were anesthetized by placing the tubes in an ice bath for 10 min and individually transferred to two consecutive *S. enterica*-free liquid diets for a total of 48 h post-AAP. Specifically, adult insects were allowed access to each sachet for 24 h. At the corresponding AAP and post-AAP, live insects were collected in sterile microcentrifuge tubes and stored at 4°C until processing for confocal microscopy. This experiment was repeated 7 times, and a total of 228 live insects (148 and 80 exposed and unexposed to *S. enterica*, respectively) were dissected and examined. A similar experiment was performed to visualize the presence and persistence of the Δ*prgH* strain in the alimentary canal of *M. quadrilineatus*. This experiment was repeated 3 times, and 132 live insects (84 and 48 exposed and unexposed to *S. enterica*, respectively) were dissected and examined. The insects were fed a liquid diet inoculated with a GFP-fluorescent Δ*prgH* (Δ*prgH*::Cm^r-pKT-kan) strain and sampled after either a 12-h AAP or a 24- and a 48-h post-AAP as described above. A supplementary set of experiments was conducted to determine whether *prgH* was expressed (GFP fluorescence) in the gut of *M. quadrilineatus* after acquisition. Following the procedures described above, the insects were fed a liquid diet consisting of either *prgH* pProbeNT *S. enterica* (10^6 CFU/ml) or sterile water mixed at a 1:1 ratio with a 10% sucrose solution, and insects were sampled after a 12-h AAP and a 24- and a 48-h post-AAP. This experiment was repeated 5 times, and a total of 182 live insects (122 and 60 exposed and unexposed to *prgH* pProbeNT *S. enterica*, respectively) were dissected and examined. In both experiments (with GFP-fluorescent and with *prgH* pProbeNT *S. enterica*), additional insects were collected at each time point and individually transferred to new, sterile microcentrifuge tubes and placed at −80°C to kill them without affecting the potential ingested *S. enterica*, and then they were homogenized in 100 μl of sterile water as previously described (6). Insect homogenate samples were then plated on LB-Kan (see below) to verify the presence of viable *S. enterica* in insect bodies.

Insect dissection and microscopy. Live *M. quadrilineatus* insects were dissected under a dissecting microscope in 1× phosphate-buffered saline (PBS) solution (pH 7.4); using two fine-tip forceps, the head was cut, and then the rear end of the abdomen, with the hindgut, midgut, and part of the foregut attached to it, was gently pulled backward in a single motion. The dissected organs were fixed in 4% paraformaldehyde overnight and washed with PBS three times for 10 min each time at 4°C on a rotator device. The fixed tissues were carefully mounted on microscope slides in ProLong Gold Antifade mounting medium (Thermo Fisher Scientific Inc.), protected from light as much as possible, and examined using a

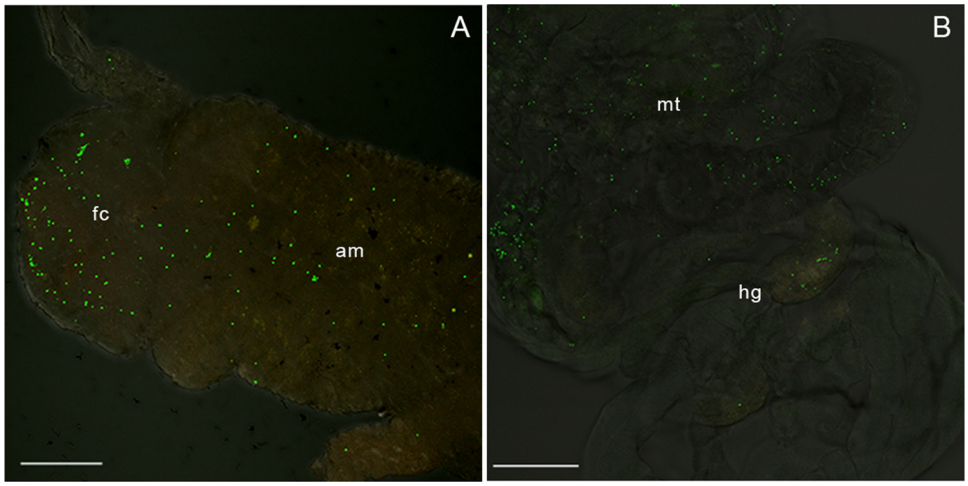

FIG 2 Persistence of ingested *S. enterica* in the alimentary canal of *M. quadrilineatus*. Shown are confocal micrographs of dissected guts of insects that were fed an *S. enterica*-inoculated liquid diet confined in Parafilm sachets for a 12-h acquisition access period and then moved to two consecutive noninoculated liquid diets for two 24-h post-AAPs each. Fluorescent *S. enterica* (green) was observed in the filter chamber (fc) and the anterior midgut (am) at the 24-h post-AAP (A) and in the hindgut (hg) and Malpighian tubules (mt) at the 48-h post-AAP (B). The experiment was repeated 7 times, and a total of 46 and 48 insects were dissected and examined at the 24- and 48-h post-AAPs, respectively. Scale bars, 100 μm.

Nikon A1R (Fig. 1A and B and 2) or a Leica SP8-X (see Fig. 5) confocal microscope. From each experiment, a subset of samples was also examined with an Olympus BX-60 epifluorescence microscope (Opelco, Dulles, VA) to ensure that fluorescent bacteria were present in the samples after tissue fixation and before confocal microscopy (Fig. 1C). Fluorescence was not observed in insects fed a noninoculated diet, independent of the strain used or sampling time (data not shown).

Competition assay. To examine the roles of T3SS SPI-1 and SPI-2 genes in *S. enterica* persistence inside the insect, *M. quadrilineatus* insects were fed an artificial liquid diet consisting of a 10% sucrose solution mixed with equal populations of the WT strain (JDB1034) and a mutant with the gene of interest deleted (Δ*prgH*::Kan^r or Δ*ssaK*::Kan^r) in a 1:1 ratio. Both *prgH* and *ssaK* are required to inject effector proteins into host cells and therefore for virulence in animal hosts (21, 22). The ratio of WT to mutant bacteria in the inoculum was verified by viable counts after plating serial dilutions of the inoculum on LB medium amended with the corresponding antibiotic (Nal for the WT and Kan for mutants). Following the procedures described above, individual insects were fed liquid diets (10^6 CFU/ml) through Parafilm sachets for a 24-h AAP and then transferred to *S. enterica*-free liquid diets for a 24-h post-AAP. Bacterial populations of both strains (the WT and the corresponding mutant), which carried distinct antibiotic markers, were enumerated from homogenized insects at a 24-h post-AAP (as described above) and from honeydew samples collected at the 24-h AAP and 24-h post-AAP time points (6). Specifically, honeydew and homogenized insect samples were dilution plated on LB-Nal (WT) and LB-Kan (mutant) to enumerate the bacterial populations. Samples were also enriched in antibiotic-amended medium in order to improve the sensitivity in detecting *S. enterica* in samples containing the pathogen even at very low concentrations. The bacterial population in samples that were negative after direct plating but positive after enrichment was arbitrarily determined as 1 CFU. These experiments were repeated 4 times ($n = 56$ insects) and 3 times ($n = 40$ insects) for the WT-*prgH* mutant and WT-*ssaK* mutant comparisons, respectively. Complementation studies were performed following the same procedures to compare the WT to the *prgH* mutant and the complemented strain (Δ*prgH*::Cm^r *prgH*-pEVS) and an empty-vector control (Δ*prgH*::Cm^r pEVS). These experiments were repeated 4 times each, and 50, 48, and 60 insect homogenate samples were evaluated for the WT-*prgH* mutant, WT-*prgH* complement, and WT–*prgH* empty-vector comparisons, respectively. The competitive index (CI) was determined as the difference between the WT population (log CFU/ml) and the compared strain (deletion mutants, complement, or empty vector) for each sample. In this case, a difference instead of a ratio was calculated because in some cases, either the WT or another strain was not recovered from honeydew or insect homogenate samples. A mean CI of 0 denotes no difference between the two population sizes.

Statistical analysis. Consistently, *S. enterica* was not recovered from noninoculated control insect homogenate or honeydew samples in any of the experiments; therefore, these data are not shown. No significant differences were found among replications of the overall experiments; thus, means from all the replicates of each experiment were combined. Statistical significance was determined by a paired *t* test relative to the CI determined for a competition between the WT population and the mutant strain population recovered from either honeydew or insect homogenate samples and by a one-sample *t* test to determine whether the mean CI differed significantly from a CI of 0 (no competitive difference between the WT and the compared strain). Moreover, McNemar's test was used to test for marginal homogeneity in contamination rates over time, based on the number of honeydew and insect homogenate samples testing positive for *S. enterica* at the 24-h AAP or the 24-h post-AAP. Comparisons were made based on individual *M. quadrilineatus* insects fed mixed inocula of *S. enterica* TTSS mutants and the WT. This approach is commonly applied as a normal approximation on paired, nominal data expressed as a dichotomous trait, with matched pairs of subjects, and is designed to determine whether marginal detection frequencies are equal. All statistical analyses were performed using R software version 2.14.1 (http://www.r-project.org).

RESULTS

Presence and persistence of *S. enterica* in the alimentary canal of *M. quadrilineatus*. To investigate the presence of ingested *S. enterica* within *M. quadrilineatus*, insects ($n = 148$) were dissected after feeding on a liquid diet inoculated with GFP-labeled *S. enterica* (WT pKT-kan) following a 12-h AAP or after being transferred to sterile microcentrifuge tubes and fed a noninoculated liquid diet for 24 or 48 h post-AAP. Insects were dissected with the purpose of evaluating the postacquisition persistence of ingested *S. enterica* within the alimentary canals of adult *M. quadrilineatus* insects. *S. enterica* was observed in 77% of insects sampled after

TABLE 2 Mean proportions of gut dissections in which ingested fluorescent *S. enterica* was observed in *M. quadrilineatus*

Strain	Sampling time[a]	Total no. of insects sampled	Proportion of insect guts with fluorescent *S. enterica*[b]
Wild-type-pKT-kan	12-h AAP	54	0.77 ± 0.09
	24-h post-AAP	46	0.29 ± 0.07
	48-h post-AAP	48	0.38 ± 0.13
Δ*prgH*::Cm^r-pKT-kan	12-h AAP	32	0.31 ± 0.04
	24-h post-AAP	24	0.08 ± 0.06
	48-h post-AAP	28	0.06 ± 0.04
prgH-pProbeNT	12-h AAP	34	0.35 ± 0.10
	24-h post-AAP	44	0.16 ± 0.05
	48-h post-AAP	44	0.18 ± 0.08

[a] Insects were fed an inoculated liquid diet for a 12-h acquisition access period (AAP) and then fed two consecutive noninoculated liquid diets for 24 h post-AAP each.
[b] Proportions are reported as mean proportion ± standard error.

the 12-h AAP and in 29% and 38% of the insects sampled after the 24- and 48-h post-AAPs, respectively (Table 2). Dissection and reference to the internal anatomy of other leafhopper species (23) allowed us to make detailed illustrations of the poorly described digestive tract of *M. quadrilineatus* (Fig. 1), which were used as reference points while identifying the organs containing fluorescent *S. enterica*. Confocal imaging of the dissected guts showed aggregates and single cells of *S. enterica* distributed along the alimentary canal of *M. quadrilineatus*. In insects dissected after the 12-h AAP, *S. enterica* was regularly observed in specific portions of the alimentary canal, such as the filter chamber and anterior midgut (Fig. 1). Close examination revealed both single cells and small aggregates. Modest aggregates of fluorescent bacteria were also observed in the filter chamber, as well as the anterior and posterior midgut regions, of insects dissected after the 24-h post-AAP (Fig. 2). In insects dissected after the 48-h post-AAP, bacteria were found mainly in the hindgut and Malpighian tubules, the insect renal system, and very few were observed in the midgut (Fig. 2). These results demonstrate the presence of ingested *S. enterica* predominantly in the middle and posterior parts of the alimentary canal of *M. quadrilineatus* and persistence inside the insect for up to 48 h post-AAP. The passage of *S. enterica* in the alimentary canal of *M. quadrilineatus* appears to proceed from the filter chamber and anterior midgut to the posterior midgut to the hindgut and Malpighian tubules.

The SPI-1 T3SS is important for persistence of *S. enterica* in the alimentary canal of *M. quadrilineatus*. CI analysis is a popular method in *Salmonella*-animal model research that uses mixed infections to determine virulence attenuation caused by a given mutation (24). To evaluate the potential role of SPI-1 and SPI-2 T3SSs in *S. enterica* persistence inside *M. quadrilineatus*, we determined the CI of the WT versus the Δ*prgH* (SPI-1) and the WT versus the Δ*ssaK* (SPI-2) strains by comparing the populations of viable cells of each strain recovered from either honeydew or insect homogenate samples. No differences were observed between the WT and the *prgH* mutant populations in honeydew samples collected immediately after acquisition (AAP) or 24 h later (post-AAP) (Fig. 3A). However, the *prgH* mutant was recovered at sig-

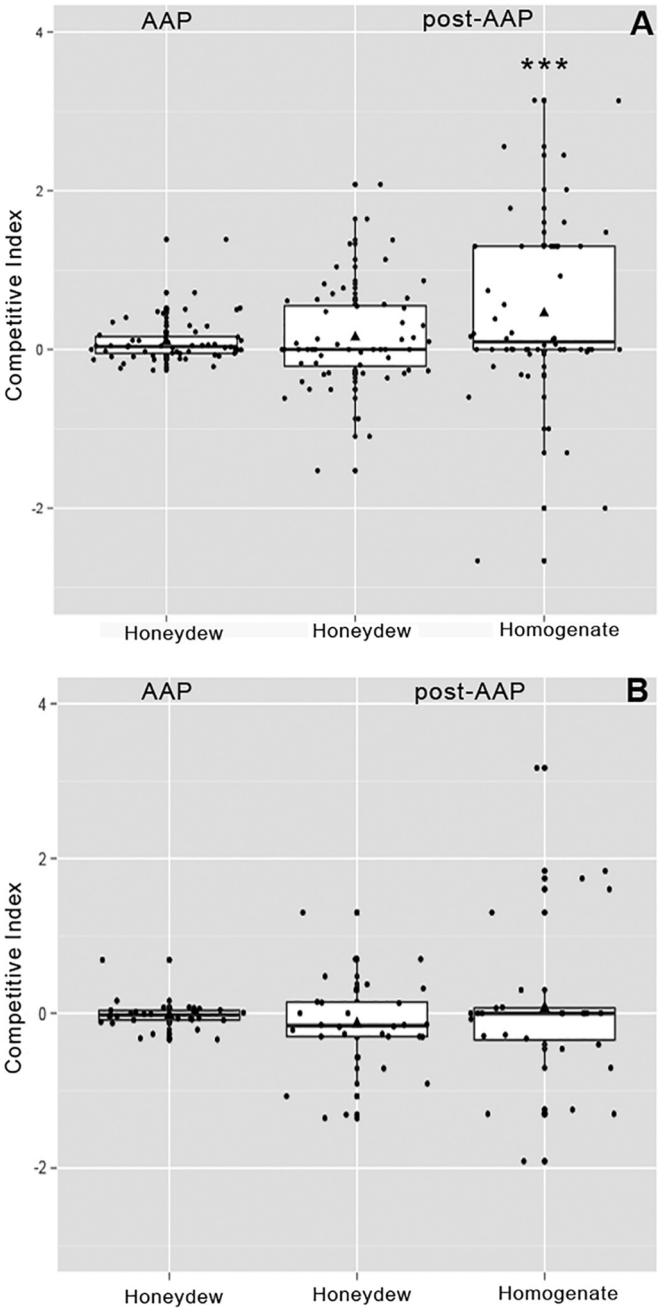

FIG 3 Competition assay in *M. quadrilineatus* fed mixed inocula of *S. enterica* WT and the *prgH* mutant (A) or the WT and the *ssaK* mutant (B). Insects were fed an inoculated liquid diet confined in Parafilm sachets for a 24-h AAP and then moved to a noninoculated liquid diet for a 24-h post-AAP. Bacteria were recovered from the honeydew at the 24-h AAP and 24-h post-AAP and from insect homogenates at the 24-h post-AAP. The sizes of the boxes are determined by the 25th and 75th percentiles, and the horizontal line and triangle within each box represent the median and mean, respectively. The CI represents the difference between the WT and the mutant strain populations (log CFU per milliliter). A mean CI (triangle) significantly different than 0 (***, $P < 0.05$) denotes a competitive difference between the WT and the mutant. The data shown are from 4 ($n = 56$ samples) (A) and 3 ($n = 40$ samples) (B) independent, experimental replications.

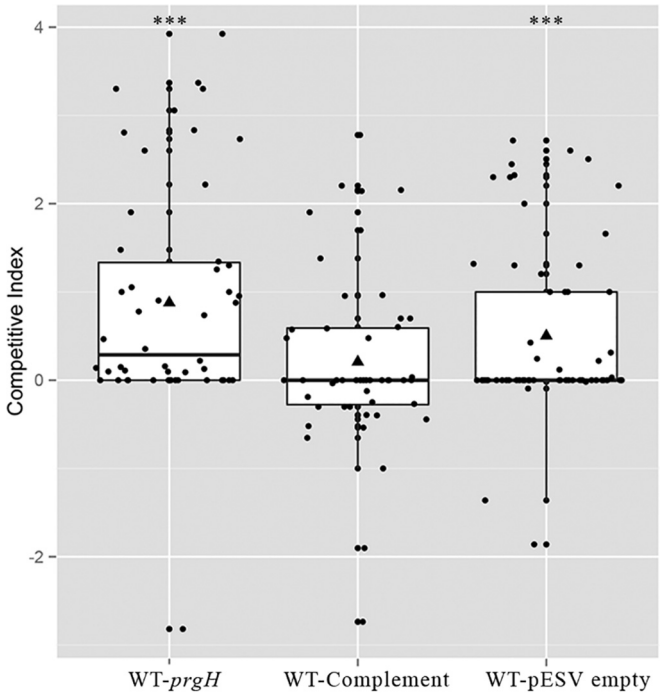

FIG 4 Competition assay in *M. quadrilineatus* fed mixed inocula of *S. enterica* WT and *prgH* mutant, WT and *prgH* complement strain, or WT and *prgH* transformed with empty pESV143 vector. The insects were fed an inoculated liquid diet confined in Parafilm sachets for a 24-h AAP and then moved to a noninoculated liquid diet for a 24-h post-AAP. Bacteria were recovered from insect homogenates at the 24-h post-AAP. The size of the box is determined by the 25th and 75th percentiles, and the horizontal line and triangle within each box represent the median and mean, respectively. The CI represents the difference between the WT and the mutant, the complement, or the compared strain populations (log CFU per milliliter). A mean CI (triangle) significantly different than 0 (***, $P < 0.05$) denotes a competitive difference between the WT and the mutant, the complement, or the empty-vector-transformed strain. The data shown are from 4 independent experimental repetitions (WT-*prgH*, $n = 50$; WT-complement, $n = 48$; WT-*prgH*–pESV143 empty, $n = 60$ insect homogenate samples).

nificantly lower levels than the WT (the mean CI was significantly different than 0; $P < 0.05$) in insect homogenate samples collected after the 24-h post-AAP (Fig. 3A). In the case of the WT-*ssaK* mutant comparison, no differences in recovered bacteria were observed in honeydew or homogenized insect samples collected at either the AAP or post-AAP (the mean CI was not significantly different than 0; $P > 0.05$) (Fig. 3B). A complemented *prgH* mutant strain (Δ*prgH*::Cm^r *prgH*-pEVS) was constructed and used to further evaluate the role of *prgH* in the survival of *S. enterica* inside *M. quadrilineatus*. Consistent with previous results, the *prgH* mutant was recovered at significantly lower levels than the WT (the mean CI was significantly different than 0; $P < 0.05$) in insect homogenate samples collected after the 24-h post-AAP (Fig. 4). However, no differences in recovered bacteria were observed between the WT and the complemented strain in insect homogenate samples (the mean CI was not significantly different than 0; $P > 0.05$), indicating that complementation with *prgH* in *trans* restored *S. enterica* persistence in *M. quadrilineatus* (Fig. 4). Additionally, the *prgH* mutant transformed with the empty vector (Δ*prgH*::Cm^r-pEVS empty) was recovered at significantly lower levels than the WT (the mean CI was significantly different than 0; $P < 0.05$) in insect homogenate samples, similar to the mutant without the plasmid (Fig. 4), demonstrating that carriage of the plasmid does not assist in colonization of the insect. These results demonstrate that *prgH*, and therefore a functional SPI-1 T3SS, is important for persistence of *S. enterica* inside *M. quadrilineatus*.

The SPI-1 T3SS influences excretion and attenuates the persistence of *S. enterica* inside *M. quadrilineatus*. To investigate if *S. enterica* excretion correlated with recovery from within the insect, we performed matched-pair comparisons of individual *M. quadrilineatus* insects for the presence of *S. enterica* at the 24-h AAP or post-AAP in honeydew or insect homogenate samples (Table 3). The comparisons examined the proportions of samples

TABLE 3 Matched-pair comparisons of correlated proportions of the presence of *S. enterica* in honeydew and/or homogenate samples of *M. quadrilineatus* fed mixed inocula of *S. enterica* T3SS mutants and the WT[a]

Matched pair of samples[b]	Proportion[c]			
	−−	−+	+−	++
Wild type-Δ*prgH*[d]				
Wild type				
Honeydew 24-h AAP–honeydew 24 h post-AAP	0.01	(0.00	0.13)	0.86
Honeydew 24-h AAP–insect homogenate	0.00	**(0.01**	**0.26)**	0.73
Honeydew 24-h post AAP–insect homogenate	0.01	(0.19	0.23)	0.57
Δ*prgH*::Kan^r				
Honeydew 24-h AAP–honeydew 24-h post-AAP	0.01	**(0.00**	**0.20)**	0.79
Honeydew 24-h AAP–insect homogenate	0.00	**(0.01**	**0.49)**	0.50
Honeydew 24-h post-AAP–insect homogenate	0.07	**(0.14**	**0.41)**	0.38
Wild type-Δ*ssaK*[e]				
Wild type				
Honeydew 24-h AAP–honeydew 24-h post-AAP	0.00	(0.00	0.05)	0.95
Honeydew 24-h AAP–insect homogenate	0.00	**(0.00**	**0.30)**	0.70
Honeydew 24-h post-AAP–insect homogenate	0.00	(0.05	0.30)	0.65
Δ*ssaK*::Kan^r				
Honeydew 24-h AAP–honeydew 24-h post-AAP	0.00	(0.00	0.07)	0.93
Honeydew 24-h AAP–insect homogenate	0.00	**(0.00**	**0.27)**	0.73
Honeydew 24-h post-AAP–insect homogenate	0.02	(0.05	0.25)	0.68

[a] Insects were fed an inoculated liquid diet for a 24-h AAP and then fed a noninoculated liquid diet for a 24-h post-AAP.
[b] Matched-pair samples correspond to honeydew and insect homogenate samples obtained from individual insects fed a particular diet. Matched-pair comparisons are only in rows.
[c] The number listed is the proportion of the total samples observed with the given wild-type or mutant *S. enterica* contamination pattern. If the number of +− samples was significantly different than the number of −+ samples based on McNemar's test ($P \leq 0.01$), the values are shown in boldface, and direct comparisons are made within parentheses only. The −− and ++ proportions are provided for reference purposes only and are not part of the chi-square comparison.
[d] Insects ($n = 56$) fed a mixed inoculum of wild-type and *prgH* mutant strains.
[e] Insects ($n = 40$) fed a mixed inoculum of wild-type and *ssaK* mutant strains.

that were positive only for either the insect or honeydew samples at specific time points. High proportions of honeydew samples that were positive after both the AAP and the post-AAP (++) were observed across strains. These results are consistent with our previous work (6) and demonstrated that insects that have access to an inoculated diet can excrete ingested bacteria during acquisition and even 24 and 48 h post-AAP. For the WT and the *ssaK* mutant, there were no differences between the proportions of honeydew samples that were positive only after the AAP (+−) or the post-AAP (−+). However, in the *prgH* mutant, a higher proportion of honeydew samples were positive after the 24-h AAP and negative after the 24-h post-AAP (0.20) in comparison to samples in which the honeydew was negative after the AAP and positive after the post-AAP (0), demonstrating that the ingested *prgH* mutant was rapidly eliminated in insect excretion even during the acquisition period.

The comparison of *S. enterica* excretion and persistence inside *M. quadrilineatus* indicated that overall, insects that tested positive after homogenization had also excreted honeydew that was contaminated. This idea was reflected in the high proportion of double-positive samples (honeydew and insect homogenate) observed across strains. Moreover, the proportion of samples in which only the honeydew samples at 24 h AAP were positive was significantly higher than the proportion in which only the insect homogenate samples were positive. These results further demonstrate that not all of the ingested *S. enterica* bacteria are retained inside the insect and that a high proportion of bacteria are excreted in the honeydew. For the WT and the *ssaK* mutant, there were no differences in bacterial recovery at the 24-h post-AAP between the proportions in which exclusively the honeydew (+−) or the insect homogenate (−+) samples were positive. However, in the case of the *prgH* mutant, the proportion in which the honeydew but not the paired insect homogenate samples were positive was significantly higher than the proportion in which only the insect homogenate samples were positive, proving that the ingested *prgH* mutant was shed in the honeydew at a high frequency even by insects in which bacteria were not detected in insect homogenate samples.

***prgH* is expressed in the mid- and hindgut of *M. quadrilineatus*.** To further investigate the involvement of *prgH* in *S. enterica* survival inside *M. quadrilineatus*, a *prgH* pProbeNT strain was constructed to monitor *prgH* expression *in vivo*. Insects ($n = 122$) were fed a *prgH* pProbeNT-inoculated liquid diet and dissected after either the 12-h AAP or the 24-and 48-h post-AAPs. Fluorescent cells were observed in 35% of insects sampled after the 12-h AAP and in 16% and 18% of the insects sampled after the 24- or 48-h post-AAP, respectively (Table 2). Confocal imaging of the dissected guts showed the presence of fluorescent bacteria in the mid- and hindguts of insects that were fed the *prgH* pProbeNT-inoculated liquid diet (Fig. 5). These data confirm the expression of *prgH* in cells found in the mid- and hindguts of insects long after the acquisition period and passage of the inoculated sucrose solution.

To determine if *prgH* played a role in the localization of *S. enterica* in the alimentary canal of *M. quadrilineatus*, insects ($n = 84$) were fed a GFP-labeled *prgH* mutant (Δ*prgH*::Cm^r-pKT-kan)-inoculated liquid diet and dissected after either the 12-h AAP or the 24- and 48-h post-AAPs. Similar to our observations in the experiment with the WT (Fig. 1), fluorescent cells were mainly observed in the mid- and hindgut of *M. quadrilineatus*. However,

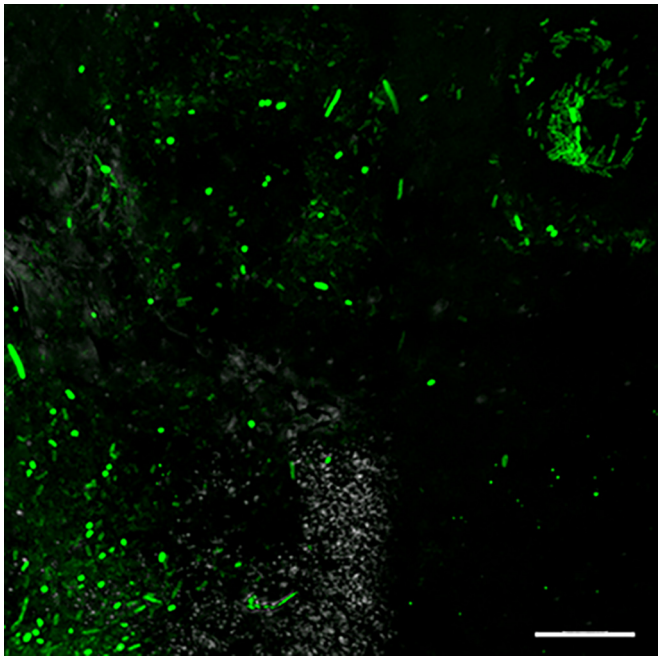

FIG 5 Expression of *S. enterica prgH* in the gut of *M. quadrilineatus*. Shown is a confocal micrograph of the dissected gut of an insect fed an *S. enterica*-inoculated liquid diet confined in a Parafilm sachet for a 12-h AAP and then moved to two consecutive noninoculated liquid diets for 24-h post-AAP each. Fluorescent *S. enterica* cells were observed in a portion of a midgut dissected after a 48-h post-AAP due to the green fluorescent protein transcribed from the *prgH* promoter from the plasmid pProbeNT. The experiment was repeated 5 times, and a total of 44 insects were dissected and examined. Scale bar, 20 μm.

the *prgH* mutant was observed at lower frequencies of 31% after the 12-h AAP and 8% and 6% after the 24- and 48-h post-AAPs, respectively, than the WT (Table 2). These findings show that *prgH* influences the retention of *S. enterica* during passage through the gut of *M. quadrilineatus* and confirm our earlier conclusion of its importance for persistence. Because the fluorescent *prgH* mutant was observed throughout the experiment, these data suggest that *prgH* is not the sole factor that determines *S. enterica* localization inside the insect gut.

DISCUSSION

In this study, we demonstrated the presence and persistence of ingested *S. enterica* in the alimentary canal of *M. quadrilineatus*. The alimentary canal of insects is a continuous tube that connects the mouth to the anus through which ingested microorganisms are moved in the company of food. However, the harsh conditions encountered in the insect midgut (such as extreme pH, presence of digestive enzymes, redox potential, and ionic strength) and the activation of defense mechanisms in response to foreign organisms normally inhibit the establishment and survival of ingested microorganisms (25, 26). Previous studies have shown successful gut migration of *S. enterica* in synanthropic and coprophagic insects (27–29). However, the fates of ingested bacteria varied significantly among species of insects, indicating that only certain species offer favorable conditions that allow movement and retention of *S. enterica* in the alimentary canal. Here, insect dissection revealed the presence of ingested *S. enterica* in the mid- and posterior portions of the alimentary canal of *M. quadrilineatus*, in-

cluding organs such as the filter chamber, anterior and posterior midgut, and Malpighian tubules, even 48 h post-AAP. These results, and culture recovery of viable bacteria from honeydew and insect homogenates, indicate that *S. enterica* can survive passage through the midgut of this phytophagous hemipteran and that it is released in honeydew. Thus, the ability to survive internalization, gut transit, excretion, and deposition by *M. quadrilineatus* allows plant-associated *S. enterica* to maintain a cyclical lifestyle between insects and plants, which may enhance the maintenance of the human pathogen in agricultural environments.

Plant-associated *S. enterica* may establish temporary associations with *M. quadrilineatus* that allow retention within the insect and release onto plant surfaces. *S. enterica* was observed in the mid- and hindgut of *M. quadrilineatus* immediately after acquisition and up to 48 h post-AAP. Similar studies have also reported the occurrence of the enterobacteria *P. stewartii* and *Erwinia tracheiphila* in the mid- and hindguts of their vectors, the corn flea beetle and the striped cucumber beetle, *Acalymma vittatum*, respectively (30, 31). It is noteworthy that there was a significant reduction of *S. enterica* inside the insect during and after acquisition, evidenced by the differences in the frequencies of fluorescent bacteria observed in the guts of *M. quadrilineatus* dissected after a 12-h AAP or a 24- or 48-h post-AAP. We posit that this reduction could have been an effect of a lack of pathogen replication within the insect and its possible constant discharge in the honeydew. Overall, the persistence of *S. enterica* at low levels in *M. quadrilineatus* suggests that individual contaminated insects may serve as a point source of the human pathogen in association with plants over a short period, but the risk of long-term transmission by individual insects is low. Nevertheless, the aggregation behavior and short-distance dispersal on lettuce (32) indicate that *S. enterica*-contaminated leafhoppers could contaminate multiple leaves and adjacent plants even in a short period. Additionally, the fact that infestation with *M. quadrilineatus* enhanced the epiphytic survival of *S. enterica* on lettuce leaves (8) and that its honeydew can serve as a growth medium for the bacterium (J. P. Soto-Arias, R. L. Groves, and J. D. Barak, presented at the Food Research Institute annual meeting, Madison, WI, 21 to 22 May 2014) increases the chances of insect contamination, and therefore further dispersal of the pathogen on and among plants. Thus, this study further confirms that the most likely mode of transmission of ingested *S. enterica* by *M. quadrilineatus* implicates survival of the pathogen during passage through the alimentary canal and release in the environment in contaminated honeydew.

The presence of *S. enterica* in the Malpighian tubules up to 48 h post-AAP raises the possibility that persisting bacteria might escape from the insect digestive tract into the hemocoel. Various phytopathogenic bacteria have been shown to be capable of surviving and multiplying in specific hindgut regions of the pea aphid (*Acyrthosiphon pisum*), resulting in insect poisoning (33–36). We did not measure pathogen replication directly; however, the populations of fluorescent bacteria that were observed in the alimentary canal up to 48 h post-AAP lead us to hypothesize that *S. enterica* does not grow appreciably in the alimentary canal, and therefore, the potential pathogenicity of this human pathogen to *M. quadrilineatus* seems unlikely. Additionally, it is hypothesized that escaped bacteria will be successfully cleared in the *M. quadrilineatus* hemolymph, based on our observation that in some cases fluorescent bacteria that were in contact with the hemolymph after dissection were phagocytosed by insect hemocytes (data not shown). Previous studies have demonstrated the ability of various insects to clear bacteria, such as *Staphylococcus aureus*, *Listeria* spp., *Escherichia coli* K-12, and *Bacillus subtilis*, from the hemolymph after bacterial injection into the hemocoel (37–39). *S. enterica* infection via septic challenge and persistence in the hemolymph of live leafhoppers warrant closer examination.

The complex microbiota of *M. quadrilineatus* may influence the fate of ingested *S. enterica*. *M. quadrilineatus* possesses two obligate symbionts (*Sulcia muelleri* and *Nasuia deltocephalinicola*) that provide essential amino acids that the insect is unable to synthesize or obtain in sufficient quantities from plant phloem (40). Recent studies of endosymbiotic microbiota in natural populations of *Macrosteles* leafhoppers (*Macrosteles striifrons* and *Macrosteles sexnotatus*) have revealed the coexistence of obligate and facultative endosymbionts with phytopathogenic phytoplasmas, as well as other enteric bacteria (41), suggesting that these endosymbionts may encourage leafhopper colonization by enterics. In contrast, insect resistance to various pathogens and parasites and activation of an immune response against foreign intruders have been associated with the presence of endosymbiotic bacteria (42–44), so endosymbionts can limit the survival of enterics, such as *S. enterica*, within leafhoppers. The presence and persistence of ingested *S. enterica* in the alimentary canal of *M. quadrilineatus* suggests that the pathogen may interact with coexisting endosymbionts, and future studies can reveal whether these interactions can positively or negatively affect the retention and transmission of *S. enterica* by the leafhopper.

The SPI-1 T3SS allows *S. enterica* persistence, colonization, and pathogenicity in multiple hosts. The SP1-1 family of T3SSs has been found in several animal/human (*S. enterica*, *E. coli*, *Shigella* spp., and *Yersinia pestis*)- and plant (*Erwinia* spp., *Xanthomonas* spp., and *P. stewartii*)-pathogenic bacteria (45). Additionally, the presence of homologs of the SPI-1 T3SS in insect symbionts, including *Hamiltonella defensa* and *S. glossinidius*, implies that SPI-1 may be necessary for persistence inside insect hosts (10, 13, 45). Similarly, *P. stewartii* carries an SPI-1 T3SS required for persistence in the gut of its insect vector and successful transmission of the pathogen to its ultimate plant host (14). In the case of *S. enterica*, the SPI-1 T3SS has multiple roles: (i) invasion of mammalian host epithelial cells (13) and (ii) suppression or activation of plant defenses of the model plants *Arabidopsis thaliana*, *Nicotiana tabacum*, and *Medicago* spp. (16, 46, 47). In this study, the *prgH* mutant, which lacks a functional SPI-1 T3SS (21), was defective in persistence in *M. quadrilineatus*. Thus, our results suggest that, in addition to animal pathogenicity and plant defense regulation, the SPI-1-encoded T3SS also impacts the retention of *S. enterica* inside its potential vector, *M. quadrilineatus*. In contrast, the SPI-2 T3SS is not involved in this vector interaction.

S. enterica prgH is expressed in the mid- and hindgut of *M. quadrilineatus* and appears to be required for persistence in this vector. PrgH, in combination with PrgK and InvG, has been shown to compose the essential multiring base structure of the T3SS needle complex necessary to export effector proteins into host cells (48). Both PrgH and PrgK, in addition to PrgI, PrgJ, OrgA, and OrgB, are required for *S. enterica* entry into host cells (21). It is possible that in this study the lack of *prgH*, and thus the absence of the T3SS needle structure, precluded *S. enterica* from delivering effector proteins necessary to manipulate and invade insect cells. However, this hypothesis may be improbable based on the fact that populations greater than 10^4 CFU/ml of the WT strain

were only occasionally recovered from homogenized insects post-acquisition. Thus, *S. enterica* may be retained at low titers without invasion of specific organs, in a manner similar to some insect symbionts, such as *H. defensa* (10). Additionally, it is noteworthy that the disruption of the *prgH* gene and replacement with the insertion of the Cm resistance cassette could have had a polar effect affecting the transcription of the downstream genes that share the same operon (*prgIJK*). However, the fact that we were able to complement the mutant phenotype with *prgH* alone and not the whole operon suggests that *prgIJK* are expressed normally in the *prgH* mutant (Δ*prgH*::Cm^r) and there is no polar effect or that these other genes have no effect on the persistence of the pathogen in this vector. Besides a role in host invasion, *S. enterica* SPI-1 has also been associated with bacterial adhesion and clumping in media, which demonstrated the possibility of T3SS-mediated bacterial aggregation outside eukaryotic host cells (49). Induction of *prgH* expression was observed in the mid- and hindgut of *M. quadrilineatus* for up to 48 h post-AAP, and the *prgH* mutant had low retention during gut passage and persistence postacquisition compared to the WT. These results suggest that the SPI-1 T3SS needle may influence *S. enterica* attachment and the formation of modest cell aggregates on the surfaces of insect organs and tissues, preventing the elimination of bacteria in insect excretions. Interestingly, match-paired comparison of individual *M. quadrilineatus* insects demonstrated that the *prgH* mutant was shed in the honeydew at a high frequency even in insects from which *S. enterica* was not recovered after insect homogenization. This finding suggests that the SPI-1 T3SS needle may affect transmission, since the absence of the needle complex increased shedding up to 24 h post-AAP, which might decrease the chance of the insect spreading the bacterium to other plants due to premature shedding of the bacterium prior to movement. Further studies are necessary to investigate the roles of specific effector proteins that are delivered by the SPI-1 T3SS in bacterial attachment, aggregation, and persistence inside *M. quadrilineatus* and transmission to and among plants.

Overall, this study provides novel evidence of the presence and persistence of *S. enterica* in a potential insect vector and sheds light on the possibility that in agricultural environments, plant-associated human pathogens may establish opportunistic associations with phytophagous insects that ultimately allow them to reach and colonize new host plants. However, future studies are necessary to advance our knowledge of the potential symbiotic interaction between plant-associated human bacterial pathogens and phytophagous insects, the implications of this association in bacterial and insect environmental fitness, and the contribution to fresh produce contamination and human disease.

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