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The Bacterium *Pantoea stewartii* Uses Two Different Type III Secretion Systems To Colonize Its Plant Host and Insect Vector

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Plant- and animal-pathogenic bacteria utilize phylogenetically distinct type III secretion systems (T3SS) that produce needle-like injectisomes or pili for the delivery of effector proteins into host cells. *Pantoea stewartii* subsp. *stewartii* (herein referred to as *P. stewartii*), the causative agent of Stewart's bacterial wilt and leaf blight of maize, carries phylogenetically distinct T3SSs. In addition to an Hrc-Hrp T3SS, known to be essential for maize pathogenesis, *P. stewartii* has a second T3SS (*Pantoea* secretion island 2 [PSI-2]) that is required for persistence in its flea beetle vector, *Chaetocnema pulicaria* (Melsh). PSI-2 belongs to the Inv-Mxi-Spa T3SS family, typically found in animal pathogens. Mutagenesis of the PSI-2 *psaN* gene, which encodes an ATPase essential for secretion of T3SS effectors by the injectisome, greatly reduces both the persistence of *P. stewartii* in flea beetle guts and the beetle's ability to transmit *P. stewartii* to maize. Ectopic expression of the *psaN* gene complements these phenotypes. In addition, the PSI-2 *psaN* gene is not required for *P. stewartii* pathogenesis of maize and is transcriptionally upregulated in insects compared to maize tissues. Thus, the Hrp and PSI-2 T3SSs play different roles in the life cycle of *P. stewartii* as it alternates between its insect vector and plant host.

The type III protein secretion systems (T3SS) of Gram-negative bacterial pathogens inject effector proteins into the cytosol of eukaryotic cells in order to modulate host cell defenses, enabling successful pathogen colonization and growth (45, 50). The host defense and surveillance systems impose intense selective pressures on T3SSs, resulting in a host-pathogen coevolutionary arms race with diversifying selection acting on the effectors (26, 50). The T3SS apparatus is composed of 20 to 25 structural proteins organized into three substructures: a transmembrane channel, a cytoplasmic domain, and an external injection needle, collectively known as an injectisome (4). Five T3SS families, adapted to colonize either animals or plants, can be distinguished (54). The T3SSs of animal pathogens are grouped into three families: the Inv-Mxi-Spa family, which enables bacterial cell invasion and survival; the Ssa-Esc family, which is required for intracellular replication of bacterial pathogens; and the Ysc family, which allows extracellular manipulation of animal cells by bacteria. The *hrc-hrp*-encoded T3SSs of plant pathogens cluster into two families, Hrc-Hrp1 and Hrc-Hrp2 (54), which generate thin pili designed to traverse the thick plant cell wall, enabling pathogen colonization of susceptible hosts (32). Interestingly, some groups of bacterial pathogens possess two T3SS clusters, each playing distinct roles in the same host at different phases of pathogenesis (13, 48). However, the roles of multiple T3SSs in bacterial pathogenesis and ecology, especially with regard to multiple hosts, are not well understood.

Pantoea stewartii subsp. *stewartii* Smith (referred to here as *P. stewartii*), the causative agent of Stewart's bacterial wilt and leaf blight of maize (*Zea mays* L.), requires a Hrc-Hrp1 family T3SS for plant pathogenesis (23, 27). Although this pathogen can be mechanically transmitted to maize under laboratory conditions by wounding, in nature it is largely dependent on its maize flea beetle vectors, predominantly *Chaetocnema pulicaria* (Melsh) and *Chaetocnema denticulate* (Illiger), for transmission, dissemination, and overwintering (15, 16). *P. stewartii* does not have a saprophytic phase in its life cycle, and it is thought to persist in the alimentary tracts of adult flea beetles that overwinter in the soil, thus enabling its transmission to new plants in the spring (16). *P. stewartii* is also suspected to overwinter as an endophyte within alternative grass hosts (16). Although several studies on *P. stewartii* transmission by its insect vector have been conducted (15, 16, 18), the genetic mechanisms that enable this bacterium-insect association are unclear.

In this study, we report that *P. stewartii* carries a second T3SS that is required for persistence in the gut of its flea beetle vector. Inactivation of this second T3SS reduced the bacterium's ability to persist within the insect vector so drastically that the beetles were subsequently unable to transmit an effective inoculum dose to maize plants. Complementation studies partially reversed these phenotypes. Our results demonstrate that this T3SS plays an important role in successful transmission of *P. stewartii* by its insect vector and suggest different roles for the two known *P. stewartii*

Received 19 March 2012 **Accepted** 26 June 2012
Published ahead of print 6 July 2012
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This publication was assigned no. HCS09-10 by the OARDC.
Supplemental material for this article may be found at http://aem.asm.org/.

doi:10.1128/AEM.00892-12

TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype and/or phenotype	Source or reference
E. coli		
DH10B	F$^-$ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80d*lacZ*ΔM15 Δ*lacX74 endA1 recA1 deoR*Δ (*ara*,*leu*)*7697 araD139 galU galK nupG rpsL* λ$^-$	Gibco BRL
HB101	F$^-$ *thi-1 hsd20* (r_B^- m_B^-) *supE44 recA13 ara-14 leuB6 proA2 lacY1 rpsL20* (Sm^r) *xyl-5 mtl-1*	5
S17-1	*pro thi*, chromosomal *tra* operon and *recA* from plasmid RP4	49
P. stewartii		
DC283	A Nal^r derivative of wild-type strain SS104	8
DC440	Wild type, isolated in 1999 in Wooster, OH	10
DM223	*wceG cpsA* glycosyl transferase mutant; EPS partial mutant	9
DM5044	DC283 *yhiN*::mini-Tn*5-gus* Nal^r Km^r	This study
DM5106	DC283 *sapD*::mini-Tn*5-gus* Nal^r Km^r	This study
DM5107	DC283 *mtlA*::mini-Tn*5-gus*	This study
DM5108	DC283 *sicA*::mini-Tn*5-gus* Nal^r Km^r	This study
DM5115	DC283 *ysaG*::mini-Tn*5-gus* Nal^r Km^r	This study
DM5121	DC283 *psaN1*::mini-Tn*5-gus* Nal^r Km^r	This study
DM5123	DC283 *lepA*::mini-Tn*5-gus* Nal^r Km^r	This study
DM5125	DC283 *pilD*::mini-Tn*5-gus* Nal^r Km^r	This study
DM 5126	DC283 *ospC*::mini-Tn*5-gus* Nal^r Km^r	This study
DM5127	DC283 *kuP*::mini-Tn*5-gus* Nal^r Km^r	This study
DM5130	DC283 *ackA*::mini-Tn*5-gus* Nal^r Km^r	This study
DM7003	DC283 *psaN2*::*aphA3* Nal^r Km^r	This study
ESΔR	Hypermucoid, *esaR*	3
P. ananatis DC131	Wild type, isolated from maize in Missouri in 1976	10
D. dadantii EC16	Wild type	35
Plasmids		
pBluescript SK(+)	ColE1 α-*lacZ* Ap^r	Stratagene
pDM3007	1.4-kb BamHI/KpnI fragment containing P_{lac}-*ysaN*$^+$ cloned into pRK415; Tc^r	This study
pLD55	Suicide vector with R6K *ori*, α-*lacZ*, *tetAR*, Ap^r	42
pMM6	pRK415 containing P_{lac}-*hrpL*$^+$	41
pRK415	IncP *a*-*lacZ*, Tc^r	34
pRK2013::T*n7*	ColE1 *mob*$^+$ tra_{RK2} Δrep_{RK2} *repE kan*::Tn*7* Tp^r Sm^r Sp^r	19
pUC18K	pUC18 with promoterless, terminatorless *aphA* cassette; Km^r	39
pUTmini-Tn*5-gus*	Mini-Tn*5-gus* in suicide vector pGP704; Ap^r Km^r	22

T3SSs in the life cycle of this pathogen as it alternates between insect and plant hosts.

MATERIALS AND METHODS

Bacterial strains and plasmids. Bacterial strains and plasmids used in this study are listed in Table 1. *P. stewartii* strains were grown at 28°C in Luria-Bertani (LB) broth or on agar supplemented with appropriate antibiotics [nalidixic acid (Nal), 20 μg/ml; kanamycin (Km), 50 μg/ml; and tetracycline (Tc), 25 μg/ml]. *Dickeya dadantii*, *Pantoea ananatis*, and *Escherichia coli* were grown as previously described (10, 35).

Virulence and hypersensitivity assays. Maize seedlings (*Zea mays* var. *rugosa*, cv. 'Seneca Horizon') and tobacco (*Nicotiana tabacum* cv. 'Bottom Special') were grown and maintained in growth chambers at 30°C with an 18-h/6-h day/night cycle. Virulence assays for *P. stewartii* mutants in maize seedlings and hypersensitivity assays were performed as previously described (23, 27).

Collection of maize flea beetles. Flea beetles (*C. pulicaria*) cannot be reared in the laboratory and were collected for each experiment in and around maize fields in Wooster, OH (40°46′3″N, 81°54′20″W), throughout the growing season. In each collection, subsamples of beetles were used for confirming species identity. Prior to *P. stewartii* colonization, persistence, and transmission experiments, beetles were held for at least 10 days on healthy maize at 27°C and 50% relative humidity with a 16-h photoperiod. Only beetles kept on plants that remained free from Stewart's wilt symptoms were used in subsequent experiments.

Detection of *P. stewartii* in field-collected flea beetles using viable cell counts and cytology. In order to estimate the proportion of flea beetles naturally carrying *P. stewartii*, flea beetles were sampled (n = 25 to 60 for each date) (Table 2) from the fields described above in spring, summer, late summer, and fall of 2007 and 2008. Single insects were ground in 300 μl 0.01 M potassium phosphate buffer, pH 7, for 15 s. Serial dilutions of the homogenates were plated on Ivanoff's semiselective medium for *P. stewartii* (325 mM glycerol, 35 mM ferric ammonium citrate, 256 mM NaCl, 17.6 mM Na_2SO_4, 14.4 mM K_2HPO_4, 0.9 mM $CaCl_2$, 0.4 mM $MgSO_4 \cdot 7H_2O$, 5.57 mM sodium taurocholate, 1.5% agar) (31), and the number of CFU was determined. *P. stewartii* identity was verified for three randomly selected yellow colonies per insect by species-specific PCR (8). Using toothpicks, single colonies were dipped into 25-μl reaction mixtures containing 1× Green GoTaq reaction buffer (Promega, Madison, WI) (1.5 mM $MgCl_2$, 2.5 mM deoxynucleoside triphosphates [dNTPs]), 20 pmol of each primer (ESR1-f, 5′ CGAAGCGAGGACACACG 3′, and ESIG2-r, 5′ GCGCTTGCGTGTTATGAG 3′), and 2.5 U *Taq* DNA polymerase. PCR conditions were: 3 min at 94°C followed by 35 cycles of 1 min at 94°C, 1 min at 55°C, and 3 min at 72°C. PCR products were separated in a 1% agarose gel. Additional insect samples (n = 139) were dissected and

TABLE 2 Proportion of field-collected flea beetles (FLB) naturally infected with *Pantoea stewartii*

Collection date	No. of *P. stewartii*-positive FLB/total (%)	Gut region	No. positive/total (%)
June 2007	5/30 (16.7)[a]	ND[c]	ND
August 2007	15/60 (25.0)[a]	ND	ND
Sept. 2007	1/30 (3.3)[a]	ND	ND
Sept./Oct. 2007	3/91 (3.3)[b]	Foregut	0/23 (0)
		Midgut	0/24 (0)
		Hindgut	2/23 (8.7)
		Mt[d]	1/21 (4.8)
April 2008	2/25 (8.0)[a]	ND	ND
Sept./Oct. 2008	1/48 (2.0)[b]	Foregut	0/12 (0)
		Midgut	1/14 (0.07)
		Hindgut	0/11(0)
		Mt	0/11 (0)

[a] *P. stewartii* was detected using semiselective medium and verified using PCR.
[b] *P. stewartii* was detected using immunofluorescence confocal microscopy of beetle guts.
[c] ND, not determined.
[d] Mt, Malpighian tubules.

analyzed by immunofluorescence confocal microscopy (see below) (Table 2).

Transposon mutagenesis, high-throughput genetic screen, and sequencing. Transposon mutagenesis was performed by filter mating of 2×10^9 S17-1 *pir* (pUT mini-Tn*5-gus*) cells with 4×10^9 DC283 cells for 18 h at 28°C on LB, followed by plating of the mating mixture onto 150-mm-diameter LB plates containing Nal, Km, and X-Gluc (50 μg/ml 5-bromo-4-chloro-3-indolyl-ß-D-glucuronic acid) to obtain ca. 500 to 1,000 colonies on each plate. After 4 days at 28°C, colonies were selected using a Genetix QPix robotic colony picker (Genetix USA, Boston, MA) and arrayed in 384-well microtiter plates containing 50 μl of buffered LB plus 4.4% glycerol per well. A charge-coupled device (CCD) camera (Hamamatsu Photonics, Hamamatsu City, Japan) was calibrated to preferentially pick blue colonies expressing *uidA* in order to eliminate from the screen most gene fusions that were not constitutively expressed in a rich medium. About 10,000 GUS^+ DC283::mini-Tn*5-gus* mutants were stored at −20°C after growth in the microtiter plates at 28°C for 48 h. To genetically screen DC283::mini-Tn*5-gus* strains for repression of the gene fusion by HrpL, each microtiter plate was replicated onto an LB Nal Km X-Gluc plate (plate A) and onto an LB plate containing a lawn of *E. coli* DH10B (pMM6) and HB101 (pRK2013::Tn*7*) mixed in a 1:1 ratio. This was done to introduce pMM6, which constitutively expresses $hrpL^+$ in *P. stewartii*, into the mini-Tn*5-gus* mutants by triparental mating. pMM6 transconjugants were then selected by replica mating on LB Nal Kan Tc X-Gluc agar (plate B). Downregulated (GUS^-) yellow mutants observed in plate B were selected from plate A after alignment and visual comparison. This resulted in the selection of 28 GUS^- mutants that were checked again for downregulation of GUS on LB Nal Km Tc X-Gluc agar plates.

The transposon-genome junctions on each side of the mini-Tn*5-gus* insertion site for the 28 candidate HrpL-down-regulated mini-Tn*5-gus* insertion mutants were sequenced with the primers 5′GUS (5′CATTTC ACGGGTTGGGGTTTCT3′) and O-END-mTn5 (5′CCGCACTTGTGT ATAAGAGTCAG3′) using an ABI 3700 automated DNA sequencer at the Plant-Microbe Genomic Facility, The Ohio State University. Junction sequences were BLAST searched against the unannotated *P. stewartii* genome sequence (Human Genome Sequencing Center, Baylor College of Medicine [https://www.hgsc.bcm.edu/content/pantoea-stewartii]), and the full-length wild-type amino sequences from each corresponding gene found in the Baylor genome sequence were searched against the GenBank nonredundant (nr) database using BLASTp. (In 2012, 65 contigs from the *P. stewartii* genome project, which were obtained by whole-genome shotgun sequencing, were deposited in GenBank under accession numbers AHIE01000001 to AHIE01000065, along with most of the protein sequences resulting from this search.)

Cloning and site-directed mutagenesis of *psaN*. The *psaN* gene was amplified by PCR using primers psaN-5′-Eco (GGAATTCCACGGCCA CCTGGAGCTTCGC) and psaN-3′-Xho (CCGCTCGAGCGGCGATAT CCTGCTGTAATACCTG). The 1,457-bp amplicon was verified by sequencing. It was then cut with EcoRI and XhoI and ligated into the corresponding sites of pBluescript SK. The BamHI/KpnI-cut insert from this plasmid was then recloned into pRK415 to make plasmid pDM3007, which was then used for complementation studies. A nonpolar *psaN* mutation was constructed by ligating the *aphA3* cassette from pUC18K (39) into the HpaI site of pDM3004, which is located 335 bp from the start of *psaN*. The *psaN*::*aphA3* mutation was then subcloned into suicide plasmid pLD55 as a BamHI/KpnI fragment. The resulting plasmid was used to exchange the mutant allele into the *P. stewartii* DC283 chromosome, as previously described (41), to create strain DM7003. The replacement was confirmed by PCR using primers to amplify the *psaN* gene.

Antibody production. Antigen was prepared as described by Tsuchiya et al. (55). Wild-type *P. stewartii* DC283 was grown in LB Nal broth to an optical density at 600 nm (OD_{600}) of ~1.0. Cells were washed three times in phosphate-buffered saline (PBS) [0.01 M potassium phosphate, 0.15 M NaCl (pH 7.4)] to remove exopolysaccharide slime and capsule and then resuspended in PBS at an OD_{600} of ~0.6. Bacteria were fixed in 0.5% formaldehyde overnight at 4°C, washed once with PBS, then resuspended in sterile saline, and stored at −80°C. Antibodies were produced in two New Zealand White rabbits as described previously (28). Each rabbit was injected with a mixture of 500 μl of whole-cell suspension and 500 μl of Freund's incomplete adjuvant (Sigma-Aldrich, St. Louis, MO). Two booster infections were done at 2-week intervals, with 7 ml blood collected just prior to the booster injections for testing antibody titers by Western blotting. Animals were exsanguinated at 9 weeks after the first injection, and the resulting sera were centrifuged at 1,200 × *g* for 15 min at 4°C. The clarified antisera were cross-absorbed with a 1:1:1 mixture of *Dickeya dadantii* EC16, *Pantoea ananatis* DC131, and *E. coli* HB101 as described previously (47). Antisera were diluted in a 1:1 (vol/vol) with 100% glycerol, lyophilized, and stored at −20°C.

Beetle dissection and *P. stewartii* immunolocalization. To assess *P. stewartii* colonization of beetle intestinal tracts, beetles collected from the field were quarantined for at least 10 days on healthy maize (see above) and then were exposed to plants infected with wild-type *P. stewartii* or the *P. stewartii psaN* mutant for 2 days for acquisition and then transferred to healthy maize every 2 days. Individual beetles ($n = 7$ to 9) were harvested at 2 h and 4, 8, and 12 days after transfer to healthy plants for dissection of their intestinal tracts. Beetles were dissected under a stereomicroscope in 0.01 M potassium phosphate buffer, pH 7, using two fine-tip forceps. The head, with the foregut and part of the midgut attached to it, was pulled forward and separated from the thorax. Then, the rear end of the abdomen, with the hindgut and the rest of the midgut attached to it, was pulled backward and freed from the cuticle surrounding the abdomen. The dissected organs were fixed, washed, and permeabilized as described previously (1). For labeling with antibody, insect organs were immersed in blocking buffer [phosphate-buffered saline plus 0.1% Triton X-100 (PBS-T), containing 5% goat serum] for 30 min and then incubated with incubation buffer [1:500 (vol/vol) dilution of *P. stewartii* antiserum, containing 1% goat serum] for 3 h at room temperature. Tissues were washed three or four times with PBS-T, incubated for 1 h in a 1:600 dilution of goat anti-rabbit IgG conjugated with Alexa Fluor 488 (Molecular Probes, Eugene, OR), washed three or four times with PBS-T, and then stained for 5 min with 3 nM propidium iodide (Molecular Probes). Samples were examined using a confocal laser scanning microscope (Leica TCS SP), with parameters set as previously described (1). The percentage of infected insects was compared between the wild type and mutants. For a more quantitative estimate of bacterial infection, the number of fluorescent bacterial foci was estimated using a scale of 0 to 4, in which 0 indicates

no fluorescent foci, 1 indicates few weak foci, 2 indicates bright independent foci, 3 indicates dense distribution of bright independent foci, and 4 indicates wider fluorescence areas. The experiment was conducted three times in total. Statistical analysis was carried out with MINITAB (Minitab Inc.). Differences among treatments were examined using one-way analysis of variance (ANOVA), and Fisher's protected least significant difference test (FLSD) was used for means separation.

Assessment of *P. stewartii* persistence in beetles using viable cell counts. Beetles were collected and held on maize seedlings for 10 days. About 80 flea beetles were placed on 15 to 20 maize seedlings that had been infected with either mutant or parental strains of *P. stewartii* (8) for 2 days to allow bacterial acquisition. To prevent reacquisition of bacteria, beetles were moved to healthy seedlings every 2 days. At 0, 4, 8, and 10 days after acquisition, 5 to 10 insects per treatment were starved for 2 h and then were ground in 0.01 M potassium phosphate buffer, pH 7. Serial dilutions were plated on LB agar amended with appropriate antibiotics: nalidixic acid (20 μg/ml) for wild-type and bacterial mutants; a combination of nalidixic acid and kanamycin (50 μg/ml) for the mini-Tn*5-gus* and *aphA3* insertion mutants (*sapD*, *psaN*, and Δ*psaN*); and nalidixic acid plus kanamycin and tetracycline (5 μg/ml) for the *psaN*$^{+}$ complemented strain. The number of CFU per insect was calculated after incubation of LB plates at 28°C for 2 days. Three independent repetitions of the experiment were done. The $\log_{10}$ CFU was used for statistical analysis (MINITAB), using one-way analysis of variance (ANOVA). Fisher's protected least significant difference test (FLSD) was used for means separation.

Semiquantitative RT-PCR. Total RNA was isolated from *P. stewartii* cells cultured overnight on LB medium ($OD_{600} \sim 0.6$), *P. stewartii*-infected maize, and insects fed on *P. stewartii*-infected plants at 4 days postinfection. A two-step RT-PCR was conducted with primer pairs specific for the Hrp ATPase (hrp-F, 5′GCCCTTATCACACCCCTTTATCTC 3′, and hrp-R 5′TTTTGCCCTCAGCACGAAAC3′) and the *Pantoea* secretion island 2 (PSI-2) PsaN ATPase (psaN-F, 5′AATGTCTGGTTCAT CTCGCACAC3′, and psaN-R, 5′GCTCCTCAACAAACTCCGTCAC3′). Two hundred nanograms of total RNA from *P. stewartii* cultures and from maize extracts and 400 ng of total RNA from beetles were used for first-strand cDNA synthesis. PCR was carried out for 20, 30, 35, and 40 cycles. 16S rRNA gene primers (ESR1-f, 5′ CGAAGCGAGGACACACG 3′, and ESIG2-r, 5′ GCGCTTGCGTGTTATGAG 3′) (10) were used as positive controls, and RT-PCRs without the addition of reverse transcriptase were included as negative controls to assess potential DNA contamination.

Transmission studies. Subsamples of insects from the above colonization experiment were used to assess subsequent transmission of the pathogen to maize seedlings. Individual 10-day-old seedlings were exposed to five beetles each from day 0 or day 8 after acquisition of bacteria for a 2-day transmission period. A total of five seedlings per treatment were used, and the experiment was repeated three times. Plant growth conditions were as described above. *P. stewartii* infection of seedlings was assessed at 10 days postinoculation by estimating the number of CFU in 0.3 g of symptomatic tissue or tissues surrounding flea beetle feeding sites. Leaves were cut into ca. 2- to 3-mm strips and incubated in 2 ml of 0.01 M potassium phosphate buffer (pH 7) for 15 min, and then serial dilutions were plated onto LB agar amended with 20 μg ml nalidixic acid to determine the total number of CFU. Differences among treatments were examined using one-way analysis of variance (ANOVA). Fisher's protected least significant difference test (FLSD) was used for means separation with the MINITAB package (Minitab Inc.).

Phylogenetic analyses. Protein sequences of the ATPases of the injectisomes and FliL, the ATPase of the *E. coli* flagellum, were aligned using Muscle (14), and a tree was generated using the neighbor-joining algorithm (gap excluded) of ClustalX with 1,000 bootstraps (53). The output file was viewed in Dendroscope (30), and names were added in Adobe Photoshop CS3 (Adobe Systems Inc.). FliL was selected as an outgroup. Accession numbers of T3SS islands of bacterial species (in alphabetical order) were as follows: *Aeromonas salmonicida* subsp. *salmonicida* AscN, CAD56760; *Bradyrhizobium japonicum* RhcN, AAG60799; *Bordetella pertussis* Tohama I BscN, CAC79575; *Burkholderia pseudomallei* K96243 SctN, YP_111406; *B. pseudomallei* K96243 SpaL, YP_111547; *Chlamydia trachomatis* A/HAR-13 SctN, AAX50947; *Chlamydophila pneumoniae* AR39 StcN, AAF37934; *Chromobacterium violaceum* ATCC 12472 CsaV, NP_902273; *Desulfovibrio vulgaris* DP4 BscN, YP_961187; *Erwinia amylovora* HrcN, AAB06001; *Erwinia tasmaniensis* Et1/99 SpaL, YP_001907837; *Escherichia coli* EivC, ACD01068; *E. coli* EscN, AAK26715; *E. coli* IAI39 FliL, CAR17249; *Mesorhizobium loti* MAFF303099 HrcN, BAB52652; *Pantoea agglomerans* HrcN, CAC43015; *Pantoea stewartii* HrcN, ABB77414; *P. stewartii* PsaN, GQ249669; *Pectobacterium carotovorum* subsp. *carotovorum* HrcN, ABZ05778; *Photorhabdus luminescens* LscN, AAO18044; *Pseudomonas aeruginosa* PscN, AAB86534; *Pseudomonas cichorii* HrcN, ABA47275; *Pseudomonas syringae* pv. tomato HrcN, AAG33879; *Ralstonia solanacearum* GMI1000 HrcN, CAD18021; *Rhizobium* sp. NGR234 HrcN, AAB91948; *Salmonella enterica* subsp. *enterica* serovar Choleraesuis str. SC-B67 SsaN, AAX65342; *S. enterica* subsp. *enterica* serovar Choleraesuis str. SC-B67 SpaL, YP_217813; *Shigella flexneri* SpaL, NP_085312; *Sodalis glossinidius* InvC, AAS66861; *Vibrio parahaemolyticus* RIMD 2210633 VpaN, NP_798047; *V. parahaemolyticus* RIMD 2210633 YscN, NP_800848; *Xanthomonas campestris* pv. vesicatoria str. 85-10 HrcN, CAJ22063; *Xanthomonas oryzae* pv. oryzae HrpB6, AAN46406; *Yersinia enterocolitica* YscN 1, AAK69223; *Yersinia enterocolitica* YsaN 2, AAB69192; *Yersinia pestis* CO92 YscN, CAB54917; and *Yersinia pseudotuberculosis* YscN, AAA20119.

Nucleotide sequence accession number. Sequence information for PSI-2 has been deposited in GenBank under accession no. GQ249669.

RESULTS

***P. stewartii* resides in the midgut and hindgut of field-collected beetles.** Although it has been suggested that *P. stewartii* overwinters in flea beetles (16), the specific site(s) where *P. stewartii* is located within the beetles was unknown. To investigate this, beetles were collected in and around maize fields at various times from April to October during 2007 and 2008. *P. stewartii* was detected in field-collected flea beetles by culturing beetle extracts on a semiselective medium, and the identities of *P. stewartii*-like single colonies were confirmed by PCR using *P. stewartii*-specific primers. *P. stewartii* was detected in some beetles collected in April, prior to local maize planting, but *P. stewartii*-positive insects were most frequent in late August (Table 2), consistent with previous observations (18). To examine the location of *P. stewartii* inside the beetles, *C. pulicaria* individuals that were collected in September and October 2007 and 2008 were subjected to immunofluorescence microscopy analyses using *P. stewartii*-specific antibodies. Labeling was detected in the beetle's midgut and hindgut, but not in the foregut (Table 2). These results indicate that *P. stewartii* predominantly colonizes the middle and posterior parts of the gut in *C. pulicaria*.

Identification of *P. stewartii* genes that are downregulated by the HrpL alternative sigma factor. To find genes that might be important for bacterium-host interactions, *P. stewartii* DC283 was randomly mutated with the mini-Tn*5-gus* transposon (22) and screened for genes that were both up- and downregulated by the alternative sigma factor HrpL, which activates the Hrp regulon *in planta* (40). The screen for HrpL-activated genes identified mostly known *hrp-hrc* and *wts* genes and several candidate effector genes that were subsequently shown to be defective (M. Merighi, D. R. Majerczak, and D. L. Coplin, unpublished data). We also hypothesized that HrpL might be indirectly involved in the repression of genes that were either nonessential for or detrimental to plant pathogenesis. Initially, we did not expect that this screen would also turn up the genes described in this paper that promote

TABLE 3 Homology searches of transposon junctions of 12 HrpL-downregulated mini-Tn*5-gus* insertion mutants

Insertion mutant	Gene name in *P. stewartii*[a]	Putative gene function in *P. stewartii*	Closest homologue (GenBank accession no.)[b]	Protein sequence length (aa); identity (E-value)[c]
DM5106	*sapD*	Peptide transport system ATP-binding protein	*Pantoea ananatis SapD* (YP_00519557)	331; 99 (0.0)
DM5107	*mtlA*	PTS system, mannitol-specifc EII ABC component	*P. ananatis MtlA* (AER34649)	644; 98 (0.0)
DM5108	*pchA**	Type III secretion chaperone protein	*Yokenella regensburgei lcrH-sycD* (EHM45774)	184; 86 (4e-92)
DM5115	*psaG**	Type III secretion apparatus protein	*Y. regensburgei mxiH* (EHM45790)	86; 85 (1e-37)
DM5121	*psaN**	Type III secretion cytoplasmic ATP synthase	*Y. regensburgei invC* (EHM45782)	431; 89 (0.0)
DM5123	*lepA*	GTP-binding protein	*P. ananatis lepA* (ADD78062)	599; 99 (0.0)
DM5125	*pilD*	Leader peptidase (prepilin peptidase)/*N*-methyltransferase	*Yersinia enterocolitica pulO* (CAC83039)	162; 98 (6e-40)
DM 5126	*ospC1*	Type III secretion effector	*Shigella flexneri ospC1* (AAL72322)	480; 54 (6e-161)
DM5127	*kup*	Kup system potassium uptake protein	*P. ananatis kup* (ADD77115)	622; 97 (0.0)
DM5130	*ackA*	Acetate kinase A/propionate kinase 2	*P. ananatis ackA* (YP_005194842)	400; 99 (0.0)
DM5044	*yhiN*	FAD/NAD(P)-binding oxidoreductase	*P. ananatis yhiN* (AER30810)	394; 97 (0.0)

[a] GenBank accession numbers for *P. stewartii* DC283 query sequences: *sapD* EHU00723; *mtlA*, EHT98766; *pchA*, ACT68037; *psaG*, ACT68022; *psaN*, ACT68029; *lepA*, EHT99695; *ospC1*, EHT98016; *kup*, EHU00817; *ackA*, EHT99841; and *yhiN*, EHT99058. *, gene within PSI-2 (Fig. 1A).

[b] Nucleotide sequences of genome regions flanking the mini-Tn*5-gus* were compared with protein-coding genes of the *P. stewartii* genome sequence in ASAP (24) using BLASTx. The full-length protein sequence of each *P. stewartii* gene was then BLAST searched against the GenBank nr protein database using BLASTP, and the closest homologue in another species is indicated.

[c] aa, amino acids.

insect colonization. The mini-Tn*5-gus* transposon makes *uidA* transcriptional fusions and contains stop codons in all reading frames just upstream of the β-glucuronidase (GUS) reporter gene. To screen for HrpL-downregulated genes, ca. 10,000 mini-Tn*5-gus* insertion mutants that exhibited GUS activity in a rich medium (LB) were screened for decreased GUS activity following introduction of a plasmid that constitutively expressed the wild-type *hrpL* (*hrpL*$^+$) gene. Downregulated mutants were selected by visualizing the difference in GUS activity between the mutants with and without the *hrpL*$^+$ plasmid on plates containing X-Gluc, resulting in the selection of 28 potential mutants. Sequencing the DNA flanking the mini-Tn*5-gus* insertion sites indicated that we had 12 independent insertions and that 10 of them corresponded to open reading frames in the incomplete *P. stewartii* genome sequence at Baylor College of Medicine.

Database searches revealed that in 11 of the mutants, the transposon had disrupted genes encoding proteins with similarities to the T3SS components SycD, MxiH, SpaL, and OspC1, ABC transporter components SapD/DppD and MtlA, prepilin peptidase PulO, acetate kinase AckA, potassium uptake protein Kup, FAD/NAD(P)-binding oxidoreductase YhiN, and translation elongation factor LepA (Table 3). The 12 independent mutants were fully virulent on maize, produced a hypersensitive reaction on tobacco, did not express GUS activity in *hrp*-inducing minimal medium, and grew normally in a minimal salts-glucose medium (see Table S1 in the supplemental material) and in LB (data not shown), indicating that these genes are not required for virulence in plants and that the mutations did not cause any growth defects.

Some HrpL-downregulated genes are located in a T3SS pathogenicity island. We searched the *P. stewartii* DC283 genome to determine the genomic context of the four T3SS genes identified in the transposon screen and found a gene cluster for a complete T3SS with gene order and sequence similarity to the *Yersinia enterocolitica* Ysa pathogenicity island (21), which is involved in the gastrointestinal phase of bacterial infection in mammals (56), and the *Sodalis glossinidius* SSR-1 pathogenicity island (11), which is required for bacterial invasion of insect gut cells (12) (Fig. 1). The transposon mutant screen identified three genes in the *P. stewartii* island with homology to *S. glossinidius ysaG* and *Y. enterocolitica sycD* and *ysaN* (Fig. 1; Table 3). We named this novel secretion system *Pantoea* secretion island 2 (PSI-2). The *P. stewartii* PSI-2 gene with similarity to *Y. enterocolitica sycA* and *Yokenella regensburgei sycD* was named *pchA*, the one with similarity to *S. glossinidus ysaG* was named *psaG*, and those similar to other *ysa* and *ysp* genes were named *psa* and *psp*, respectively (Fig. 1). *P. stewartii* PSI-2 *pspB*, *pspC*, and *pspD* are homologs of *Shigella flexneri ipaB*, *ipaC*, and *ipaD*, which are required for internalization and T3SS effector delivery (17, 39). PSI-2 is flanked by an IS*630* insertion sequence and an *rve* integrase (Fig. 1), suggesting that PSI-2 integrated into the *P. stewartii* genome following horizontal gene exchange. The twelfth HrpL-downregulated transposon insertion in mutant DM5113 was located in the intergenic region between *psa9* and *rve*.

The genetic similarity of PSI-2 with T3SSs of other bacteria was examined using phylogenetic analysis of *P. stewartii* PsaN (GenBank accession no. ACT68029.1), which belongs to a conserved family of T3SS ATPases located in the bacterial inner membrane that are essential for building the pilus and for secretion of effectors (38, 44, 54). PsaN grouped phylogenetically with YsaN of *Y. enterocolitica*, SpaL of *S. flexneri* and InvC of the insect symbiont *S. glossinidius* (Fig. 2). In contrast, the HrcN ATPase homolog of the *P. stewartii* Hrc-Hrp system clustered with the HrcN homologs of other plant pathogens (Fig. 2). These findings indicate that *P. stewartii* PSI-2 belongs to the Inv-Mxi-Spa group of bacterial T3SSs required for bacterial invasion of animal cells (54).

In addition to the three PSI-2 genes, the mini-Tn*5-gus* screen identified a homolog of the *S. flexneri ospC1* type III effector gene (Table 3) that is involved in postinvasion pathogenesis (60). A homolog of MxiE/YsaE, which activates *ospC1* and other T3SS genes in *Salmonella* and *Yersinia* (33), was also present in *P. stewartii* PSI-2 (Fig. 1). OspC1 is involved in postinvasion pathogenesis of *S. flexneri* (60). In the *P. stewartii* genome, *ospC1* is flanked

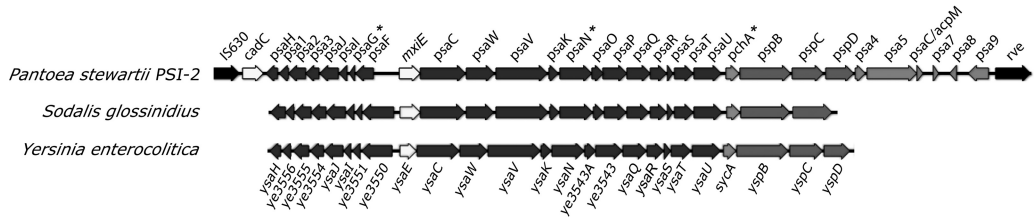

FIG 1 Organization of the *Pantoea stewartii* type III secretion system (T3SS), PSI-2. PSI-2 is aligned with T3SS pathogenicity islands from *S. glossinidius* (11, 12) and *Y. enterocolitica* (20, 21). PSI-2 genes that are downregulated by HrpL (Table 3) are indicated by asterisks. Putative gene functions include recombination and transposition (IS*630* and *rve*), transcriptional regulators (*cadC*, *mxiE*, and *ysaE*), secretion apparatus structural (dark gray) and regulatory (light gray) functions (*psa*, *ysa*, and *ye3543A*), secreted effector chaperones (*pchA* and *sycA*), and secreted effectors (*psp* and *ysp*).

by a gene similar to the integrase gene *rve* and by genes encoding hypothetical proteins that do not have similarities to protein sequences in the GenBank nr database. This suggests that, similar to PSI-2, *OspC1* lies on a genomic segment that may have been introduced into the *P. stewartii* genome by horizontal gene exchange. The mini-Tn*5-gus* screen also identified a homolog of *Salmonella enterica* serovar Typhimurium *ackA* (Table 3), which encodes an acetate kinase that is required for producing the formate necessary for inducing the T3SS pathogenicity island 1 (29). Together these data suggest that *P. stewartii* has a second functional T3SS, as well as several unlinked genes that that are similarly downregulated by HrpL. We hypothesized that some of these genes might play a role in colonization of flea beetles by *P. stewartii*.

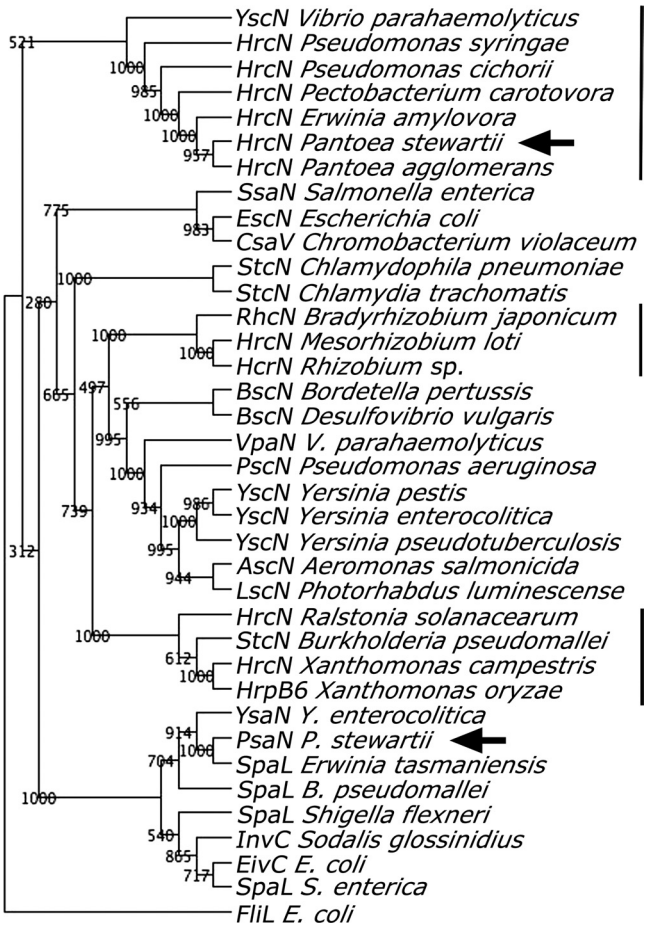

FIG 2 Phylogenetic analysis of type three secretion system (T3SS) ATPases from animal- and plant-associated microbes. Accession numbers for the gene and species names shown are given in Materials and Methods. Numbers to the left of the branches are bootstrap values for 1,000 replications, as outlined in Materials and Methods. Plant-associated microbes are designated by a solid line. The positions of the *Pantoea stewartii hrcN* from the *hrc-hrp* T3SS and *psaN* from PSI-2 are indicated by arrows.

Expression of the PSI-2 *psaN* gene is increased in insects compared to maize tissues. Our finding that *P. stewartii psaN*::mini-Tn*5-gus* has decreased GUS expression in a rich medium (LB broth) upon introduction of wild-type *hrpL* or during growth in a *hrp*-inducing minimal medium suggests that *psaN* is highly expressed in a nutrient-rich environment, such as the insect gut, but not under conditions similar to those in the plant apoplast. Indeed, *psaN* transcripts were readily detected in wild-type DC283 grown in LB broth (see Fig. S1 in the supplemental material). In addition, investigation of the relative expression levels of PSI-2 *psaN* and Hrc-Hrp *hrcN* in *P. stewartii*-infected flea beetles and plants showed relatively higher *psaN* than *hrcN* expression in flea beetles, whereas the *hrcN* expression level was higher than that of *psaN* in maize (see Fig. S1). It was known that the Hrp-Hrc T3SS was expressed in plants but not in rich media. Therefore, we expected that it would not be highly expressed in the insect intestine. These results suggest that PSI-2 is less active in plants and is expressed in the insect and that it may have an important role in the insect host.

The PSI-2 T3SS is required for *P. stewartii* persistence in flea beetle guts. To further investigate PSI-2 involvement in *P. stewartii* flea beetle colonization, flea beetles were allowed to feed on maize infected with the *psaN*::mini-Tn*5-gus* mutant (DM5121) and DC283 (wild-type). Immediately after feeding on infected maize plants (day 0), both mutant and wild-type bacteria were detected in the lumen of all gut regions by immunofluorescence confocal microscopy, including the foregut, midgut and hindgut of 71 to 90% of beetles (Fig. 3). However, 8 days later, the *psaN* mutant was detected in significantly fewer beetles than was the wild-type strain, and infested beetles had lower levels of mutant bacteria in the midgut and hindgut than wild-type bacteria (Fig. 4).

To obtain independent confirmation of these findings, we measured viable cell populations of the T3SS *psaN* mutant and the wild-type parent strain (DC283) during colonization of flea beetles. For comparison, we included a second, more recently isolated *P. stewartii* wild-type strain (DC440), since DC283 could have lost some of its ability to colonize insects after more than 40 years in storage. Since resistance to antimicrobial peptides might be a col-

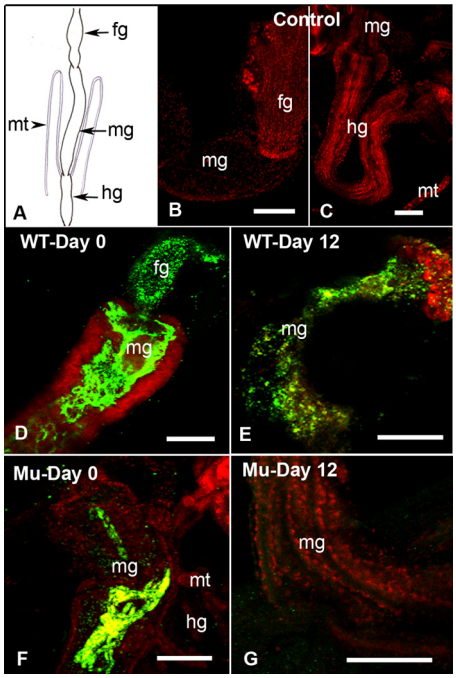

FIG 3 The PSI-2 *psaN* is required for *P. stewartii* persistence in the flea beetle gut. (A) Diagram of flea beetle foregut (fg), midgut (mg), hindgut (hg), and Malpighian tubules (mt). (B to G) Immunochemical localization of *P. stewartii* (green fluorescence) in propidium iodide-stained guts (red fluorescence). Beetles fed on healthy control plants for 2 h (B) and 12 days (C), beetles fed on wild-type (DC283) *P. stewartii*-infected plants for 2 h (day 0) (D) and 12 h (E), and beetles fed on *psaN*-deficient (DM5121) *P. stewartii* for 2 h (day 0) (F) and 12 h (G) are shown. Bars, 100 μm.

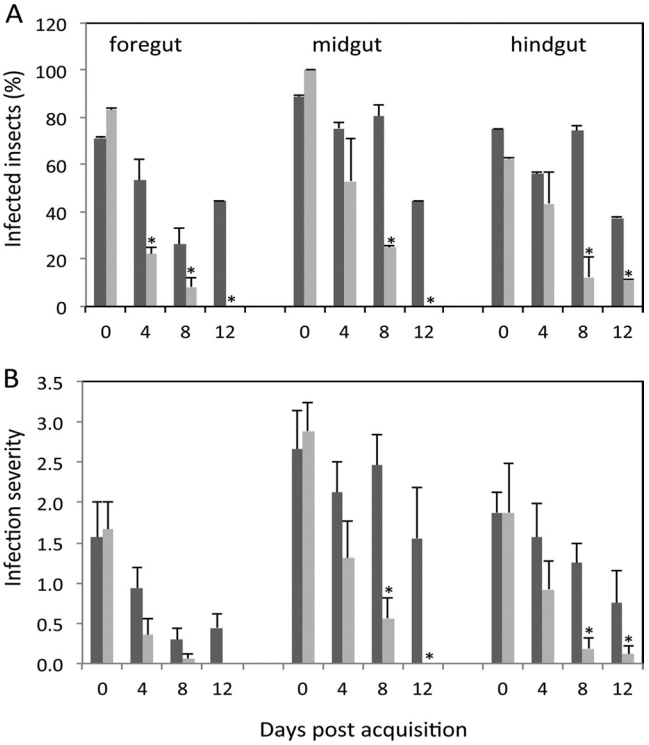

FIG 4 Persistence of wild-type and *psaN*-deficient *P. stewartii* in the flea beetle gut. Beetles were allowed to feed on maize infected with wild-type (DC283; dark gray) or *psaN*-deficient (DM5121; light gray) strains of *P. stewartii* for 2 days and then moved to healthy maize, as outlined in Materials and Methods. (A) Percentage of *P. stewartii*-infected beetles; (B) relative abundance of bacterial foci in beetle guts (rated on a scale of 0 to 4). Data are mean ± standard errors (SE) for 7 to 9 individuals from three biological replicates. *, significantly different ($P < 0.05$).

onization factor, we also included the *P. stewartii sapD*::mini-Tn*5-gus* (DM5106), which has a mini-Tn*5-gus* insertion in the Sap (sensitivity to antimicrobial peptides) ABC transporter gene *sapD*. The initial infection levels of the two mutants and the two wild-type strains were similar in beetles immediately after acquisition and increased by an average of 25% over a period of 10 days for DC283, DC440 and the *sapD* mutant (Fig. 5A). In contrast, the initial population level of the *psaN* mutant decreased to 72% on day 4, to 32% on day 8, and no viable cells were detected in the beetles on day 10. Thus, PSI-2 *psaN* appears to be required for *P. stewartii* persistence but not initial colonization of flea beetles, whereas *sapD* appears to have no apparent involvement in *P. stewartii* colonization and persistence.

The *psaN* gene is located in the middle of an operon that contains a total of 13 genes. Thus, the mini-Tn*5-gus* insertion in the T3SS *psaN*::mini-Tn*5-gus* mutant could affect expression of seven genes downstream of *psaN*. To investigate whether the observed mutant phenotype was associated with disruption of the *psaN* gene itself, a nonpolar *aphA3* cassette was inserted into *psaN* after the codon for amino acid 111, then recombined into the DC283 chromosome to generate the nonpolar *psaN* mutant DM7003 *psaN*::*aphA3*. After initial colonization of beetles, populations of both the T3SS *psaN* polar (*psaN*::mini-Tn*5-gus*) and nonpolar (*psaN*::*aphA3*) mutants started to decline at day 4 and were reduced to 27% and 20%, respectively, on day 8, whereas the infection levels of wild-type DC283 remained the same over 8 days (Fig. 5B). In contrast, a *P. stewartii* mutant in which the nonpolar *psaN* mutation in *psaN*::*aphA3* was genetically complemented with plasmid pDM3007 that constitutively expresses wild-type *psaN* (generating *P. stewartii psaN*::*aphA3*/*psaN*$^+$) had a population level of 63% that of the wild-type on day 8 (Fig. 5B), indicating that complementation with *psaN*$^+$ alone can restore *P. stewartii* persistence in the flea beetles. However, persistence was not restored to full wild-type levels (Fig. 5B). Possible explanations for this may be that constitutive overexpression of *psaN* from a plasmid turns down PSI-2 expression by feedback repression, high levels of PsaN negatively affect growth, or pDM3007 may be slightly unstable in the insect gut. It was previously reported that the PsaN homologs from *Yersinia* (20) and *Xanthomonas* (38), YsaN and HrcN, respectively, are required for a functional T3SS. Thus, loss of PsaN probably disables the PSI-2 T3SS.

Reduced vector transmission of *psaN* mutants. We also investigated if PSI-2 is involved in *P. stewartii* transmission by flea beetles to maize. Shortly after acquisition on day 0, the *psaN*::mini-Tn*5* polar mutant, the *psaN*::*aphA3* nonpolar mutant, the *psaN*$^+$-complemented nonpolar mutant, and wild-type strain DC283 were transmitted equally well by the beetles to maize plants at 90 to 100% efficiency (Fig. 6). However, at 8 days after acquisition, the T3SS *psaN* polar and nonpolar mutants were transmitted at 10% and 12.5% efficiency, respectively, and wild-type *P. stewartii* and *P. stewartii psaN*::*aphA3*/*psaN*$^+$ were transmitted at 80% and 60% efficiency, respectively (Fig. 6). Thus, vector transmission of *P. stewartii* can also depend on a functional PSI-2.

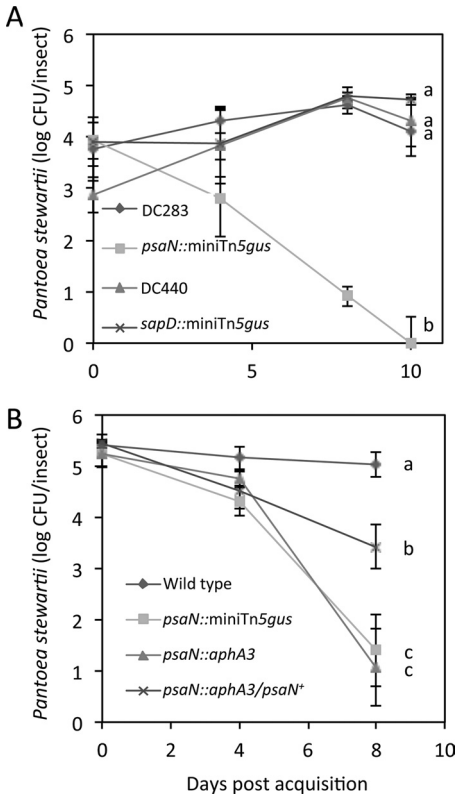

FIG 5 Mutation of *psaN* reduces persistence of colonization of *P. stewartii* in flea beetles. Beetles were allowed to feed on maize infected with two wild-type strains of *P. stewartii* (DC283 and DC440) or a *psaN* (DM5121) or *sapD* (DM5107) mutant strain. (B) Genetic complementation of the *P. stewartii* nonpolar *psaN*::*aphA3* mutant (DM7003) with a plasmid carrying wild-type *PsaN* (pDM3007) increases *P. stewartii* persistence in flea beetles. The number of viable *P. stewartii* cells was estimated as CFU from two or three beetle samples in each of three independent experiments. Data are mean log CFU per insect ± SE. Letters (a, b, and c) indicate significant differences in the number of CFU on day 10 (A) or 8 (B) ($P < 0.05$).

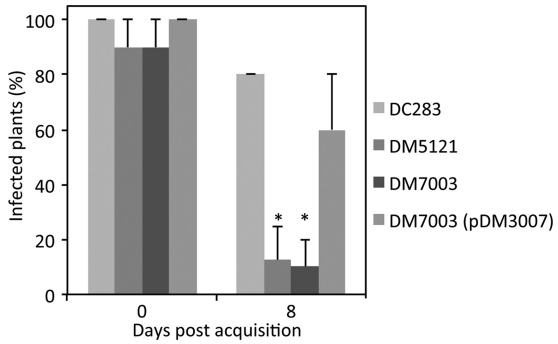

FIG 6 The PSI-2 *psaN* is required for persistent transmission of *P. stewartii* by flea beetles. Beetles were allowed to feed on maize plants infected with wild-type (DC283), *psaN*-defective strains (DM5121 and DM7003), or a rescued *psaN2*::*aphA3*/*psaN*$^+$ strain [(DM7003(pDM3007)] for 2 days. Individual maize seedlings were then exposed to five beetles each from day 0 or day 8 after acquisition of bacteria for a 2-day transmission period. Data are means ± SE for five seedlings per treatment in three independent experiments. Asterisks indicate means significantly lower than unmarked bars ($P < 0.05$).

DISCUSSION

In this study, we demonstrated that, in addition to the Hrc-Hrp1 family T3SS necessary for plant infection (23, 27), *P. stewartii* contains an Inv-Mxi-Spa type T3SS that is required for bacterial persistence in its insect vector and subsequent transmission by the vector to host plants. Other bacteria, including *Yersinia*, *Salmonella*, and *Sodalis* spp., contain multiple T3SSs in their genomes, but these T3SSs additively contribute to successful invasion of a single host (12, 13, 48, 57, 58). In contrast, the two T3SSs of *P. stewartii* enable it to colonize multiple hosts belonging to different kingdoms—i.e., plants and insects. The PSI-2 cluster is not needed for plant pathogenicity, and its expression in plants may be detrimental to pathogenesis (6). Thus, strict regulation of the two T3SS clusters is probably necessary, and both may involve HrpL. The HrpL sigma factor is a positive regulator of the plant pathogenicity *hrc-hrp* T3SS that is controlled by a regulatory cascade that senses plant and environmental signals (40). The mini-Tn*5*-*gus* mutant screen provided evidence that HrpL also downregulates several PSI-2 genes (*pchA*, *psaG*, and the *ospC1* homologue *ackA*) and several other genes (Table 3). Since HrpL activates transcription as an alternate sigma factor, it is most likely that it acts indirectly on PSI-2 and the other genes by activating expression of one or more negative transcriptional regulators, global regulators, or feedback mechanisms.

In nature, *P. stewartii* is not transmitted by wind, rain, or cultural practices, cannot survive as an epiphyte or in plant debris, and is only rarely seed-borne. Consequently, it must persist in the vector gut until it can be successfully transmitted to maize. Since *P. stewartii* resides primarily in the flea beetle midgut and hindgut lumen, the most likely mode of transmission involves *P. stewartii* passage through the hindgut into feces, followed by mechanical introduction into plants via feeding wounds (7, 43). This is similar to the transmission paths of other insect-transmitted plant pathogens, e.g., *Xanthomonas axonopodis* and *Erwinia tracheiphila* (43, 59). Since this mode of transmission is primarily mechanical, it is unlikely that PSI-2 is required for the actual transfer of bacteria from feces to the interior tissues of the plant. Therefore, the inability of flea beetles to transmit the *psaN* mutants probably indicates that high gut populations are required for the insect to deliver an effective inoculum.

Bacterial genes that are involved in interactions with insect vectors (2, 36, 51) and insect hosts (25, 52) have been identified and recently reviewed by Nadarasah and Stavrinides (46). However, the involvement of a separate T3SS required for persistence in an insect vector and subsequent transmission has not previously been reported for plant or vertebrate pathogens. Genome sequencing has revealed multiple T3SSs in Gram-negative bacteria associated with (in)vertebrate animals and plants (12, 37, 48, 54). The nonpathogenic epiphytic bacterium *Erwinia tasmaniensis* Et1/99, which is closely related to *P. stewartii*, is found on apple and pear flower surfaces. It has a bipartite Inv-Mxi-Spa-like T3SS, in addition to an Hrc-Hrp T3SS (37), suggesting that it may have an (in)vertebrate animal host. However, this Inv-Mxi-Spa gene cluster is missing a structural gene (37) and hence may be nonfunctional. Some plant-pathogenic *Xanthomonas* spp. carry animal-type T3SSs and persist in insect vectors, but it is not known whether these T3SSs enable these bacteria to invade (in)vertebrate animals (59). Conversely, the human pathogens *Vibrio parahaemolyticus* and *Burkholderia* spp. harbor plant-type T3SS Hrc-Hrp genes, suggesting that these bacteria may use plants as alternate

hosts (54). Our results suggest that the life cycles of these and other bacterial pathogens may include currently unknown alternate host species that are relevant to food security.

ACKNOWLEDGMENTS

We thank Kristen Willie, Angela Strock, Jane Todd, Mark Zimmerman, and Cameron Exner for excellent technical support, David Mackey, J. C. Jang, Allan Downie, Eric Stockinger, Xiaodong Bai, and Sophien Kamoun for their helpful comments and suggestions on earlier versions of the manuscript, and Eric Cabot and Nicole Perna for submission of the PSI-2 sequence to GenBank.

This work was supported by USDA, CSREES, National Research Initiative grants 2002-35319-11562 to D.L.C., 2005-35319-15328 to D.L.C. and S.A.H., and 2008-35319-04506 to D.L.C. and D. Mackey and by state and federal funds appropriated to The Ohio State University (OSU), Ohio Agricultural Research and Development Center (OARDC).

Preliminary genomic sequence data were obtained from Baylor College of Medicine Human Genome Sequencing Center website at http://www.hgsc.bcm.tmc.edu. The genomic DNA sequencing project of *Pantoea stewartii* DC283 was supported by National Research Initiative of the USDA Cooperative State Research, Education and Extension Service, grant number 0198694 to Nicole T. Perna, at the University of Wisconsin—Madison.

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