

Salmonella Interactions with Plants and Their Associated Microbiota

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ABSTRACT

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The increase in the incidence of gastroenteritis outbreaks linked to the consumption of foods of plant origin has ignited public concern and scientific interest in understanding interactions of human enteric pathogens with plants. Enteric disease caused by nontyphoidal *Salmonella* is a major public health burden, with the number of cases of illness linked to fresh produce, spices, and nuts surpassing those linked to foods of animal origin. Mounting evidence supports the hypothesis that colonization of plants is an important part of the life cycle of this human pathogen. Although plant responses to human pathogens are distinct from the more specific responses to phytopathogens, plants appear to recognize *Salmonella*, likely by detecting conserved microbial patterns, which subsequently activates basal defenses. Numerous *Salmonella* genes have been identified as playing a role in its colonization of plant surfaces and tissues, and in its various interactions with other members of the phyto-microbial community. Importantly, *Salmonella* utilizes diverse and overlapping strategies to interact with plants and their microflora, and to successfully colonize its vertebrate hosts. This review provides insight into the complex behavior of *Salmonella* on plants and the apparent remarkable adaptation of this human pathogen to a potentially secondary host.

Additional keywords: foodborne pathogen, fruit, vegetable, phyllosphere, rhizosphere, enteric illness, microbe-microbe interactions.

Salmonellosis caused by nontyphoidal salmonellae is the largest cause of foodborne gastroenteritis. Nontyphoidal strains of *Salmonella* are estimated to infect over 1 million people per year in the United States alone (<http://www.epi.ufl.edu/?q=ranking+therisks>) (10). The public health burden is significant, accounting for several billion dollars in medical costs and ≈400 deaths per

year (10,57). *Salmonella* is a major causal agent of foodborne outbreaks for which etiological agents have been determined and is ranked as the most burdensome foodborne pathogen in the United States (10). The link between salmonellosis and foods of animal origin is well known and has received considerable regulatory attention. Although the number of illness cases related to the consumption of meats has declined in recent years, the overall salmonellosis outbreak rate has remained steady due to increased risk from nontraditional sources of the pathogen. These include fresh fruit and vegetables, spices, and nuts, and underscores the importance of plants as potential sources of the pathogen (10,26,57,58). From 1998 to 2007 produce was linked to more outbreaks than beef, pork, or poultry with fresh produce potentially being the riskiest food (10,26,58). In addition to the significant public health burden, outbreaks of gastroenteritis linked to the consumption of produce significantly reduce demand and impact the produce industry economically (77).

Despite the magnitude of the problem, relatively little is known about traits and mechanisms that allow *Salmonella* to persist outside of vertebrate animals. This paucity of information is stark: over 72,000 studies in PubMed are indexed under *Salmonella*, with less than 100 of them regarding *Salmonella*-plant interactions. The majority of these publications result from studies over the last two decades, highlighting a growing interest in understanding behavior of *Salmonella* outside of its animal hosts.

***Salmonella* persistence on plants.** Plants may be valuable alternate hosts for enteric pathogens by providing a refuge after excretion from the animal intestinal tract. The ability to colonize edible plants may be an effective survival strategy for *Salmonella* as it provides a direct route from its excretion in the environment back to its numerous herbivorous and omnivorous hosts (Fig. 1) (57). Field studies revealed that *Salmonella enterica* sv. Typhimurium was capable of persisting in manure-amended soils for up to 231 days, and that the pathogen was detected on aboveground parts of lettuce and parsley, and on carrots and radishes grown in these amended soils for 2 to 3 months (40,41). In addition to its transfer from manure to plants, *Salmonella* deposited onto lettuce and parsley seedlings via irrigation water survived on plants until harvest (40,41), thus corroborating the results of earlier field studies in which *Salmonella* Typhi was shown to survive on lettuce from the seedling stage to maturity after contamination with overhead water (29).

A three-step food-chain experiment by Semenov et al. (74) further supports the hypothesis that plants can serve as alternative

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hosts for human enteric pathogens. The authors demonstrated that *Salmonella* and *Escherichia coli* O157:H7 colonized seedlings sown into soil amended with pathogen-containing manure; cows, mice, and snails who ate these seedlings shed the pathogens in their excrements; and the shed pathogens persisted in manure or soil for at least 2 weeks (74). Consistent with the studies of Semenov et al. (74), Schikora et al. (73) demonstrated that *Salmonella* Typhimurium inoculated into and recovered from *Arabidopsis* leaf homogenates was as virulent as the inoculum grown in a nutrient-rich culture medium. *Salmonella* cells from leaf homogenates invaded the spleen and caused mortality in mice (73). Collectively, the above observations and the increase in salmonellosis outbreaks linked to the consumption of produce provide evidence that plant colonization by *Salmonella* can be part of its life cycle.

Early studies on the fitness of *Salmonella* in the phyllosphere revealed that this pathogen has the ability to multiply and form microcolonies on leaves, although its population sizes are often exceeded by those of plant-associated bacterial species (18). Comparative studies on lettuce leaves of different ages showed that *Salmonella* and *E. coli* O157:H7 achieved 10-fold greater population sizes on young leaves ('heart') than on the older middle leaves. Given that middle leaf exudates contained less total N, but not less total C, than those of young leaves and that lower growth of the pathogens on middle leaves could be complemented by addition of N, but not of C, to the inoculum suspension, colonization of middle leaves may have been limited by low N availability (16). Thus, it is likely that the lower fitness of human pathogens compared with that of plant-associated bacteria is partly rooted in the low abundance and restricted range of nutrients that they can assimilate on plant surfaces. However, plant surfaces are not homogenous and contain various microsites that represent oases of available nutrients (54) and which may support multiplication of human pathogens after contamination events. Because these sites are also attractive to plant-associated microbes, cells of enteric pathogens likely must interact and compete with indigenous microbial communities in order to occupy such preferred sites (14).

Laboratory studies demonstrated that *Salmonella* is capable of colonizing plants through multiple routes including wetting of leaves, contaminated soil, roots, seeds or flowers (18,24,36). For colonizing bacteria, aerial plant surfaces are a challenging environment, presenting stresses such as desiccation, UV irradiation, and starvation, with only patchy nutrient availability (38,55). Human pathogens on leaves have been shown to preferentially move towards stomata and colonize the vein areas, the bases of trichomes and lesions or other surface irregularities (2,7,15,16, 18,48–50), which may provide shelter from these stresses and increased nutrient and water availability. Hence, these microsites may offer physico-chemical conditions that are conducive not only to survival but also may be exploited by *Salmonella* for growth and where the human pathogen may behave partly like many epiphytic bacteria, including plant pathogens.

***Salmonella* genes involved in plant colonization.** If the hypothesis that plants can serve as alternate hosts for enteric pathogens is correct, *Salmonella* should not behave solely as a transient immigrant with a restricted residence time in the plant habitat, but should harbor traits allowing for its interaction with plants and their colonization. This may include also the ability to effectively use available nutrients, evade recognition by the plant defense system and/or resist or suppress plant defense responses. Thus, it should be possible to find genotypic and phenotypic evidence of *Salmonella* adaptation to its life in and on plants.

Screens of *Salmonella* mutant libraries for those unable to attach to alfalfa sprouts or colonize tomato fruits identified 20 and 55 unique non-overlapping genes, respectively (4,61) (Box 1). Even though screens in these studies were not saturating, the predicted functions of the *Salmonella* genes involved in plant colonization are distinct from those typically used by this pathogen for the infection of animal models, and were also distinct from those used by phytopathogens in plants (77). It is of note that the *Salmonella* virulence genes located on pathogenicity islands (SPIs) appear to have different roles during interactions with different plant species: in tomatoes, SPI mutants were as fit as the wild type (61), whereas in alfalfa and lettuce, SPI mutants have phenotypes that are distinct from those of the wild-type

GLOSSARY

- Apoplast:** the space outside of the plant cell membrane and between cells where water diffuses freely.
- Biotrophic:** a plant pathogen that obtains nutrients from living cells.
- Curli:** bacterial thin aggregative fimbriae formed by amyloid fibrils; produced by various members of the Enterobacteriaceae.
- Enteric illness:** human illness caused by ingestion of food that is contaminated with a pathogenic microbe or a toxic chemical.
- HEp-2 cells:** eukaryotic cell line derived from a carcinoma; used in studies that investigate pathogen invasion of host cells.
- Infectious dose:** minimum number of pathogen cells required to cause disease in a host.
- Lumen:** inner open space of an organ, e.g., of the intestine.
- Macrophage:** white blood cell that phagocytose; act in both innate and adaptive immunity in vertebrates.
- MAMP:** microbe-associated molecular pattern, a conserved microbial surface component recognized by the plant innate immunity.
- Nontyphoidal salmonellae:** cause of most salmonellosis cases; include most pathogenic *Salmonella enterica* serovars, except Typhi and Paratyphi, which cause typhoid fever in humans and other primates.
- Salmonella* fimbriae:** typically proteinaceous appendages with multiple functions, most notably in attachment. In addition to aggregative fimbriae (structures homologous to *E. coli* curli) encoded by the *agf* genes, *Salmonella* encode Lpf, Sef, Pef, and Fim fimbriae. The latter are not found in closely related lineages of enterics.
- Soft rot:** macerated plant tissue due to the degradation of the plant cell wall via pectinolytic activity of various bacterial plant pathogens.
- SPI:** *Salmonella* pathogenicity island, a chromosomal cluster of genes involved in *Salmonella* virulence. Subspecies of *Salmonella enterica* differ in the number of SPIs, some containing up to five SPIs.
- SPI-1:** *Salmonella* pathogenicity island 1, harbors genes encoding a type 3 secretion system required for invasion of epithelial and macrophage cells. The acquisition of SPI-1 separated *Salmonella* from the common ancestor with *E. coli*. All subspecies of *Salmonella* carry SPI-1.
- SPI-2:** *Salmonella* pathogenicity island 2, involved in replication in host cells, codes for a type 3 secretion system.
- SPI-3:** *Salmonella* pathogenicity island 3, encodes genes with various functions, including *mgtCB* which are required for survival in host cells and virulence.

strain (27,39,73). These differences in the roles for the SPI genes could be due to the differences in the interactions of *Salmonella* with plant vegetative and reproductive organs, which were sampled in these studies.

rdar phenotype in *Salmonella* colonization of plants. The involvement of the rdar-like phenotype (Box 2) in the persistence in and on plants seems to be conserved in human enteric pathogens. Aggregative fimbriae, encoded by the *agf* operon, contribute to biofilm formation on HEp-2 cells and in the chicken intestine (53). *Salmonella agfB* and *rpoS* mutants (defective in the production and regulation of aggregative fimbriae, respectively) were deficient in initial attachment to the root surface of sprouts (4). *agf* genes also played a significant role in the colonization of the parsley phyllosphere following irrigation with *Salmonella*-contaminated water (52). Laboratory studies with isogenic non-rdar *Salmonella* mutants suggest that their fitness within tomato fruits is significantly increased compared with rdar strains (93). *E. coli* O157:H7 variants lacking curli fimbriae are readily recovered from *E. coli* O157:H7 populations associated with produce outbreaks (21). Carter et al. (21) reported that *E. coli* O157:H7 curli-positive variants (equivalent to *Salmonella* rdar) have greater survival under low nutrient stress conditions than their curli-negative variants (equivalent to non-rdar *Salmonella*), whereas the opposite trend is observed for acid stress (21). Increased acid stress-resistance in curli-negative variants of *E. coli* O157:H7 is due to the presence of a functional RcsB, which positively regulates acid resistance in *E. coli* but negatively regulates curli production (21). Thus, while the molecular and physiological basis of the increased competitive fitness of *Salmonella* non-rdar mutants over their rdar strain in tomato fruit is not clear, it is possible that like in *E. coli* O157:H7, the selection for the non-rdar mutant cells over the wild-type cells results from enhanced tolerance of the non-rdar strain to acid stress in tomato fruit tissue and from a lack of nutrient limitation, which otherwise would favor the rdar genotype.

Cellulose production contributes to the rdar phenotype along with aggregative fimbria (Box 2) and is produced at different levels among *Salmonella* strains recovered from outbreaks related to fruits and vegetables (68,93). This surface polymer, which was first identified as an important host plant attachment factor in

Agrobacterium tumefaciens (60), and later as a fitness determinant in *Pseudomonas fluorescens* in the sugar beet rhizosphere and phyllosphere (32), also plays a role in the binding of *Salmonella* and *E. coli* to alfalfa sprouts (6,59). In this Focus Issue, Kroupitski et al. (47) report that *Salmonella* mutants in *bcsA*, *misL*, and *gidR*, encoding a cellulose synthase catalytic subunit, an adhesin of the autotransporter family expressed from SPI-3, and a putative ATP/GTP-binding protein, were impaired in attachment to and persistence on lettuce leaves stored at cold tempera-

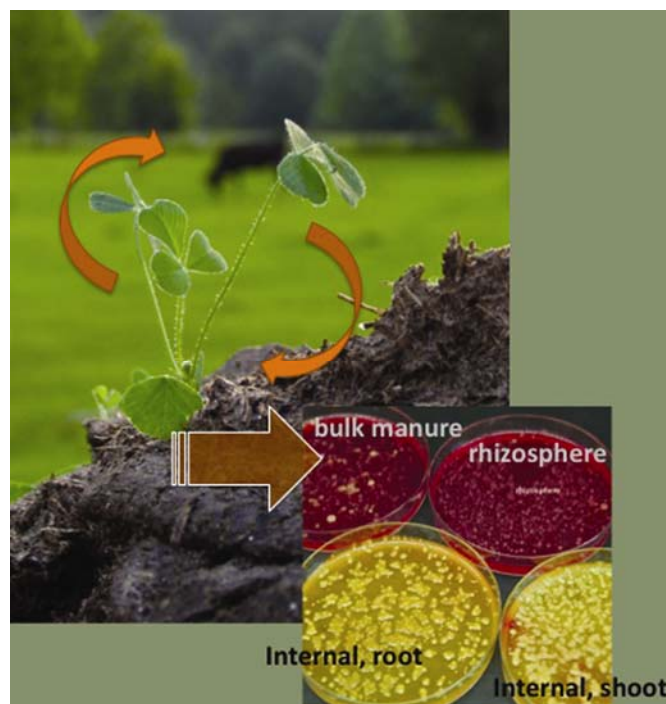
BOX 1

In planta monitoring of *Salmonella* gene regulation: technological considerations.

To better understand how *Salmonella* interacts with plants and to test whether its persistence in plants is a co-evolved part of its lifecycle, it will be important to define the factors that allow *Salmonella* to colonize its various animal and plant hosts. There is a need for sensitive tools to define *Salmonella* gene regulation in planta and its responses to specific plant metabolites. While mutant screens are ideally suited for the identification of genes required for a specific step in the interactions of enterics with plants, e.g., attachment to surfaces (6), they will likely miss more complex phenotypes, in which multiple inputs are involved in modulating a behavior. Various promoter-probe screens are better suited for the identification of the genes that are differentially regulated during attachment or persistence within plants, and thus contribute to the complex phenotype without necessarily being required for persistence in or on plants. For example, a differential fluorescence induction (DFI) screen combined with fluorescence-activated cell sorting (FACS) technology led to the identification of ~50 unique genomic fragments that induced the differential fluorescence of the reporter (61). These differentially regulated promoters functions include *Salmonella* metabolism within plants and its ability to recognize specific plant metabolites. Single mutants in the genes corresponding to the promoters identified with DFI-FACS had no fitness defect in plants, consistent with the properties of DFI screens (61).

FIGURE 1

Lifecycle of enteric bacteria. Excretion from a host and subsequent colonization of plants may be part of the lifecycle of enteric bacteria. Plants germinating from cow manure are not uncommon, as in this photograph of an alfalfa seedling growing on manure in a grazed pasture in Archer, FL. Inset: samples from the cow manure, rhizosphere, and surface-sterilized shoot and root tissues of the alfalfa seedling in the photograph were homogenized in phosphate-buffered saline and plated on xylose lysine deoxycholate agar (XLD) medium (Oxoid, Hampshire, UK) and incubated at 42°C to detect fecal coliforms (yellow colonies on XLD agar). Relatively few coliforms were detected in the aged bulk manure and in the rhizosphere; however, substantial populations of presumed fecal coliforms were detected inside the plant tissue.



tures (47). It is noteworthy that MisL also affects binding of *Salmonella* to fibronectin in animal hosts (28). Hence, *Salmonella* appears to use some of its virulence factors targeted at mammalian hosts to interact with plants, and also relies on factors that are commonly used in phytobacteria for attachment to plants.

Motility genes. Motility factors, such as flagella, play a role in the survival of *Salmonella* on plants. Genes involved in flagella synthesis (but not those involved in chemotaxis) were required for attachment of *Salmonella* serovar Typhimurium to lettuce leaves and serovar Senftenberg to basil leaves, and mutations affecting *Salmonella* motility and chemotaxis significantly inhibited its penetration into stomata (12,48). This is consistent with the observation that *E. coli* O157:H7 genes involved in motility and chemotaxis were strongly upregulated within the first 15 min of exposure to lettuce leaf lysates (51). *Salmonella* also chemotaxes toward lettuce root exudates (44) and movement of the pathogen up the xylem in *Arabidopsis thaliana* roots was eliminated and invasion decreased in flagella- and motility-minus mutants (24). However, within red ripe tomatoes (infected with *Salmonella* through shallow wounds), mutations in neither the *Salmonella flhDC* (master regulator of the flagellar regulon) nor *fliF* (resulting in a non-flagellated mutant with a functional motor) had an effect on competitive fitness (61). Additionally, *fliB* and the *fli* and *flg* operons, which code for flagellar synthesis in *Salmonella*, were downregulated during its colonization of soft rot lesions caused by *Dickeya dadantii* on cilantro and lettuce (34). Therefore, motility and chemotaxis are likely to be required during the early stages of the interactions of these enteric pathogens with plants, but not once they gain entry into plant tissues where nutrients may be plentiful. Flagella may also function as microbial-

associated molecular patterns (MAMPs) in induction of plant defenses since nonflagellated mutants of *Salmonella* had an increased endophytic fitness in alfalfa roots (39).

Salmonella genes STM0278 and STM0650, which are involved in multicellular surface spreading (“swarming”), were required for colonization of seedling surfaces (4). While it is not known how these genes affect swarming, it is tempting to speculate that swarming has other roles in addition to locomotion toward the preferred colonization sites in the rhizosphere (27,39). Differentiation of *Salmonella* into multicellular surface swarms is associated with global physiological changes (88), including increased resistance to antibiotics mediated by the *cysB* gene (83,84). Interestingly, *cysB* was differentially regulated inside tomatoes of different varieties, with the strongest expression of *cysB* in tomatoes of cv. Hawaii 7997, which is resistant to certain races of *Ralstonia solanacearum* (61). It is tempting to surmise that this enhanced resistance to the phytopathogen is at least partly mediated by plant basal defense antimicrobials that may have upregulated *cysB*.

The role of plant genotype in interactions with *Salmonella*. While scientific consensus on the issue of plant-associated gene regulation in human enteric pathogens is beginning to emerge, the role of plant genotype in *Salmonella*–plant interactions remains significantly less understood. Several research groups demonstrated that the outcome of interactions with *Salmonella* depends on the plant species and genotype (7,8,42,44,61). Indeed, internalization of *Salmonella* into plant tissue varies greatly among plant species (42) and crop colonization differs among cultivars of a given species (7,8,44). There was an approximately 100-fold difference in the phyllosphere populations of *Salmonella* on four tomato varieties, with *Solanum pimpinellifolium* variety WVa700 supporting the lowest number of bacteria (7). Of note, WVa700 was also significantly less susceptible to bacterial speck caused by *Pseudomonas syringae* pv. tomato (7). The plant genotype has an important role also in the proliferation of *E. coli* O157:H7 in the lettuce phyllosphere (64). These observations suggest that specific genetic factors pertaining to the plant response to microbial colonization drive the outcome of the interaction of enteric pathogens with plants, or that differences in physicochemical properties, such as availability of certain nutrients or surface morphology, associated with different crop genotypes impact proliferation of *Salmonella* and *E. coli* on and inside plants. These discoveries point to the potential of breeding for resistance to colonization by enteric pathogens, although the economic feasibility of breeding for resistance to these organisms is not yet clear (77). It remains unknown whether there is a correlation between plant basal immune responses to phytopathogens and to human

BOX 2

rdar phenotype. Under laboratory conditions on agar surfaces, most wild-type strains of *Salmonella* form rough and dry colonies, which can absorb Congo Red dye. This phenotype is known as “rdar”. It requires multiple regulatory inputs, which converge to control the production of cellulose and aggregative fimbriae (curl) (67). Mutations in any of the corresponding genes result in a variety of non-rdar phenotypes (e.g., bdar, saw, and pdar). Interestingly, non-rdar mutants are more common in collections of *Salmonella* isolates recovered from produce-related outbreaks than in those of clinical strains or those recovered from meats (76,93) (Fig. 2).

FIGURE 2

Phenotypes of *Salmonella enterica* isolates on a Congo Red-containing plate. The characteristic red wrinkled appearance (rdar phenotype) is seen in colonies of the wild-type *S. Typhimurium* 14028 (right, middle row, also see notations on the grayscale inset). *S. Newport* (from a tomato field on the Eastern Shore of Virginia, top left), two *S. Braenderup* isolates from clinical patients in a tomato outbreak (bottom left and top right corners, indicated with “B”), *Salmonella* Agona (left, middle row; “A”), Montevideo (center, “Mo”) and Michigan (middle, top row, “Mi”) are non-rdar. Spontaneous non-rdar mutants can arise when rdar strains are passaged through tomatoes, e.g., the two non-rdar spontaneous mutants (93) of *S. Typhimurium* 14028 in the forefront.



pathogens. Such a correlation would provide an opportunity to integrate breeding for increased basal resistance of crops to both plant and human enteric pathogens.

Evidence of *Salmonella* recognition by plants. Once *Salmonella* has gained entry into plant tissue, such as the leaf mesophyll, its presence in the plant apoplast may trigger sophisticated plant defenses aimed at inhibiting microbial multiplication and potential invasion by plant pathogens. There is increasing evidence that plants respond to *Salmonella* via basal defense pathways. In support of this hypothesis, transcriptome analysis of *Arabidopsis thaliana* leaves infiltrated with *E. coli* O157:H7 revealed that the human pathogen upregulated PAMP-inducible genes (82). Suppression of plant defense functions by phytopathogenic bacteria relies partly on type 3 secretion system (TTSS). Endophytic colonization of *Medicago* spp. roots by *Salmonella* was enhanced in mutants lacking flagella and SPI-1 TTSS indicating that when present, these bacterial surface components may be perceived by the plant, thereby inhibiting *Salmonella* colonization via activation of plant innate immunity (39). Shirron and Yaron (75) suggested that an increase in the oxidative burst of tobacco protoplasts during co-incubation with a *Salmonella invA* mutant (defective in SPI-1 TTSS) resulted from a lack of suppression of the tobacco defense response (75). In a similar fashion, *Salmonella* mutants in *invA*, *prgH*, *ssaV*, and *ssaI*, all of which are defective in SPI-1 or SPI-2 TTSS structures, showed reduced colonization of *Arabidopsis thaliana* leaves compared with the wild type, possibly due to a lack of plant defense suppression (73). Recently, it was reported that SseF, a TTSS effector of *S. enterica* can induce the hypersensitive response after transfer into tobacco by *Agrobacterium tumefaciens* or *Xanthomonas campestris* pv. *vesicatoria*, which may indicate a nonhost response by the plant basal defense (85).

The O-antigen of *Salmonella* Enteritidis is implicated in attachment and colonization of alfalfa sprouts (6). Comparative studies in *Arabidopsis thaliana* showed that the O-antigen also plays a role in eliciting a plant response since *Salmonella* serovars expressing this antigen (e.g., Senftenberg) caused leaf chlorosis and wilting; on the contrary serovars of a different serogroup did not induce such symptoms despite considerable colonization by all of the tested serovars after their infiltration into the leaves (11). Therefore, plants may sense the presence of human pathogens with pathways additional to those involved in recognition of common plant-associated microbes.

Although *Salmonella* can multiply in the apoplast and was observed intracellularly in *Arabidopsis thaliana* protoplasts and in tobacco cultured cells (72,75), its ability to infect intact cells of whole living plants has not been demonstrated. However, as implied above, there is increasing evidence that plants have the ability to recognize enteric pathogens, including their MAMPs with basal defense signaling pathways. A defense-related PR1 protein was upregulated after inoculation with *Salmonella* in both *Arabidopsis thaliana* and lettuce (39,44). Salicylic acid-dependent and -independent plant defenses were triggered by flagella and components of the TTSS (39). In this Focus Issue, Roy et al. (69) report that *E. coli* O157:H7 induces greater levels of expression of the plant defense gene PR1 in *Arabidopsis thaliana* leaves than *Salmonella* (69). They additionally corroborate previous findings by Kroupitski et al. (50) that *Salmonella* triggers weak stomatal closure in lettuce and provide evidence of a stronger stomatal immunity against *E. coli* O157:H7 (50). This weaker immune response to *Salmonella* compared with that to *E. coli* O157:H7 may explain their finding that *Salmonella* has a greater ability to colonize the leaf apoplast.

Exposure of *Arabidopsis thaliana* to *Salmonella* Typhimurium 14028 and *E. coli* elicited measurable and temporally distinct transcriptomic responses in the plant. In total, 114 plant genes ($\approx 10\%$ of those activated in response to *Salmonella*) were acti-

vated in response to *Salmonella* at 2 and 24 h after inoculation (73). One hundred and sixty *Arabidopsis thaliana* genes were commonly up-regulated in response to *Salmonella*, *E. coli* K12, and *P. syringae*; however, the magnitude of specific responses to *Salmonella* or *E. coli* was significantly (50 to 100 \times) less than to *P. syringae* (73). In another study, inoculation of *Arabidopsis thaliana* with *E. coli* O157:H7 elicited responses that are distinct from those elicited by the plant pathogen *P. syringae* pv. tomato DC3000, but similar to those elicited by its attenuated mutants (82). The latter included genes belonging to hormone and stress response pathways with two exceptions: genes encoding a jasmonic acid methyl transferase and a putative anthocyanidin synthase, which were up-regulated only in response to TTSS mutants of DC3000 (82). These observations suggest that plants recognize and respond to enteric pathogens as "general" endophytes, and the defenses mounted by plants in response to phytopathogens or symbionts are distinct from those elicited by these human enteric pathogens.

Proliferation of *Salmonella* and *E. coli* O157:H7 in some plant tissues has been reported to cause disease-like symptoms. For example, root inoculation of lettuce with 10^5 cells of *Salmonella* Dublin stunted growth of the seedlings and led to a modest reduction in plant biomass upon extended cultivation (>12 days) (44). In *Arabidopsis*, immersion of seedlings into a dense suspension of *Salmonella* or infiltration of leaves with the pathogen can elicit chlorosis, wilting, or tissue necrosis (11,72). Infiltration of the wild-type *Salmonella* into *Arabidopsis* leaves elicited chlorosis to the same extent as the infiltration of $MgCl_2$ solution; however, lesions elicited by *Salmonella* SPI-1 and SPI-2 mutants were approximately twice as large as the controls (73). The appearance of plant disease symptoms in *Arabidopsis* was associated with the ability of *Salmonella* to overcome jasmonate-mediated plant defenses (72), whereas studies by Iniguez et al. (39) implicated Salicylic acid-induced defense pathways in the response of *Arabidopsis* to *Salmonella* (39). These findings suggest that the human pathogen is recognized by plants and that general host defenses are induced, although the physiological consequences of these defenses are not consistent from one study to another and need to be better understood.

Interactions of *Salmonella* with phyto bacteria. In animal studies, the ability of *Salmonella* to colonize the intestine is greatly dependent on its success in becoming established among the host gut microflora, either by manipulating the host's physiology or by utilizing nutrients that are not used efficiently by the native microbes (80,91). Several recent studies have also explored potential mechanisms used by *Salmonella* to interact with members of the native plant-associated microflora.

The importance of phyto bacteria in the persistence of human enteric pathogens on plants first came to light from supermarket produce surveys that demonstrated that 60% of produce showing symptoms of soft rot also harbored presumptive *Salmonella* (89). Later laboratory studies revealed that plant tissue macerated by pectinolytic pathogens such as *D. dadantii* (*Erwinia chrysanthemi*) and *Pectobacterium carotovorum*, promoted growth of *Salmonella* Typhimurium and *E. coli* O157:H7 to population densities approximately 10 times greater than on healthy plants; the sudden increase in proliferation of the human pathogens coincided with the appearance of soft rot symptoms (15,34,62,92) (Fig. 3). Transcriptomic studies by Goudeau et al. (34) revealed that *Salmonella* cells colonizing lettuce and cilantro leaf soft rot lesions caused by *D. dadantii* utilize a broad range of nutrients made available through the pectinolytic activity of the plant pathogen (34). These include fucose and rhamnose, two substrates for the production of propanediol, which along with ethanolamine, can serve as carbon sources under anaerobic conditions (34). Propanediol utilization is required for *Salmonella* replication in macrophages and colonization of the chicken lumen (37,45).

Ethanolamine utilization confers a competitive advantage onto *Salmonella* in the lumen of the inflamed intestine in the mouse colitis model (81). Commonalities between soft rot lesions and the host intestine such as anaerobic conditions and nutritional resources indicate an important overlap in ecological niche and may explain the adaptation of *Salmonella* to macerated leaf tissue (34).

Biotrophic plant pathogens, like *P. syringae* and *Xanthomonas campestris*, were also shown to promote growth or survival of *Salmonella* and enterohaemorrhagic *E. coli* on plants (2,3,9). Formation of lesions on leaves by both these phytopathogens was associated with an increase in availability of total sugars, specifically, inositol and sucrose (3). While it is tempting to speculate that the increased leakage of these compounds favors proliferation of the human pathogens at the lesion sites, other substrates and factors may also be involved since *E. coli* O157:H7 is unable to utilize inositol, and most *Salmonella* serovars are unable to utilize sucrose. Furthermore, the increase in the availability of these carbon sources in plant lesions caused by *X. campestris* did not account fully for the increased proliferation of *Salmonella* and EHEC in the lesions caused by either *X. campestris* or *P. syringae* (3). An increase in growth similar to that observed in response to the biotrophic phytopathogens was observed on lettuce leaves that were mechanically damaged or showed symptoms of tip burn (dry lesions on leaf margins resulting from a physiological disorder) (2,15).

Phyllosphere bacterial communities are diverse, both functionally and structurally. In addition to erwinias, xanthomonads, and pseudomonads, which are ubiquitous on leaf surfaces, *Salmonella* may reside with closely related coliforms that are frequently present on plants, and with β - and α -proteobacteria, firmicutes, bacteroidetes, and actinobacteria (56,65). Metagenomic studies revealed that the decreased abundance in ready-to-use carbon and ammonium in a biofilm composed of spinach epiphytes likely resulted in increased competition of the enteric pathogen *E. coli* O157:H7 with other spinach leaf microbes capable of converting unavailable C and N to their bio-available forms (22). Competition for nutrients with members of the Enterobacteriaceae appeared to significantly reduce the fitness of *Salmonella* and *E. coli* O157:H7 in plant-associated ecological niches (23,24,56). In contrast, the presence of a member of the *Burkholderiales* that utilizes different carbon sources than *Salmonella* does modestly increase proliferation of *E. coli* O157:H7 in the lettuce phyllosphere (23). A similar trend was observed with other phytopathogens that stimulated growth of *E. coli* O157:H7 in vitro and in planta (56). In leaf tissue macerated by *D. dadantii*, a phytopathogen belonging to the Enterobacteriaceae, *Salmonella* underwent high growth rates and its populations sizes were highly correlated with those of the soft rot pathogen throughout disease

development (34). Goudeau et al. (34) suggested that this apparent lack of competition with the plant pathogen stems from the extensive activity of the *Salmonella* propanediol catabolic pathway, along with the synthesis of its co-factor, cobalamin, which are both absent in *D. dadantii* (34). Thus, although *D. dadantii* makes the necessary substrates for propanediol synthesis and catabolism available to *Salmonella* through pectinolysis, the plant pathogen itself utilizes the oligogalacturonides released from the plant cell wall, thereby creating a nutritional environment with resources partitioned for both bacterial species.

Competition for nutrients is unlikely to be the sole mechanism by which enteric pathogens can be excluded from, or become minor members of, plant-associated bacterial communities (56). On plant surfaces, they may be exposed to phages and antibiotic-producing phytopathogens. For example, a strain of *P. syringae* (with previously demonstrated fungicidal activities) reduced growth of *E. coli* O157:H7 on wounded apples by 10- to 1,000-fold (43). These discoveries led to experiments on biological control of human enteric pathogens in produce, such as those by Fett (30) who showed that a well-characterized biocontrol strain of *Pseudomonas fluorescens* 2-79 effectively reduced *Salmonella* populations on alfalfa sprouts (30). Likewise, bacteriophages can considerably reduce the contamination of various produce with enteric pathogens (78). This suggests that phages and known biocontrol bacteria may be useful as potential tools for controlling enteric pathogens throughout the produce production cycle. It is important, however, to consider that zero tolerance for most human pathogens on fresh fruit and vegetables implies that even the most effective biocontrol agent would need to be integrated as one of several hurdle technologies in a general control strategy.

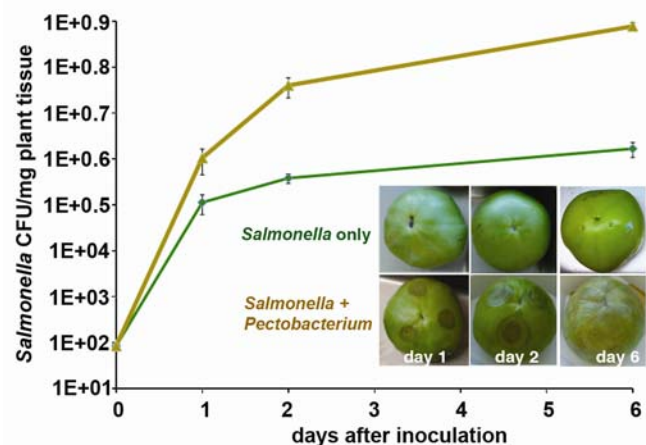
Besides mechanisms of metabolic cooperation or competition between phytopathogens and *Salmonella*, cell-to-cell signaling in multi-species microbial consortia on plants may also occur. The contribution of signaling via quorum sensing circuits mediated by either *N*-acyl homoserine lactones (AHL) or the autoinducer-2 (AI-2) signal to the behavior of *Salmonella* in plant-associated bacterial communities has been tested. Even though the *Salmonella* AHL receptor encoded by *sdiA* was involved in the responses of this bacterium to AHLs from phytopathogens (Box 3), in vivo expression technology and fitness studies conclusively demonstrated the lack of the role for SdiA and its regulon in interactions with pectobacteria in soft rots on several crop species (62). Despite the fact that the *Salmonella luxS* gene was expressed during its invasion of a soft rot, AI-2-based signaling in *Salmonella* did not appear to have an important role during its interactions with the plant pathogen *P. carotovorum* on tomato fruit, as demonstrated by Cox et al. (25) in this Focus Issue.

Salmonella has the ability to form single- and mixed-species aggregates in the phyllosphere (18,19). As reported by Poza-

FIGURE 3

Soft-rot bacteria promote proliferation of *Salmonella* in plants. An increased proliferation of *Salmonella* and *Escherichia coli* in plants infected with soft-rot bacteria has been observed in the market place (89) and under laboratory conditions (15,19,34,62).

In this experiment (J.T. Noel, unpublished data), *Salmonella* Typhimurium 14028 (≈ 100 to 500 cells) was co-inoculated with ≈ 3 million cells of hypervirulent *Pectobacterium carotovorum* SR38 by injection into the tomato pericarp and incubated at 22°C. For enumeration, tomatoes were macerated in phosphate-buffered saline and dilution-plated onto xylose lysine deoxycholate agar. The brown and green lines represent the growth of *Salmonella* with and without *Pectobacterium* soft rot, respectively. Inset: appearance of representative tomatoes throughout the experiment.



Carrion et al. (63) in this Focus Issue, aggregates formed by common epiphytes affect the fitness of the human pathogen in the phyllosphere. *Salmonella* cells that landed in preexisting aggregates of *P. syringae*, *P. fluorescens*, and two *Erwinia* species had a greater probability of surviving dry conditions on lettuce and cilantro leaves than as solitary cells (63). These observations suggest that human pathogens may find refuge not only in particular physical microsites on plants but also in microbial conglomerates where protection from adverse conditions outweighs potential competition and antibiosis from other plant colonists. In general, *Salmonella* and EHEC appear to be successful secondary colonists, benefiting from the action of phytopathogens, e.g., suppression of plant defense and plant tissue damage (lesions, water soaking, and soft rots). The ability to outcompete or to avoid nutrient competition with phytopathogens, and to resist the action of antimicrobials released by phytopathogens would be important in determining the outcome of the interactions of these enteric bacteria with plant-associated microorganisms.

Interactions of *Salmonella* with plant-associated protists.

Within human hosts, *Salmonella* utilizes sophisticated systems to invade nonphagocytic cells, and establish the *Salmonella*-containing vacuole (SCV), which it exploits for survival and replication (31). In the environment, *Salmonella* predation by protists results in its entrapment in a food vacuole where it experiences

conditions that overlap with those in the SCV (66). The human pathogen *Legionella pneumophila* is known to interact with *Acanthamoeba* and survives in its cysts, a process that increases the infectivity of *Legionella* (71). *Salmonella* also appears to resist digestion by *Acanthamoeba* and certain ciliates commonly present in agricultural soils and on preharvest and postharvest vegetables, which may serve as additional environmental reservoirs (20,35,79). Tolerance of *Salmonella* to digestion in *Tetrahymena* vacuoles results in its excretion in the protist's fecal pellets where the human pathogen survives at greater rates than as single cells (20). The release of fecal pellets containing intact *Salmonella* cells was observed on plants in the laboratory (35). Passage through *Tetrahymena* induces a large number of regulatory changes affecting between 989 and 1,282 genes or approximately 25% of the *Salmonella* genome (66). Many genes that were differentially regulated are involved in anaerobiosis, virulence, stress response (oxidative, osmotic, acid, and antimicrobial stress, as well as SOS response), indicating similarities in the physiology of *Salmonella* cells residing in *Tetrahymena* vacuoles to those in macrophages and epithelial cells. The acid resistance genes, *adiA* and *adiY*, were strongly upregulated and played a role in *Salmonella* resistance to digestion by *Tetrahymena* (66). Rehfuß et al. (66) reported that the induction of acid stress response genes in *Tetrahymena* vacuoles imparts an enhanced resistance to subsequent acid stress upon *Salmonella*, and suggested that it may improve the pathogen's ability to survive the acidic stomach pH of its hosts. Such pre-adaptation may reduce the infectious dose of *Salmonella* in humans.

The passage of *Salmonella* through the amoeba *Acanthamoeba polyphaga* is associated with survival within contractile vacuoles, a process that relies on the *sseC*, *ssaU*, and *phoP* genes (13,79). These genes are part of SPI-2, which is responsible for intracellular replication in macrophages. Once established within the contractile vacuole, the bacteria entered logarithmic growth producing a population of over 200 cells that were able to persist for at least 4 days (33). The surviving *Salmonella* are subsequently able to multiply on the amoeba's waste products. Passage also induces a filamentation response which appears to provide protection from predation, although the mechanisms involved are unclear.

Interactions of *Salmonella* with plant-associated fungi. Fungi are prevalent members of plant microbial communities. Thus, it is highly likely that *Salmonella* encounters and interacts with fungi during its residence on plants. Various types of interactions between bacteria and fungi, ranging from antagonistic to beneficial, have been described (46). The attachment of *Salmonella* cells to fungal species in the cilantro phyllosphere has been observed (14). Furthermore, laboratory studies demonstrated the formation of large and dynamic *Salmonella* biofilms on *Aspergillus niger*, a common colonizer of plant surfaces, whereas *E. coli*, *Pseudomonas agglomerans*, and *Pseudomonas chlororaphis* were unable to attach to the fungus and produce biofilms (17). Differences in colonization of *Aspergillus* were mirrored by differential binding of the bacterial species to chitin, an important component of fungal cell walls. Cellulose production in *Salmonella* was identified as the attachment factor mediating this relationship. It remains unclear whether *Salmonella* benefits directly from its association with *Aspergillus*, or other fungi, but it seems probable that the hyphae may vector the attached bacteria to new habitats or that their exudates provide additional nutrients to the human pathogens.

Fungi may also benefit human pathogens through habitat modification. Co-inoculation of tomato, potato, and onion tissue with *Salmonella* and *Rhizopus* caused a significant increase in *Salmonella* population sizes compared with its inoculation alone (90). Similarly the postharvest fungal pathogens *Alternaria alternata* and *Cladosporium* spp. enhanced the growth of *Salmonella* in

BOX 3

Peculiarities of *Salmonella* quorum sensing (QS).

Similarly to most Gram-negative bacteria, *Salmonella* can detect certain N-acyl homoserine lactones (AHL) and autoinducer-2. Exceptionally, the *Salmonella* AHL receptor, SdiA, does not have a cognate AHL synthase, nor does *Salmonella* (or its close relatives *E. coli*, *Enterobacter*, *Citrobacter*, *Chronobacter*, and *Klebsiella*) produce AHLs (1). Phylogenetic analyses revealed that SdiA likely originated from the *Pseudomonas* RhlR, which was horizontally acquired as the *rhlR-rhlI* cluster by the common progenitor of enterics, including *Salmonella*, *Erwinia*, and *Pantoea*. The *rhlR-rhlI* cluster further evolved to *expR-exlI* (*phzR-phzI*) within this common progenitor, while *Salmonella*, *E. coli*, *Enterobacter*, *Citrobacter*, *Chronobacter*, and *Klebsiella* lost the AHL synthase gene (70), but retained the AHL receptor, SdiA. In all these organisms, SdiA is encoded upstream of the *gacA* ortholog. In *Salmonella*, the ability of SdiA to regulate the downstream genes in the presence of AHLs is temperature-dependent. Unlike phytopathogens, where AHL-mediated QS controls a number of genes involved in virulence, SdiA upregulates less than a dozen genes whose functions are currently unknown (1).

Salmonella possesses a second potential signaling system based on the AI-2 molecule produced via the synthase LuxS. *Salmonella* receives the signal via the *lsrACDBFG* operon, an ABC transporter with homology to the *rls* ribose transporter of *E. coli*. The sole known function of the *lsrACDBFG* operon in *Salmonella* is the uptake and processing of AI-2. However, the primary role of *luxS* appears to be degradation of toxic intermediates in the activated methyl cycle (AMC), which makes distinguishing between metabolic changes related to the *luxS* genotype and those resulting from AI-2 signal exchange difficult. Microarray studies have shown that only a small portion of the *luxS*-responsive genes (7.9% in *Salmonella*, 1.9% in *E. coli* and 9.2% in *Streptococcus mutans*) also respond to exogenous AI-2 and an inability of exogenous AI-2 to rescue the *luxS* mutation in *Salmonella*. This underscores the complexity and uncertainties associated with role of *luxS* in AI-2 signaling in *Salmonella*.

ripe tomato fruit, likely via alkalization of the plant tissue resulting from their proteolytic activity (86,87). Hence it appears that fungi may not only provide enhanced access to growth substrates by degrading the plant tissue but may additionally reduce environmental stresses that inhibit *Salmonella*.

CONCLUSIONS

Outbreaks of gastroenteritis linked to the consumption of fresh fruits and vegetables have provided the rationale for investigating the biology of *Salmonella* on plants. Numerous studies in this new multidisciplinary field of research have yielded important discoveries that continue to challenge the dogma that *Salmonella* is best defined as an enteric colonist. Key studies, including those in this Focus Issue, point to the ability of this human pathogen to interact with plants and with the plant-associated microflora. It is clear that *Salmonella* can sense subtle environmental cues brought about by the genotype or physiological state of its plant host and respond with distinct patterns of gene expression accordingly. Plants also recognize *Salmonella* and activate basal defenses in response to the human pathogen when at high densities and in close contact with plant cells in the apoplast. It is still unclear, however, whether *Salmonella* is a clever opportunist that shows sufficient versatility under rare conditions in the plant environment to proliferate to infectious doses, or if its behavior on plants results from an evolutionary adaptation to use plants as an important vector to infect vertebrate hosts through their dietary intake.

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