Escherichia coli O157:H7 Induces Stronger Plant Immunity than *Salmonella enterica* Typhimurium SL1344

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Accepted for publication 30 December 2012.

ABSTRACT

Roy, D., Panchal, S., Rosa, B. A., and Melotto, M. 2013. *Escherichia coli* O157:H7 induces stronger plant immunity than *Salmonella enterica* Typhimurium SL1344. *Phytopathology* 103:326-332.

Consumption of fresh produce contaminated with bacterial human pathogens has resulted in various, sometimes deadly, disease outbreaks. In this study, we assessed plant defense responses induced by the fully pathogenic bacteria *Escherichia coli* O157:H7 and *Salmonella enterica* serovar Typhimurium SL1344 in both *Arabidopsis thaliana* and lettuce (*Lactuca sativa*). Unlike SL1344, O157:H7 induced strong plant immunity at both pre-invasion and post-invasion steps of infection. For instance, O157:H7 triggered stomatal closure even under high relative

humidity, an environmental condition that generally weakens plant defenses against bacteria in the field and laboratory conditions. SL1344 instead induced a transient stomatal immunity. We also observed that *PRI* gene expression was significantly higher in *Arabidopsis* leaves infected with O157:H7 compared with SL1344. These results suggest that plants may recognize and respond to some human pathogens more effectively than others. Furthermore, stomatal immunity can diminish the penetration of human pathogens through the leaf epidermis, resulting in low bacterial titers in the plant apoplast and suggesting that additional control measures can be employed to prevent food contamination. The understanding of how plant responses can diminish bacterial contamination is paramount in preventing outbreaks and improving the safety of food supplies.

The number of serious cases of food contamination leading to death has been increasing, and outbreaks associated with fresh produce have emerged as an important public health concern. It is estimated that 76 million cases of foodborne diseases occur yearly in the United States (4). In particular, *Escherichia coli* and *Salmonella enterica* appear to be two of the most common causal agents of food poisoning associated with the consumption of fresh leafy vegetables (28). The enterohemorrhagic *E. coli* O157:H7 (hereafter referred to as O157:H7) causes bloody diarrhea and hemolytic uremic syndrome and *S. enterica* serovar Typhimurium causes gastroenteritis. These human pathogens are not known to be plant pathogens (1). Nonetheless, they can survive on and penetrate into plant tissues and maintain their population in the mesophyll, causing serious foodborne disease outbreaks. Both surface and interior contamination of leaves with human pathogens can be dangerous but internal contamination is difficult, if not impossible, to remove by standard disinfection procedures. Therefore, the understanding of the initial contamination process is paramount in preventing foodborne diseases.

Several studies have shown association of *Salmonella* spp. and O157:H7 with stomata and naturally occurring lesions (3,8,14, 17). Wounds also provide a nutrient-rich path toward the plant interior (9). However, in the absence of wounding, a major unsolved issue is how human pathogens internalize plant tissue (7) and cope with the active innate immune system against non-pathogens. To fight infection, plants rely on the innate immunity consisting of two main branches (15), pathogen-triggered immunity (PTI) and effector-triggered immunity (ETI). In PTI, conserved molecular signatures of microorganisms called pathogen- or microbe-associated molecular patterns (PAMPs or MAMPs)

are recognized by plant receptors called pattern recognition receptors. Recognition leads to activation of defense signaling pathways in the plant cell, including plant hormone-dependent as well as hormone-independent signaling pathways (6). The hormones jasmonate, salicylic acid, and ethylene are particularly important (10). Successful virulent phytopathogens are able to overcome PTI by different virulence strategies such as the production of type III secretion system effectors and phytotoxins (20). In incompatible interactions, effectors released by the pathogen into the plant cell are recognized by specific nucleotide-binding leucine-rich repeat proteins encoded by resistance genes, which results in ETI, a strong response leading to hypersensitive reaction that limits pathogen spread to other tissues.

PTI is considered to be the first line of defense against microbial attack. One of the important components in this initial defense against pathogens is stomatal immunity. The stomatal pore is a prevalent opening in the leaf epidermis (21) that is crucial in allowing gas exchange necessary for photosynthesis and is also a major route for bacterial penetration into plant tissue. Stomata function as active innate immunity gates against invasion of bacteria into *Arabidopsis* leaves (29). Previous studies suggest that O157:H7 cannot overcome stomatal closure, resulting in prolonged activation of the stomatal immune response (22). Interestingly, however, a recent study documented a remarkable ability of *S. enterica* serovar Typhimurium SL1344 (hereafter referred to as SL1344) to migrate toward stomata and enter plant tissues without triggering the stomatal immune response (17). This finding is both unexpected and exciting, raising the possibility that not only plant pathogens (11,12,22,30) but also some human pathogens (SL1344) have evolved mechanisms to subvert stomatal defense to enter plant tissues. However, it is equally possible that this bacterium is able to evade recognition by the plant immune system. In either case, the response of plants toward infection by SL1344 seems to be different than that toward O157:H7. At this point, this observation remains an intriguing phenomenon; the underlying mechanism is not understood. Here,

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<http://dx.doi.org/10.1094/PHYTO-09-12-0230-FI>
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we provide evidence that SL1344 may not subvert plant immunity but, instead, induces weaker responses compared with O157:H7.

MATERIALS AND METHODS

Plant material and growth conditions. *Arabidopsis thaliana* (L. Heyhn.) seed were sown in a 1:1:1 (vol/vol/vol) mixture of growing medium (Redi-earth plug and seedling mix; Sun Gro), fine vermiculite, and perlite. Plants were grown in controlled environmental chambers equipped with humidity control (Percival H2X Two Atomizer Humidifiers; Percival Scientific, Inc., Perry, IA) at 22°C, 60 ± 5% relative humidity (RH), and a 12-h photoperiod under light intensity of 100 $\mu\text{mol.m}^{-2}.\text{s}^{-1}$. For all experiments, 4- to 5-week old plants were used. The ecotype Columbia (Col-0; ABRC stock CS60000) was used as a wild-type plant. The *Arabidopsis* mutant *ost1-2* (23) and its wild-type Landsberg *erecta* (Ler) were a kind gift from Dr. J. Leung at CNRS, France. Hydroponically grown, naturally pest-free lettuce plants (*Lactuca sativa* 'Butter Lettuce') were obtained from a local grocery store and kept at 4°C until prior to experiments.

Bacterial strains and culturing conditions. Wild-type bacterial cells of *E. coli* O157:H7 strain 86-24 (27) and *S. enterica* serovar Typhimurium SL1344 (17) were grown in Luria-Bertani medium (tryptone at 10 g.liter⁻¹, yeast extract at 5 g.liter⁻¹, and NaCl at 5 g.liter⁻¹; pH 7.0) at 30°C for all experiments. Cells were streaked on solid medium from frozen glycerol stocks for inoculum preparation. Medium was supplemented with spectinomycin (100 $\mu\text{g.ml}^{-1}$) to grow SL1344.

Stomatal assay. To assess the effect of RH on stomatal immunity, plants were acclimated under 100 $\mu\text{mol.m}^{-2}.\text{s}^{-1}$ light, 25°C temperature, and varying RH (60 ± 5% or >95%) for 12 h inside a growth chamber equipped with humidity control (Percival H2X Two Atomizer Humidifiers). RH was monitored with a digital hygrometer (Traceable; VWR International, Inc., West Chester, PA). Stomatal assays were conducted as previously described (5), except that unstained, whole leaves were imaged with a Nikon Eclipse 80i fluorescent microscope (Nikon Corporations, Shinagawa-ku, Tokyo) equipped with long-distance objectives to measure the stomatal aperture width. For stomatal assays performed with dark-closed stomata, plants were inoculated in the morning prior to turning on the lights and kept in the dark for the duration of the experiment.

Bacterial inoculations. Bacterial strains were cultured at 30°C in liquid Luria-Bertani medium supplemented with appropriate antibiotics until an optical density at 600 nm of 0.9 to 1.0 was reached. Bacteria were collected by centrifugation and resuspended in water to the final concentration of 10⁸ CFU.ml⁻¹ containing 0.03% Silwet L-77 (Lehle Seeds Co., Round Rock, TX) for dip inoculation of plants. Highly humid conditions were obtained by keeping well-watered plants covered with plastic domes in controlled environmental chambers. The level of humidity was monitored with a digital hygrometer (Traceable; VWR). Inoculated plants were immediately incubated under the following conditions: 25°C, 60 ± 5% or >95% RH, and 12 h of daily light (100 $\mu\text{mol.m}^{-2}.\text{s}^{-1}$) and kept there for the duration of the experiment. Leaves were surface sterilized in 70% ethanol for 2 min and

Fig. 1. Human pathogens induce unique stomatal movements. **A**, *Arabidopsis* and **B**, lettuce heads were incubated with O157:H7 or SL1344 under varying relative humidity (RH). Results are shown as mean of stomatal aperture width ($n = 50$ to 70) ± standard error. Statistical significance of the difference in the means (mock versus bacterium treatment) was detected with two-tailed Student's *t* test (***) indicates $P < 0.001$. **C**, Hydroponically grown lettuce heads incubated at 60% or >95% RH. Roots of all plants were kept moist for the duration of the experiment.

bacterial population in the plant apoplast was determined as previously described (16).

Gene expression analysis. Total RNA was extracted from leaves using the RNeasy Plant Mini Kit (Qiagen Inc., Valencia, CA) and quantified using a NanoDrop spectrophotometer (Thermo Scientific, Rockford, IL). Total RNA (1 µg) was synthesized into cDNA using the Takara RNA PCR kit (AMV) version 3.0 (Clontech, Mountain View, CA) and diluted to a final concentration of 50 ng.µl⁻¹. Quantitative PCR (qPCR) reaction (20 µl) was performed with 10 µl of iTaq Fast SYBR Green Supermix (Bio-Rad, Hercules, CA), 2 µl of cDNA template from the reverse transcriptase reaction described above, and 200 nM reverse and forward gene-specific primers. Reactions were carried out in an Applied Biosystems 7300 thermocycler (Applied Biosystems, Foster City, CA) using the following cycling parameter: 1 cycle of

95°C for 5 min and 40 cycles of 95°C for 10 s and 60°C for 30 s. Pathogenesis-related 1 (*PR1*) (At2g14610) gene expression levels relative to the control samples were calculated using the $\Delta\Delta C_t$ method (18) considering the expression of the housekeeping gene *ACT8* (At1g49240) as an internal control. *ACT8* primers used were 5'-TTCCGGTTACAGCGTTTGGAGAGA-3' (forward) and 5'-AACGCGGATTAGTGCCTCAGGTAA-3' (reverse) and *PR1* primers were 5'-CTTGTTCTTCCCTCGAAAGCTCAAGATAGC-3' (forward) and 5'-GAGCATAGGCTGCAACCCTCTC-3' (reverse). Two biological replicates and three technical replicates were performed.

Statistical analysis. Statistical significance of data from the stomatal assay, bacterial counts in the apoplast, and gene expression was calculated using two-tailed Student's *t* test. All experiments reported here were repeated at least two times (biological replicates) using a minimum of three technical replicates.

RESULTS

Human pathogens induce unique stomatal movements.

Because leafy vegetables, especially lettuce, are normally maintained at high RH prior to consumption, we checked for the possibility of Arabidopsis and lettuce to mount stomatal immunity against O157:H7 and SL1344 under varying air RH (Fig. 1). The average stomatal aperture width in mock-inoculated plants was wider under >95% RH than 60% RH. Nonetheless, live O157:H7 bacterium induced strong stomatal closure in both Arabidopsis (Fig. 1A) and lettuce (Fig. 1B) plants under both RH levels, and most stomata remained closed for the duration of the experiment (4 h). Interestingly, the SL1344 bacterium induced only a transient stomatal closure at 2 h and stomatal aperture width returned to measurements similar to that of the water control in both plants under both RH at 4 h post-inoculation (Fig. 1).

We next determined bacterial titers in the apoplast of dip-inoculated leaves, which allows bacterial penetration through the stomata, closely mimicking natural infection conditions. O157:H7 and SL1344 did not proliferate inside Arabidopsis and the bacterial population declined as the plant started to senesce. The O157:H7 population, however, drastically declined (100-fold) $\approx$ 15 days after inoculation, whereas the SL1344 population reduced 10-fold in the same time period (Fig. 2A). Because these bacteria did not multiply aggressively in the apoplast and we observed a very similar kinetics of stomatal movement under both RH (Fig. 1), we expected that apoplastic bacterial enumeration would be also very similar independent of the RH. Although this was true for O157:H7, high RH favored a significantly larger ($P < 0.05$) apoplastic bacterial population of SL1344 in Arabidopsis (Fig. 2B). Unlike Arabidopsis plants, lettuce heads cannot be maintained at 60% RH for a period longer than 24 h because they start to wilt; therefore, we stopped the experiment at that time point. Nonetheless, the same trend in bacterial population counts in response to RH levels was observed in both plants (Fig. 2B and C).

SL1344 cannot actively open stomata. The results above suggest that SL1344 could be able to overcome stomatal immunity and actively open the pore, similar to phytopathogenic bacteria such as *Pseudomonas syringae* (20,22) and *Xanthomonas campestris* (11). We tested the hypothesis that SL1344 can actively open stomata by performing a stomatal assay under darkness at 60% RH. This bacterium was unable to open dark-closed stomata (Fig. 3); therefore, we reasoned that this SL1344 triggers a weak immunity in the plant and the light stimulus is quickly prioritized by the guard cell, resulting in re-opening of the stomata (Fig. 3), and O157:H7 induces a strong stomatal immune response that cannot be overcome by light (Fig. 1).

To support the idea that guard cells can prioritize their response when challenged with biotic and abiotic stresses, we performed stomatal assays with flg22, a conserved 22-amino acid peptide of

Fig. 2. High relative humidity (RH) favors penetration and survival of SL1344 but not O157:H7. **A and B**, Arabidopsis and **C**, lettuce. Bacterial enumeration in the leaf apoplast at different days (d) after dip inoculation with O157:H7 or SL1344 under varying RH. Results are shown as the mean ($n = 12$) $\pm$ standard error. Statistical significance of the difference in the means (60% versus >95% RH at each time point) was detected with two-tailed Student's *t* test (* and ** indicate $P < 0.05$ and 0.01 , respectively).

the flagellin subunit and a general inducer of a plant's innate immune responses (31). Arabidopsis and lettuce stomata significantly closed in response to flg22 compared with the water control (Fig. 4). However, we observed that high RH (>95%) diminished the effect of flg22 at a low concentration (2 μ M). Increasing the concentration of flg22 to 10 μ M caused the stomata to close to the same extent, independent of the RH level used (Fig. 4). These results suggest that strong PTI overrides the effect of high RH in opening stomata, and guard cells prioritize their response to different external stimuli showing various degrees of stomatal immunity. Therefore, it is likely that the re-opening of stomata during incubation with SL1344 (Fig. 1) represents a weak stomatal immune response to SL1344.

Stomatal immunity reduces penetration of human pathogens into leaves. Because O157:H7 induces strong stomatal closure in Arabidopsis and lettuce plants even under high RH, we could not determine whether this layer of defense is effective in diminishing contamination of plant internal tissues by human bacterial pathogens using only wild-type plants. To test this hypothesis, we conducted stomatal and pathogenesis assays (Fig. 5) with the Arabidopsis mutant *ost1-2* (23) that is unable to close stomata in response to PAMPs (22). These experiments were performed under moderate RH (60%), which does not favor bacterial penetration in the wild-type plant (Fig. 2). Because *ost1-2* plants naturally have mostly open stomata, we first determined that they can be maintained at 60% RH without wilting (Fig. 5A). The results showed that stomata in *ost1-2* plants do not close in response to O157:H7 or SL1344 (Fig. 5B) and these plants support higher bacterial titer in their apoplast compared with the wild-type plant *Ler* (Fig. 5C). Taken together, these findings provide direct genetic evidence in support of our hypothesis.

O157:H7 induces a higher level of *PR1* gene expression than SL1344. To assess whether human pathogens can also regulate Arabidopsis defense responses in whole leaves, we evaluated the expression level of the Arabidopsis marker gene *PR1* (At2g14610) that is associated with immunity against bacteria. Reverse-transcription qPCR analysis indicated that both O157:H7 and SL1344 induce an early expression of the *PR1* gene; however, O157:H7 induced a higher expression level of *PR1* than SL1344 (Fig. 6). This result suggests that the plant may activate its defense more effectively against O157:H7 than SL1344. It also may explain, at least in part, the fact that the SL1344 population persists at ≈ 1 log higher than the O157:H7 population in the apoplast of Arabidopsis at 15 to 22 days after inoculation even though the inoculum concentration (10^8 CFU.mL⁻¹) was the same for both bacteria (Fig. 2A).

Fig. 3. SL1344 cannot actively open stomata. Arabidopsis plants were dip inoculated with SL1344 and maintained at 60% relative humidity in the dark or under light for the duration of the experiment; hpi = h post-inoculation. Results are shown as mean of stomatal aperture width ($n = 50$ to 70) $\pm$ standard error. Statistical significance of the difference in the means (mock versus bacterium treatment at specific time points) was detected with two-tailed Student's *t* test (***) indicates $P < 0.001$).

DISCUSSION

An increasing number of reports on gastroenteritis outbreaks associated with consumption of fresh produce have been published in the last several years. Although there is no simple or single solution to this problem, one crucial aspect is to understand how human pathogens can penetrate and survive in plant tissues. In this study, we addressed the plant immune response toward two human bacterial pathogens, O157:H7 and SL1344, both at pre-invasion (i.e., stomatal immunity) and post-invasion stages of the infection.

Previously, we have demonstrated that O157:H7 induces strong stomatal closure in Arabidopsis leaves until 8 h post-incubation using water as vehicle to inoculate plants (22). More recently, however, Kroupitski and collaborators (17) have shown that SL1344 does not induce strong stomatal closure in lettuce compared with *P. syringae* pv. *tomato* DC3000. Because these authors have submerged lettuce leaves into saline bacterial suspension, we suspected that their finding that SL1344 does not close stomata was because the extremely wet conditions would favor stomata to stay open. Therefore, we checked stomatal response under two

Fig. 4. Strong pathogen-triggered immunity overrides the effect of high relative humidity (RH) in opening stomata. Stomatal response to different concentration of flg22 under varying RHs and constant light. **A**, Arabidopsis or **B**, lettuce leaves were floated on either water or flg22 solution and stomatal aperture was measured 4 h post-incubation. Results are shown as mean of stomatal aperture width ($n = 50$ to 70) $\pm$ standard error. Statistical significance of the difference in the means (60% versus >95% RH) was detected with two-tailed Student's *t* test (***) indicates $P < 0.001$).

levels of RH (Fig. 1C). High RH had a positive effect on stomatal opening, as shown with stomatal aperture width on water-treated plants (Figs. 1A and B and 4). Nonetheless, O157:H7 induced significant stomatal closure and SL1344 induced a transient closure in *Arabidopsis* and lettuce under both RHs (Fig. 1).

The unique stomatal movement in response to O157:H7 and SL1344 raised the immediate questions “has SL1344 evolved mechanisms to overcome stomatal immunity?” and “do plants efficiently recognize and respond to O157:H7 but not SL1344?” We have shown three pieces of evidence to support the hypothesis that stomatal re-opening in response to SL1344 is due to weak induction of PTI. First, our result shows that SL1344 cannot actively open dark-closed stomata (Fig. 3). Therefore, it is most likely that stomatal re-opening under light is a consequence of a weak stomatal immunity against SL1344, and stomata return to the open state quickly after the immune response phases out. Second, Melotto et al. (22) reported that increasing concentration of lipopolysaccharide (LPS) from *Escherichia*, *Salmonella*, and *Pseudomonas* correlates with increasing stomatal closure under light. Similarly, dose response experiments with flg22 and *P. syringae* pv. *tomato* DC3000 also positively correlated with stomatal closure (22). In a study to determine the overall transcriptional changes caused by flg22 in *Arabidopsis*, Denoux et al.

Fig. 6. O157:H7 induces higher level of pathogenesis-related 1 (*PR1*) gene expression than SL1344. Expression of the *PR1* gene (At2g14610) at 2 and 8 h post-inoculation with bacteria relative to the mock control as determined by reverse-transcription quantitative polymerase chain reaction. Statistical significance of the difference in the mean expression of *PR1* in response to SL1344 and O157:H7 relative to that in the mock control was detected with two-tailed Student's *t* test (* and ** indicate $P < 0.05$ and 0.01 , respectively).

Fig. 5. Stomatal immunity reduces penetration of human pathogens through the leaf epidermis. The *ost1-2* and wild-type *Landsberg erecta* (Ler) plants were dip inoculated with O157:H7 or SL1344. **A**, Pictures show Ler and *ost1-2* mutant plants kept at 60% relative humidity (RH) for the duration of the experiments. Note that this moderate RH does not cause plants to wilt. **B**, Stomatal aperture width was measured 2 h after inoculation with bacteria or water control. Results are shown as mean of stomatal aperture width ($n = 50$ to 70) $\pm$ standard error (SE). **C**, Bacterial enumeration in the leaf apoplast at different days (d) after inoculation. Results are shown as the mean ($n = 18$) $\pm$ SE. Statistical significance of the difference in the means (Ler versus *ost1-2*) was detected with two-tailed Student's *t* test (** and *** indicate $P < 0.01$ and 0.001 , respectively).

(6) concluded that this immune response elicitor changes the magnitude of induction of individual genes in a dose-dependent manner, and the transcriptional responses return to basal level within 24 h after treatment. In this study, we demonstrate that a low concentration of flg22 (2 μ M) at >95% RH is not as effective as 10 μ M flg22 in closing stomata (Fig. 4), suggesting that environmental conditions favoring stomatal opening such as light and high RH can override weak PTI. Consistently, penetration and survival of O157:H7 (that induces a strong response) in the apoplast of *Arabidopsis* and lettuce was not affected by the RH (Fig. 2B and C). However, high RH favored SL1344 penetration and survival in both plants (Fig. 2B and C), suggesting that >95% RH quickly diminished the effect of a weak PTI caused by SL1344. Third, SL1344 does not induce the expression of the *PR1* genes, a hallmark of plant immune response, to the same extent as O157:H7 (Fig. 6). Taken together, these findings support the idea that, unlike O157:H7, SL1344 is weakly recognized by *Arabidopsis* and lettuce, and immune responses in both mesophyll and guard cells are transient. To fully reject the alternative hypothesis that SL1344 evolved mechanisms to suppress plant immunity, it remains to be determined whether SL1344 down-regulates genes and metabolic pathways involved in plant defense responses.

We have shown that lettuce is able to mount stomatal immunity against bacteria similar to what has been observed in other plants such as *Arabidopsis*, tomato, tobacco, soybean, and common bean (11,19,22,24). These results reinforce the notion that stomatal immunity is a widespread mechanism of defense among plants extending to both plant and human pathogens. Interestingly, it seems that the molecular components involved in plant immunity against both types of pathogens are essentially the same. For instance, the guard-cell-specific OST1 kinase is required for stomatal immunity against this broad range of bacteria (22; this study). Likewise, mitogen-activated protein kinases (specifically, MPK3 and MPK6) are required for *Arabidopsis* stomatal response against *X. campestris* pv. *campestris* (11) and mesophyll immunity against *S. enterica* serovar Typhimurium 14028 (25). This unique feature of plants could be further explored to improve resistance against pathogen penetration and subsequent contamination and infection of internal tissues.

A recent study has shown that populations of several O157:H7 strains lacking cell surface structures are larger on the wild-type *Arabidopsis* and induce less *PR* gene expression than their counterparts with the cell surface structures (26). Similarly, lack of flagellin production or the type three secretion apparatus in *S. enterica* serovar Typhimurium 14028 enhances alfalfa root endophytic colonization (13). Furthermore, the type of O antigen, specifically O:1,3,19, in the LPS moiety of *S. enterica* serovars was shown to be associated with leaf wilting in *Arabidopsis*, whereas strains that lack the O antigen (rough strains) or belonging to other serogroups such as SL1344 cause no visible reaction on leaves (2). Therefore, it is possible that some bacterial species may induce stronger immunity than others, depending on how efficiently plants can recognize their MAMPs and trigger defense responses. It is also tempting to speculate that perhaps SL1344 has evolved mechanisms to avoid recognition by plants and is able to penetrate and survive as an endophyte more efficiently than O157:H7.

Finally, as mentioned above, we observed that high RH favors penetration and survival of SL1344 but not O157:H7 (Fig. 2B and C) and we reasoned that strong induction of PTI by O157:H7 may override the effect of high RH on stomatal opening. However, we cannot rule out other factors that contribute to successful penetration of SL1344 into leaves such as the ability to move on the leaf surface, chemotaxis toward nutrients exiting through the stomatal pore (17), and the overall fitness of these bacteria in the plant environment. Certainly, the ability of the plant to recognize and activate its immune system against human pathogens also contributes to the outcome and severity of plant infections.

ACKNOWLEDGMENTS

This work was supported by funding from the U.S. National Institute of Allergy and Infectious Disease (5R01AI068718) and University of Texas start-up funds to M. Melotto. We thank V. Sperandio, S. Sela, and J. Leung for providing *Escherichia coli* O157:H7, *Salmonella enterica* serovar Typhimurium SL1344, and *ost1-2* seed, respectively; and two anonymous reviewers and M. Brandl for their critiques and constructive comments.

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