Assessing the Ability of *Salmonella enterica* to Translocate Type III Effectors Into Plant Cells

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Salmonella enterica serovar Typhimurium, a human enteric pathogen, has the ability to multiply and survive endophytically in plants. Genes encoding the type III secretion system (T3SS) or its effectors (T3Es) may contribute to its colonization. Two reporter plasmids for T3E translocation into plant cells that are based on hypersensitive response domains of avirulence proteins from the *Pantoea agglomerans*–beet and *Xanthomonas euvesicatoria*–pepper pathosystems were employed in this study to investigate the role of T3Es in the interaction of *Salmonella* ser. Typhimurium 14028 with plants. The T3Es of *Salmonella* ser. Typhimurium, SipB and SifA, which are translocated into animal cells, could not be delivered by *Salmonella* ser. Typhimurium into cells of beet roots or pepper leaves. In contrast, these effectors were translocated into plant cells by the phytopathogenic bacteria *P. agglomerans* pv. *betae*, *Erwinia amylovora*, and *X. euvesicatoria*. Similarly, HsvG, a T3E of *P. agglomerans* pv. *gypsophylae*, and XopAU of *X. euvesicatoria* could be translocated into beet roots and pepper leaves, respectively, by the plant pathogens but not by *Salmonella* ser. Typhimurium. Mutations in *Salmonella* ser. Typhimurium T3SS genes *invA*, *ssaV*, *sipB*, or *sifA*, did not affect its endophytic colonization of lettuce leaves, supporting the notion that *S. enterica* cannot translocate T3Es into plant cells.

Nontyphoid *Salmonella enterica* is the causal agent of human gastroenteritis and is commonly responsible for outbreaks related to the consumption of fresh fruit and vegetables (Brandl 2006; Teplitski et al. 2009). *S. enterica* has been shown to actively attach and endophytically colonize plant tissues (Cooley et al. 2003). Similar to phytopathogenic bacteria, *S. enterica* cells can penetrate the plant through wounds and natural openings, such as hydathodes and stomata (Gu et al. 2013; Kroupitski et al. 2009) and may have to contend with the plant innate immune system. Plant and animal innate immune systems depend on a diverse assortment of cell surface and cytoplasmic receptors that detect and respond to invading pathogens (Ronald and Shirasu 2012). Several studies have shown that *S. enterica* is able

to subvert the plant immune system and colonize the plant endophytically despite the plant defense response to its presence (García and Hirt 2014). For example, flagellin is a prominent PAMP (pathogen-associated molecular pattern) component that is recognized through direct binding of a conserved N-terminal epitope called flg22 by the leucine rich repeat receptor kinase FLS2 (Zipfel et al. 2004). Previous studies suggested the existence of a different flg22 sequence in strains of *S. enterica* serovar Seftenberg that is more divergent from canonical flg22 (Berger et al. 2011). The displayed corresponding flg22 peptide (flg22-SS) reduced PTI (pattern-triggered immunity) activity in *Arabidopsis*, which would suggest that certain *S. enterica* strains may have evolved divergent flagellin sequences to avoid plant recognition (García et al. 2014). Other reports, reviewed by García and Hirt (2014), supported the observation that PAMPs of *Salmonella* spp. are less conserved than initially assumed and that sequence variations may weaken the plant immune response toward *Salmonella* spp. Therefore, it was suggested that an escape of plant detection by PAMP motifs is a strategy of *Salmonella* spp. to overcome PTI (García and Hirt 2014).

S. enterica uses two functionally distinct type III secretion systems (T3SSs), encoded on the pathogenicity islands SPI-1 and SPI-2, to transfer the type III effectors (T3Es) into animal cells. A major function of the SPI-1 secretion system is to mediate bacterial invasion of epithelial cells, whereas the principal role of SPI-2 is to facilitate the replication of intracellular bacteria within membrane-bound *Salmonella*-containing vacuoles (SCVs) (Waterman and Holden 2003). More than 30 *Salmonella* T3Es have been shown to manipulate diverse host cell functions that are important for *Salmonella* virulence in animals (McGhie et al. 2009).

The possibility that *Salmonella* T3Es may contribute to its plant colonization was examined in several studies, using a mutagenesis approach. Schikora et al. (2011) demonstrated that mutations in either SPI-1 (i.e., *invA* or *prgH*) or SPI-2 (i.e., *ssaV* or *ssaJ*) of *Salmonella enterica* serovar Typhimurium significantly reduced its proliferation in *Arabidopsis* leaves and caused increased hypersensitive response (HR)-like and chlorosis symptoms. Furthermore, a *Salmonella* ser. Typhimurium *prgH*[−] mutant, defective in a structural component of the SPI-1-encoded needle complex, triggered enhanced expression of defense genes in *Arabidopsis* (García et al. 2014). It was also demonstrated that a *Salmonella* ser. Typhimurium *invA* mutant induced stronger reactive oxygen species accumulation in tobacco BY-2 cells than the parental strain (Shirron and Yaron 2011). However, some conflicting results were reported also. For example, Iniguez et al. (2005) showed that deletion mutants

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in *spaS* and in *sipB*, which encode a structural subunit of the SPI-1 apparatus and an effector protein and translocator, respectively, resulted in increased colonization of alfalfa roots and wheat seedlings. Meng et al. (2013) failed to detect any difference in *Salmonella* population size nor did they observe any macroscopic symptoms in *Nicotiana benthamiana* or tomato due to deletion of SPI-1 components (i.e., *invA*, *prgH*) or SPI-2 components (i.e., *sseA* or *sseB*). Moreover, *Salmonella* populations increased significantly in tomato when coinoculated with *Pseudomonas syringae* pv. *tomato* but not when coinoculated with *Pseudomonas syringae* pv. *tomato* T3SS mutant (Meng et al. 2013). This may suggest that *Salmonella* spp. benefited from the immune-suppressing effects of the plant pathogen whereas its own T3SS was not functional in planta. Alternatively, nutrient release due to *Pseudomonas syringae* pv. *tomato* growth also could have caused this enhanced *Salmonella* multiplication.

While mutagenesis of T3SS structural or effector genes in *S. enterica* suggests that the T3SS may be involved in its colonization of plants, *Salmonella*-mediated delivery of effectors into plant cells, which is the hallmark of the T3SS, has not been demonstrated. Therefore, the present study was undertaken to explore whether the T3SSs of *S. enterica* can deliver T3Es into plant cells. We describe herein the use of two reporter plasmids to answer this important inquiry about the molecular interaction of a human enteric pathogen with plants.

RESULTS

Translocation assay on beet cubes.

To test whether *Salmonella* can translocate T3Es into plants, two reporter plasmids based on HR domains of avirulence proteins were employed. The first translocation assay is based on the *Pantoea agglomerans*–beet pathosystem, as described below and in Supplementary Figure S1. Two effectors from *Salmonella* ser. Typhimurium (SipB and SifA) and one effector, HsvG, from the gall-forming plant pathogen *Pantoea agglomerans* pv. *gypsophila* were selected. SipB belongs to a group of *Salmonella* translocases that are delivered through the SPI-1 to mediate the passage of effectors via the target host cell membrane (Ramos-Morales 2012). SifA belongs to a group of effectors that are delivered through the SPI-2 T3SS and trigger the production of *Salmonella*-induced filaments (Sifs) in host cells and are responsible for maintaining the SCV in infected cells (Brumell et al. 2001). HsvG is a host-specific transcription factor T3E of the gall-forming *P. agglomerans* (Nissan et al. 2006). It has a pivotal role in gall formation on gypsophila (Nissan et al. 2017). The N-terminus of each effector, which harbors the recognition domain for translocation by the T3SS (Rossier et al. 1999) was fused in frame into the pVSP-PtHis translocation cassette and was transformed into *Salmonella* ser. Typhimurium 14028, *Pantoea agglomerans* pv. *betae* 4188, *Erwinia amylovora* 238, and *Xanthomonas euvesicatoria* 85-10, as described below. Expression of the fusion constructs were validated by Western (Fig. 1) or dot blots (Supplementary

Fig. 1. Western blots of *Salmonella enterica* serovar Typhimurium harboring pVSP-PtHis fused to the N-terminal of type III effectors *sipB*, *sifA*, and *hsvG*. Empty vector denotes pVSP-PtHis without a fusion of an N-terminus. Fusions were detected with anti-His antibodies.

Fig. S2). Results illustrated in Table 1, Figure 2, and Supplementary Figure S3 indicate that SipB, SifA, and HsvG can trigger HR on beet cubes when delivered by the phytopathogenic bacteria *P. agglomerans* pv. *betae* 4188, *E. amylovora* 238, and *X. euvesicatoria* 85-10 but not by *Salmonella* ser. Typhimurium.

Table 1. Summary of translocation assays on beet root tissue

Strain ^a	HR elicitation ^b	Gall formation ^c
<i>Salmonella enterica</i> ser. Typhimurium 1408		
Empty	–	–
<i>sipB</i>	–	–
<i>sifA</i>	–	–
<i>hsvG</i>	–	–
<i>Pantoea agglomerans</i> pv. <i>betae</i> 4188		
Empty	–	+
<i>sipB</i>	+	–
<i>sifA</i>	+	–
<i>hsvG</i>	+	–
<i>Erwinia amylovora</i> 238		
Empty	–	–
<i>sipB</i>	+	–
<i>sifA</i>	+	–
<i>hsvG</i>	+	–
<i>Xanthomonas euvesicatoria</i> 85-10		
Empty	–	–
<i>sipB</i>	+	–
<i>sifA</i>	+	–
<i>hsvG</i>	+	–

^a Empty denotes that the strain harbored a translocation cassette pVSP-PtHis lacking an insertion of the effector's N-terminus. *sipB*, *sifA* and *hsvG* denote that the translocation cassette contained the N-terminus of each gene.

^b Appearance of a hypersensitive response (HR) indicates translocation.

^c Gall development was observed only by *P. agglomerans* pv. *betae* 4188 with an empty translocation cassette.

Fig. 2. Translocation assay of type III effector SipB into beet root cells by *Salmonella enterica* serovar Typhimurium and phytopathogenic bacteria. A hypersensitive response (HR) is revealed by the gray appearance of beet cells along the diagonal inoculation streak. **A**, Water control. **B**, Gall formation by *Pantoea agglomerans* pv. *betae* 4188 carrying the empty vector pVSP-PtHis (white arrows). **C**, Lack of plant reaction to *Salmonella* ser. Typhimurium 14028 harboring the translocation cassette fused to the N-terminus of *sipB*. **D**, HR elicitation by *P. agglomerans* pv. *betae* 4188 harboring the translocation cassette fused to the N-terminus of *sipB*. **E**, HR elicitation by *Erwinia amylovora* 238 harboring the translocation cassette fused to the N-terminus of *sipB*. **F**, HR elicitation by *Xanthomonas euvesicatoria* 85-10 harboring the translocation cassette fused to the N-terminus of *sipB*. Representative images from three repetitions are shown.

The time required for elicitation of HR on beet root tissue differed between the various bacteria and was 24 to 36 h for *P. agglomerans* pv. *betae* 4188, 36 to 48 h for *E. amylovora* 238, and 48 to 72 h for *X. euvesicatoria* 85-10. The possibility that the observed differential incubation periods for HR elicitation correlated with bacterial colonization rates of beet tissue was examined (Fig. 3). Equal colonization rates for all tested bacterial strains was recorded during the 24 h. However, divergent growth rates were observed thereafter and significant differences in population sizes were measured, with *P. agglomerans* pv. *betae* 4188 achieving the highest, *X. euvesicatoria* 85-10 the lowest, and *Salmonella* ser. Typhimurium and *E. amylovora* 238 exhibiting intermediate cell densities (Fig. 3).

Translocation assays on Bs2 pepper leaves.

The second translocation assay was based on the *Xanthomonas euvesicatoria*–pepper pathosystem. The *Salmonella* effectors

Fig. 3. Colonization rate of beet root tissue by various bacterial strains. Pab4188 signifies *Pantoea agglomerans* pv. *betae*; *E. amylovora* signifies *Erwinia amylovora*; S. Ty signifies *Salmonella enterica* serovar Typhimurium; Xe signifies *Xanthomonas euvesicatoria*. Results are an average of three independent experiments. Bars indicate standard deviation.

SipB and SifA and a novel effector of *X. euvesicatoria* 85-10, XopAU (Teper et al. 2016), were tested for HR elicitation on Bs2 pepper leaves. The N-termini of the effectors were fused into the translocation plasmid pAvrBs2-HR, as described below, and their expression was validated by dot blot using hemagglutinin (HA) antibodies (Supplementary Fig. S4). The reporter plasmids with fusions to the genes for the effectors SipB, SifA, and XopAU triggered distinct HR symptoms when present in *X. euvesicatoria* 85-10 inoculated onto Bs2 pepper leaves but not when transformed into *Salmonella* ser. Typhimurium (Fig. 4).

Colonization of lettuce leaves

by *Salmonella* ser. Typhimurium mutants.

To further test the possibility that mutants of the T3SSs or T3Es may affect colonization of lettuce leaves by *Salmonella* ser. Typhimurium 14028, structural mutants in *invA* and *ssaV* of SPI-1 and SPI-2, respectively, and effector mutants in *sipB* and *sifA* were tested for endophytic colonization of lettuce leaves. No significant differences ($P < 0.05$) in *Salmonella* colonization of lettuce leaves between the wild-type strain and its mutants at each time point were observed up to 15 days after inoculation (Fig. 5).

DISCUSSION

To gain information about the premise that *Salmonella* may deliver T3Es into plant cells similarly to its interaction with animal cells, we have employed two bioassay systems to assess their translocation into plant cells. Our results indicate that *Salmonella* ser. Typhimurium failed to deliver *Salmonella* or *P. agglomerans* pv. *betae* 4188 effectors into beet root cells. In contrast, the phytopathogenic bacteria *P. agglomerans* pv. *betae* 4188, *E. amylovora* 238, and *X. euvesicatoria* 85-10, which belong to three different genera of gram-negative bacteria, showed a clear translocation of *Salmonella* and *P. agglomerans* pv. *betae* 4188 effectors into beet roots, as revealed by distinct HR symptoms observed on the beet tissue upon inoculation with the transformants harboring the reporter plasmids. The lack of HR induced by *Salmonella* cells expressing the effector fusions could not be attributed to a slower growth rate on beet root tissue, because the human pathogen displayed similar and greater colonization rates on beet cubes than did *X. euvesicatoria* 85-10 and *E. amylovora* 238, respectively, both of which triggered

Fig. 4. Translocation assay of the type III effectors XopAU, sipB, and sifA into Bs2 pepper leaves by *Xanthomonas euvesicatoria* mutant in *avrBs2* (*XeΔavrBs2*) and *Salmonella enterica* serovar Typhimurium (S. Ty). Empty vector denotes the translocation plasmid AvrBs2-HR. Translocation was apparent by hypersensitive response elicitation on pepper leaves within 48 h. Representative images from two repetitions are shown.

a clear HR. Additionally, in a different translocation assay based on the *X. euvesicatoria* 85-10-pepper pathosystem, *Salmonella* ser. Typhimurium could deliver neither *Salmonella* nor *X. euvesicatoria* 85-10 effectors, as opposed to *X. euvesicatoria* 85-10, which translocated both effectors and produced standard HR symptoms on pepper leaves.

No significant differences in endophytic colonization of lettuce leaves, as also reported by Klerks et al. (2007), could be detected by various T3SS structural or effector mutants of *Salmonella* ser. Typhimurium over a period of two weeks. Thus, our results indicate a lack of involvement of T3Es in lettuce endophytic colonization by *Salmonella* ser. Typhimurium, as was also reported in tomato and tobacco (Meng et al. 2013). It is noteworthy that Üstün et al. (2012) observed that the *Salmonella* effector SseF could elicit a HR-like response in *Nicotiana benthamiana* when transiently expressed via *Agrobacterium tumefaciens* infiltration or when delivered by the T3SS of *Xanthomonas campestris* pv. *tomato* but not when leaves were infiltrated with *Salmonella* spp. harboring SseF. These observations are in accordance with our findings that *Salmonella* cannot translocate T3Es into plant cells but that its effectors trigger HR-like responses when translocated by the T3SS of plant-pathogenic bacteria.

The T3SS is an essential component of two complex bacterial machineries: the flagellum, which drives cell motility, and the nonflagellar T3SS or injectisome, which delivers effectors into eukaryotic cells and plays a vital role in virulence of gram-negative bacteria (Abby and Rocha 2012; Diepold and Armitage 2016). Evolutionary studies support the view that the injectisome-T3SS was derived from flagella ancestors (Abby and Rocha 2012; Hueck 1998). Within the injectisome, eight subfamilies that confer host specificity were distinguished (Diepold and Armitage 2016). These include Ysc, SPI-1, SPI-2, and *Chlamydia* subfamilies that are known to primarily target animal cells and Hrp1, Hrp2, and *Rhizobia* subfamilies that are known to primarily target plant cells. A more distantly related *Myxococcus* subfamily is present in free-living, nonpathogenic or symbiotic bacteria (Abby and Rocha 2012; Cornelis 2006). According to the foregoing phylogenetic studies, SPI-1 and SPI-2 of *Salmonella* evolved to

deliver T3Es into animal cells rather than into plant cells. Moreover, the injectisome structure was reported to be divided into three parts: i) a *trans* membrane export apparatus, ii) an extracellular needle in animal pathogenic bacteria, as opposed to a pilus in plant-pathogenic bacteria that conveys effectors into the cells, and iii) a translocon, which forms a pore in the host membrane or cell wall (Galán and Wolf-Watz 2006). The above information suggests that the two injectisomes of *Salmonella* phylogenetically and structurally evolved to translocate effectors into animal cells.

The apparent need of the T3SSs to deliver effectors into both animal and plant cells might have exerted an evolutionary driving force for acquisition of two or more functionally different T3SSs by the same cell. For example, *Burkholderia pseudomallei* is a highly versatile pathogen capable of infecting many species of animals and plants (Sun and Gan 2010). *B. pseudomallei* and related species can harbor up to three T3SSs; two systems, T3SS1 and T3SS2, are homologous to the Hrp2 subfamily of plant pathogens, which is closely related to the T3SSs of *Ralstonia solanacearum* and *X. campestris* (Diepold and Armitage 2016). The third system, T3SS3, is similar to SPI-1 of *S. enterica*, an animal pathogen. Thus, T3SS1 and T3SS2 are required for *B. pseudomallei* infection of tomato plants but not for that of hamsters (Lee et al. 2010), whereas T3SS3 but not T3SS1 or T3SS2 plays an essential role during infection of animals by the same pathogen (Stevens et al. 2004). Another human pathogen that harbors plant-type T3SS genes is *Vibrio parahaemolyticus*, suggesting that this bacterium may use plants as an alternate host (Troisfontaines and Cornelis 2005).

Some plant-pathogenic *Xanthomonas* spp. carry animal-type T3SSs, which may enable them to persist in insect vectors (Zandjanakou-Tachin et al. 2007), but their function in insect colonization has not yet been demonstrated. However, *Pantoea stewartii* subsp. *stewartii*, the causative agent of Stewart's bacterial wilt and leaf blight of maize, uses two phylogenetically distinct T3SSs to colonize its plant host and insect vector (Correa et al. 2012). In addition to a T3SS of the Hrp1 subfamily, known to be essential for maize pathogenesis, *P. stewartii* subsp. *stewartii* carries a second T3SS designated as *Pantoea* secretion island 2 (PSI-2) that was shown to be required for persistence in its flea beetle vector. The foregoing examples support the hypothesis that the evolutionary preference for adaptation of gram-negative bacterial pathogens to infect both plants and animals has been achieved by acquisition of phylogenetically distinct injectisomes that can interact with each kingdom separately rather than a single T3SS that can translocate T3Es into cells of both kingdoms. The latter stipulation may draw additional support from the extensive horizontal gene transfer (HGT) that likely has mediated the presence of T3SSs in bacteria (Brown and Finlay 2011), given that the approximately 20 genes that encode a functional T3SS are generally linked to chromosomal or occasionally plasmid-born pathogenicity island resulting from HGT.

The reported role of *Salmonella* T3Es in suppression of plant defenses in *Arabidopsis* (Garcia et al. 2014; Schikora et al. 2011) may be attributed to their action in the apoplast rather than through the interior of plant cells, as exemplified by harpins (Choi et al. 2013). Indeed, harpins, which are secreted but not translocated through the T3SS of plant pathogenic bacteria, are responsible for HR-like elicitation in plants and were also reported to induce mitogen-activated protein kinases in *Arabidopsis* (Desikan et al. 2001). Recently the identification of the full complement of *Salmonella* genes involved in tomato colonization was carried out using transposon insertion sequencing analysis (de Moraes et al. 2017). The requirement for *Salmonella* pathogenicity islands SPI-1 and SPI-2 T3SSs as well as SPI-3 were also shown to be important factors for plant colonization during high but not low titers of bacteria. Similarly, SPI-1 and SPI-2 T3SSs were shown to be important factors for plant

Fig. 5. Endophytic colonization of lettuce leaves by *Salmonella enterica* serovar Typhimurium 14028 and its type III secretion system (T3SS) and type III effector (T3E) mutants. Mutants disrupted in *invA* and *ssaV* genes, encoding structural proteins of T3SS-SPI-1 and T3SS-SPI-2, respectively, and mutants disrupted in the T3Es *sipB* and *sifA*, which are secreted by SPI-1 and SPI-2, respectively, were tested. Results represent the mean of three independent experiments. Bars represent standard deviation and significance ($P < 0.05$) was determined according to Student's *t* test.

colonization from high-inoculum (10^8 CFU per plant) experiments in *Arabidopsis* (Schikora et al. 2011). The possibility that proteins encoded and secreted by these genes may enhance colonization through suppression of PTI cannot be excluded but cannot be assigned to translocation of effectors.

Our results, obtained with two distinct reporter systems, indicate that neither SPI-1 nor SPI-2 of genus *Salmonella* can translocate T3Es into plant cells. However, the possibility remains that some T3Es of *Salmonella* trigger an HR-like response in *Arabidopsis* without translocation into plant cells, as was demonstrated with harpins. Given that T3SS-mediated delivery of effectors into the interior of host cells is an essential virulence component of most gram-negative animal- and plant-pathogenic bacteria, our findings are consistent with the concept that *S. enterica* is a successful plant endophyte that likely lacks the ability to trigger disease symptoms in plants.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions.

The bacterial strains and plasmids used in this study are listed in Supplementary Table S1. *P. agglomerans* pv. *betae* 4188, *E. amylovora* 238, and *X. euvesicatoria* 85-10 were grown in Luria-Bertani (LB) broth or agar at 28°C. *Escherichia coli* strains were cultured on the same medium at 37°C. Wild-type and mutant strains of *Salmonella* ser. Typhimurium 14028 were grown in LB broth or xylose-lysine deoxycholate agar (XLD) at 37°C. Antibiotics were used at the following concentrations (µg/ml): kanamycin, 50; rifampicin, 150; and spectinomycin, 50.

DNA and protein manipulation.

Isolation of genomic or plasmid DNA from bacterial strains, cloning, ligation, transformation, and other DNA manipulations were performed according to standard procedures (Ausubel et al. 1995) or as recommended by the supplier. DNA fragments were amplified by polymerase chain reaction (PCR) with T (Super Therm polymerase JMR-801; Roche, Mannheim, Germany). Primers were synthesized by Hylabs Laboratories (Rehovot, Israel) and are described in Supplementary Table S2. PCR was performed directly on bacterial colonies. Amplification was carried out, in a Biometra thermocycler (Bio-Rad Laboratories, Hercules, CA, U.S.A.), as described previously (Nissan et al. 2017). Bacterial strains were transformed by electroporation with a Gene Pulser apparatus (Bio-Rad Laboratories) according to the manufacturer's instructions. Triparental mating was performed with the *E. coli* helper plasmid pRK2073 (Ditta, G. 1986). DNA sequencing was carried out in the Instrumentation and Services Center at Tel-Aviv University. Procedures for DNA sequence homology searches and restrictions maps were performed as previously described (Nissan et al. 2017).

Construction of translocation reporters.

Delivery of T3Es into plants was carried out by two translocation assays that were constructed based on two pathosystems, i.e., the gall-forming *P. agglomerans*–beet (Nissan et al. 2017) and *X. euvesicatoria*–pepper (Roden et al. 2004). The translocation cassette of the *P. agglomerans* system was designed based on the ability of PthG, a T3E of *P. agglomerans* pv. *gypsophylae*, to elicit the HR on beet (*Beta vulgaris*) while promoting gall formation on its other host, gypsophila (*Gypsophila paniculata*) (Ezra et al. 2000, 2004). In contrast, *P. agglomerans* pv. *betae* 4188 did not harbor an active PthG and could induce galls on beet as well as on gypsophila. The PthG translocation cassette was composed of multiple cloning sites and the HR domain of the PthG (amino acids 247 to 472) fused into His-Tag. The cassette was cloned into pVS6 with expression from the *lac* promoter to yield pVSP-PthHis (Nissan et al. 2017). The N-terminus

(approximately 200 bp) of a selected effector, along with its 20-bp upstream sequence that contains the ribosome binding site (RBS), was PCR-amplified and was cloned in frame into the HR domain of PthG within the translocation cassette. The PthG translocation cassette with or without (control) the N-terminus of the tested effector was transformed into *Salmonella* ser. Typhimurium, *P. agglomerans* pv. *betae* 4188, *E. amylovora* 238, and *X. euvesicatoria* 85-10. The expression of the fusion constructs in the obtained bacterial strains was validated by Western or dot blots using anti-His antibodies.

The *X. euvesicatoria* 85-10 translocation assay was based on the AvrBs2-HR plasmid containing the HR domain of AvrBs2 (amino acids 62 to 574) fused to the HA tag, according to Roden et al. (2004) and Teper et al. (2016). The latter construct was PCR-amplified, was cloned into the broad-host range vector pBBR1MCS-2 to obtain the translocation plasmid that was then transformed into *Salmonella* and *X. euvesicatoria* 85-10. AvrBs2-HA could not be translocated into Bs2 expressing pepper plants and induce HR unless fused to an N-terminal recognition signal of a T3E. To examine if an encoded effector can be translocated into leaf cells of the Bs2 pepper plant, its N-terminal region (approximately 200 bp) along with the 20-bp upstream fragment containing the RBS was PCR-cloned and was fused in frame to the HR domain of *avrBs2* followed by the HA tag (Roden et al. 2004). The resulting plasmids were mobilized into *X. euvesicatoria* 85-10 *hrpG** Δ *avrBs2* and *Salmonella* ser. Typhimurium 14028 by triparental mating. The expression of the fusion constructs in the transformed bacterial strains was validated by dot blots using anti-HA antibodies.

Translocation assays.

The translocation assay for the *P. agglomerans* system was performed on matured swollen beet roots (*Beta vulgaris* 'Egyptian Red Beet'). The roots were soaked in 1% hypochlorite for 10 min and were then washed twice in sterile water. They were then cut into $1 \times 1 \times 0.8$ -cm cubes under sterile conditions and were placed on 1.5% water agar in a petri dish. Each of the tested bacterial strains was grown overnight on LB agar and was inoculated into five beet cubes by cross-streaking the top of the cube with a sterile toothpick carrying the bacterial cells (Nissan et al. 2017). The petri dishes were sealed with paraffin to avoid desiccation, were incubated for 5 days at 28°C, and were then examined for HR or gall formation.

Translocation assays for the *X. euvesicatoria* 85-10 system were performed as described by Teper et al. (2016). The *Salmonella* and *X. euvesicatoria* 85-10 transformants were suspended in 10 mM MgCl₂ at OD₆₀₀ = 0.1 and were infiltrated into leaves of 7-week-old pepper (ECW20R carrying the *Bs2* gene) using a needleless syringe. Translocation of T3E was positive if the tested strain containing the vector with the N-terminal fusion triggers HR on BS2 pepper within 48 h following inoculation. Bacterial transformants expressing the AvrBs2-HR domain without the fusion of an effector's N-terminal or a *X. euvesicatoria* 85-10 mutant in the T3SS apparatus served as controls.

Western and dot blot analyses.

Bacterial cells of the tested strain were grown overnight in 3 ml of LB medium, followed by transfer of 0.6 ml of cell suspension into 3 ml of fresh LB medium, and were grown further for approximately 4 h, until an OD₆₀₀ of 0.5 was reached. A sample of 50 µl of bacterial suspension was mixed with 10 µl of 6× sodium dodecylsulfate (SDS)-polyacrylamide gel electrophoresis sample buffer (375 mM Tris-HCl, pH 6.8, 6% SDS, 48% glycerol, 9% β-mercaptoethanol). For Western blot analysis, the extracted proteins were analyzed by SDS gel electrophoresis in a 12% (wt/vol) polyacrylamide gel. For dot blot analysis, 5 µl of the extracted proteins were spotted onto a nitrocellulose strip and were allowed

to dry at room temperature. The membrane blots or strips were incubated for 1 h at room temperature in 5% skimmed milk in TBST (10 mM Tris, pH 8.0, 150 mM NaCl, 0.5% Tween 20). Rabbit anti-6× His tag antibodies (1:10,000; Abcam, San Francisco) or rabbit anti-HA tag antibody (1:5,000) were added and incubated for 1 h. Membranes were washed three times for 10 min with TBST and were incubated with a 1:8,000 dilution of alkaline phosphatase-conjugated antirabbit antibodies (Abcam) for 1 h. The blots were washed three times with TBST and were developed using BCIP/NBT (5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium) phosphatase substrate (KPL, Gaithersburg, MD, U.S.A.) according to the manufacturer's protocol.

Plant inoculation and bacterial recovery.

The colonization rate of beet root tissue by various bacterial strains was performed as follows. Bacterial suspension (20 µl, 10⁶ cells/ml) was applied onto the surface of each beet cube. The cubes were then placed on 1.5% water agar under sterile conditions and were treated as described for translocation. At different time intervals, five replicate cubes per bacterial strain were macerated, each in 2 ml of sterile water, and were analyzed for bacterial density using a dilution end-point procedure on LB agar plates.

The involvement of *Salmonella* ser. Typhimurium 14028 T3SS in endophytic colonization of lettuce leaves was examined with mutants disrupted in *invA* and *ssaV*, encoding the structural proteins of T3SS-SPI-1 and T3SS-SPI-2, respectively, and mutants in T3Es, i.e., *sipB* and *sifA*. Lettuce (*Lactuca sativa* cv. Romit 936; Hishul Ltd., Ashkelon, Israel) was inoculated by submerging the leaves in a 10⁸-cell/ml suspension of the wild type and its mutants for 1 min. Endophytic growth was monitored by removing one leaf of similar size from five plants at different time intervals. The leaves were subjected to sterilization with 2% sodium hypochlorite containing 0.1% Tween 20 (Sigma-Aldrich Co., St. Louis) for 30s. The disinfectant was then removed by rinsing each leaf five times in a beaker containing 250 ml of deionized distilled water and an additional wash in sterile water. The leaves were dried, were transferred to plastic bags, and were macerated with a hammer. The resulting homogenates were then resuspended in 2 ml of sterile water, were serially diluted in sterile water, and were plated onto *Salmonella* selective agar plates (XLD; HiMedia, Mumbai). Cell number per leaf was determined after overnight growth at 37°C.

Statistical analysis.

Statistical analyses were performed using Excel software (Microsoft, Seattle) and JMP v7 (SAS Institute, Cary, NC, U.S.A.).

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LITERATURE CITED

Abby, S. S., and Rocha, E. P. C. 2012. The non-flagellar type III secretion system evolved from the bacterial flagellum and diversified into host-cell adapted systems. *PLoS Genet.* 8:e1002983.

Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. 1995. *Current Protocols in Molecular Biology*. John Wiley & Sons, Inc., New York.

Berger, C. N., Brown, D. J., Shaw, R. K., Minuzzi, F., Feys, B., and Frankel, G. 2011. *Salmonella enterica* strains belonging to O serogroup 1,3,19 induce chlorosis and wilting of *Arabidopsis thaliana* leaves. *Environ. Microbiol.* 13:1299-1308.

Brandl, M. T. 2006. Fitness of human enteric pathogens on plants and implications for food safety. *Annu. Rev. Phytopathol.* 44:367-392.

Brown, N. F., and Finlay, B. B. 2011. Potential origins and horizontal transfer of type III secretion systems and effectors. *Mob. Genet. Elements* 1:118-121.

Brumell, J. H., Rosenberger, C. M., Gotto, G. T., Marcus, S. L., and Finlay, B. B. 2001. SifA permits survival and replication of *Salmonella typhimurium* in murine macrophages. *Cell. Microbiol.* 3:75-84.

Choi, M.-S., Kim, W., Lee, C., and Oh, C.-S. 2013. Harpins, multifunctional proteins secreted by gram-negative plant-pathogenic bacteria. *Mol. Plant-Microbe Interact.* 26:1115-1122.

Cooley, M. B., Miller, W. G., and Mandrell, R. E. 2003. Colonization of *Arabidopsis thaliana* with *Salmonella enterica* and enterohemorrhagic *Escherichia coli* O157:H7 and competition by *Enterobacter asburiae*. *Appl. Environ. Microbiol.* 69:4915-4926.

Cornelis, G. R. 2006. The type III secretion injectisome. *Nat. Rev. Microbiol.* 4:811-825.

Correa, V. R., Majerczak, D. R., Ammar, D., Merighi, M., Pratt, R. C., Hogenhout, S. A., Coplin, D. L., and Redinbaugh, M. G. 2012. The bacterium *Pantoea stewartii* uses two different type III secretion systems to colonize its plant host and insect vector. *Appl. Environ. Microbiol.* 78:6327-6336.

de Moraes, M. H., Desai, P., Porwollik, S., Canals, R., Perez, D. R., Chu, W., McClelland, M., and Teplitski, M. 2017. *Salmonella* persistence in tomatoes requires a distinct set of metabolic functions identified by transposon insertion sequencing. *Appl. Environ. Microbiol.* 83:e03028-16.

Desikan, R., Hancock, J. T., Ichimura, K., Shinozaki, K., and Neill, S. J. 2001. Harpin induces activation of the *Arabidopsis* mitogen-activated protein kinases AtMPK4 and AtMPK6. *Plant Physiol.* 126:1579-1587.

Diepold, A. P., and Armitage, J. P. 2016. Type III secretion systems: The bacterial flagellum and the injectisome. *Philos. T. Roy. Soc. B.* Published online. doi.org/10.1098/rstb.2015.0020.

Ditta, G. 1986. Tn5 mapping of *Rhizobium* nitrogen fixation genes. *Methods Enzymol.* 118:519-528.

Ezra, D., Barash, I., Valinsky, L., and Manulis, S. 2000. The dual function in virulence and host range restriction of a gene isolated from the pPATH (Ehg) plasmid of *Erwinia herbicola* pv. *gypsophylae*. *Mol. Plant-Microbe Interact.* 13:683-692.

Ezra, D., Barash, I., Weinthal, D. M., Gaba, V., and Manulis, S. 2004. *pthG* from *Pantoea agglomerans* pv. *gypsophylae* encodes an avirulence effector that determines incompatibility in multiple beet species. *Mol. Plant Pathol.* 5:105-113.

Galán, J. E., and Wolf-Watz, H. 2006. Protein delivery into eukaryotic cells by type III secretion machines. *Nature* 444:567-573.

García, A. V., Charrier, A., Schikora, A., Bigeard, J., Pateyron, S., de Tazua-Moreau, M.-L., Evrard, A., Mithöfer, A., Martin-Magniette, M. L., Virlogeux-Payant, I., and Hirt, H. 2014. *Salmonella enterica* flagellin is recognized via FLS2 and activates PAMP-triggered immunity in *Arabidopsis thaliana*. *Mol. Plant* 7:657-674.

García, A. V., and Hirt, H. 2014. *Salmonella enterica* induces and subverts the plant immune system. *Front. Microbiol.* 5:141.

Gu, G., Cevallos-Cevallos, J. M., and van Bruggen, A. H. 2013. Ingress of *Salmonella enterica* Typhimurium into tomato leaves through hydathodes. *PLoS One* 8:e53470.

Hueck, C. J. 1998. Type III protein secretion systems in bacterial pathogens of animals and plants. *Microbiol. Mol. Biol. Rev.* 62:379-433.

Iniguez, A. L., Dong, Y., Carter, H. D., Ahmer, B. M. M., Stone, J. M., and Triplett, E. W. 2005. Regulation of enteric endophytic bacterial colonization by plant defenses. *Mol. Plant-Microbe Interact.* 18:169-178.

Klerks, M. M., Franz, E., van Gent-Pelzer, M., Zijlstra, C., and van Bruggen, A. H. C. 2007. Differential interaction of *Salmonella enterica* serovars with lettuce cultivars and plant-microbe factors influencing the colonization efficiency. *ISME J.* 1:620-631.

Kroupitski, Y., Golberg, D., Belausov, E., Pinto, R., Swartzberg, D., Granot, D., and Sela, S. 2009. Internalization of *Salmonella enterica* in leaves is induced by light and involves chemotaxis and penetration through open stomata. *Appl. Environ. Microbiol.* 75:6076-6086.

Lee, Y. H., Chen, Y., Ouyang, X., and Gan, Y.-H. 2010. Identification of tomato plant as a novel host model for *Burkholderia pseudomallei*. *BMC Microbiol.* 10:28.

McGhie, E. J., Brawn, L. C., Hume, P. J., Humphreys, D., and Koronakis, V. 2009. *Salmonella* takes control: Effector-driven manipulation of the host. *Curr. Opin. Microbiol.* 12:117-124.

Meng, F., Altier, C., and Martin, G. B. 2013. *Salmonella* colonization activates the plant immune response and benefits from association with plant pathogenic bacteria. *Environ. Microbiol.* 15:2418-2430.

Nissan, G., Gershovits, M., Morozov, M., Chalupowicz, L., Sessa, G., Manulis-Sasson, S., Barash, I., and Pupko, T. 2017. Revealing the inventory of type III effectors in *Pantoea agglomerans* gall-forming

- pathovars by using draft genome sequences and a machine-learning approach. *Mol. Plant Pathol.* Published online. doi.org/10.1111/mpp.12528
- Nissan, G., Manulis-Sasson, S., Weinthal, D., Mor, H., Sessa, G., and Barash, I. 2006. The type III effectors HsvG and HsvB of gall-forming *Pantoea agglomerans* determine host specificity and function as transcriptional activators. *Mol. Microbiol.* 61:1118-1131.
- Ramos-Morales, F. 2012. Impact of *Salmonella enterica* type III secretion system effectors on the eukaryotic host cell. *ISRN Cell Biol.* 2012: 787934.
- Roden, J. A., Belt, B., Ross, J. B., Tachibana, T., Vargas, J., and Mudgett, M. B. 2004. A genetic screen to isolate type III effectors translocated into pepper cells during *Xanthomonas* infection. *Proc. Natl. Acad. Sci. U.S.A.* 101:16624-16629.
- Ronald, P. C., and Shirasu, K. 2012. Front-runners in plant-microbe interactions. *Curr. Opin. Plant Biol.* 15:345-348.
- Rossier, O., Wengelnik, K., Hahn, K., and Bonas, U. 1999. The *Xanthomonas* Hrp type III system secretes proteins from plant and mammalian bacterial pathogens. *Proc. Natl. Acad. Sci. U.S.A.* 96:9368-9373.
- Schikora, A., Virlogeux-Payant, I., Bueso, E., Garcia, A. V., Nilau, T., Charrier, A., Pelletier, S., Menanteau, P., Baccarini, M., Velge, P., and Hirt, H. 2011. Conservation of *Salmonella* infection mechanisms in plants and animals. *PLoS One* 6:e24112.
- Shirron, N., and Yaron, S. 2011. Active suppression of early immune response in tobacco by the human pathogen *Salmonella* Typhimurium. *PLoS One* 6:e18855.
- Stevens, M. P., Haque, A., Atkins, T., Hill, J., Wood, M. W., Easton, A., Nelson, M., Underwood-Fowler, C., Titball, R. W., Bancroft, G. J., and Galyov, E. E. 2004. Attenuated virulence and protective efficacy of a *Burkholderia pseudomallei* bsa type III secretion mutant in murine models of melioidosis. *Microbiology* 150:2669-2676.
- Sun, G. W., and Gan, Y.-H. 2010. Unraveling type III secretion systems in the highly versatile *Burkholderia pseudomallei*. *Trends Microbiol.* 18:561-568.
- Teper, D., Burstein, D., Salomon, D., Gershovitz, M., Pupko, T., and Sessa, G. 2016. Identification of novel *Xanthomonas euvesicatoria* type III effector proteins by a machine-learning approach. *Mol. Plant Pathol.* 17: 398-411.
- Teplitski, M., Barak, J. D., and Schneider, K. R. 2009. Human enteric pathogens in produce: Un-answered ecological questions with direct implications for food safety. *Curr. Opin. Biotechnol.* 20:166-171.
- Troisfontaines, P., and Cornelis, G. R. 2005. Type III secretion: More systems than you think. *Physiology (Bethesda)* 20:326-339.
- Üstün, S., Müller, P., Palmisano, R., Hensel, M., and Börnke, F. 2012. SseF, a type III effector protein from the mammalian pathogen *Salmonella enterica*, requires resistance-gene-mediated signalling to activate cell death in the model plant *Nicotiana benthamiana*. *New Phytol.* 194:1046-1060.
- Waterman, S. R., and Holden, D. W. 2003. Functions and effectors of the *Salmonella* pathogenicity island 2 type III secretion system. *Cell. Microbiol.* 5:501-511.
- Zandjanakou-Tachin, M., Fanou, A., Le Gall, P., and Wydra, K. 2007. Detection, survival and transmission of *Xanthomonas axonopodis* pv. *manihotis* and *X. axonopodis* pv. *vignicola*, causal agents of cassava and cowpea bacterial blight, respectively, in/by insect vectors. *J. Phytopathol.* 155:159-169.
- Zipfel, C., Robatzek, S., Navarro, L., Oakeley, E. J., Jones, J. D., Felix, G., and Boller, T. 2004. Bacterial disease resistance in *Arabidopsis* through flagellin perception. *Nature* 428:764-767.