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# Requirement of Siderophore Biosynthesis for Plant Colonization by *Salmonella enterica*

**Ling-yun Hao,[a] David Kyle Willis,[a,b] Helene Andrews-Polymenis,[c] Michael McClelland,[d,e] and Jeri D. Barak[a]**

Department of Plant Pathology, University of Wisconsin—Madison, Madison, Wisconsin, USA[a]; Vegetable Crops Research Unit, Agricultural Research Service, United States Department of Agriculture, Washington, DC, USA[b]; Department of Microbial and Molecular Pathogenesis, College of Medicine, Texas A&M University System, College Station, Texas, USA[c]; Vaccine Research Institute of San Diego, San Diego, California, USA[d]; and Department of Pathology and Laboratory Medicine, College of Health Sciences, University of California—Irvine, Irvine, California, USA[e]

**Contaminated fresh produce has become the number one vector of nontyphoidal salmonellosis to humans. However, *Salmonella enterica* genes essential for the life cycle of the organism outside the mammalian host are for the most part unknown. Screening deletion mutants led to the discovery that an *aroA* mutant had a significant root colonization defect due to a failure to replicate. AroA is part of the chorismic acid biosynthesis pathway, a central metabolic node involved in aromatic amino acid and siderophore production. Addition of tryptophan or phenylalanine to alfalfa root exudates did not restore *aroA* mutant replication. However, addition of ferrous sulfate restored replication of the *aroA* mutant, as well as alfalfa colonization. Tryptophan and phenylalanine auxotrophs had minor plant colonization defects, suggesting that suboptimal concentrations of these amino acids in root exudates were not major limiting factors for *Salmonella* replication. An *entB* mutant defective in siderophore biosynthesis had colonization and growth defects similar to those of the *aroA* mutant, and the defective phenotype was complemented by the addition of ferrous sulfate. Biosynthetic genes of each *Salmonella* siderophore, enterobactin and salmochelin, were upregulated in alfalfa root exudates, yet only enterobactin was sufficient for plant survival and persistence. Similar results in lettuce leaves indicate that siderophore biosynthesis is a widespread or perhaps universal plant colonization fitness factor for *Salmonella*, unlike phytobacterial pathogens, such as *Pseudomonas* and *Xanthomonas*.**

During the past decade, salmonellosis caused by nontyphoidal serovars of *Salmonella enterica* has been among the most common bacterial food-borne illnesses in the United States. Historically, animal products were the primary source of salmonellosis. Today, more outbreaks are traced to the consumption of contaminated fresh produce, such as sprouted seeds, leafy greens, and fruit (10). Fresh produce contamination likely occurs preharvest through manure application or use of contaminated irrigation water (18). Alfalfa plants fertilized with uncomposted chicken manure and irrigated with nonpotable canal water produced seeds that tested positive for *S. enterica* and were implicated in a salmonellosis outbreak (36). Seed contamination is particularly important, since during seed germination and seedling growth, nutrients become readily available for the microbes inhabiting the seed. Seedlings, referred to as sprouts by consumers, are the most common fresh produce vehicle to cause food-borne illness (10).

Germinating seeds and their subsequent sprouts are unique among fresh produce in that *S. enterica* populations can grow quickly and extensively, with populations reaching $10^7$ CFU/g of fresh weight (11, 54). Growth requires the bacterium to either synthesize indispensable metabolites or acquire essential nutrients from its environment. Germinating seeds and the subsequent roots release soluble and insoluble products into the rhizosphere via root exudates (7). The root exudate distribution and composition vary based on many factors, including plant age, different root zones, nutrition status, and soil types. These factors can shape the microbial community associated with the root system, as bacterial species differ in their abilities to utilize and compete for various nutrient sources (24, 42). *In vitro* studies have revealed the preference of *S. enterica* for the root over the seedling phyllosphere, most likely due to the more readily available nutrients from root exudates (3, 11). The numerous sprout-implicated outbreaks are a testament to *Salmonella*'s ability to inhabit and use plants, seedlings in these cases, as vectors to humans (54).

In the absence of seed contamination, *S. enterica* in the environment can sense available plant nutrients and use these signals to actively move toward plants for subsequent attachment and colonization (4, 30). *S. enterica* moved by chemotaxis toward lettuce root exudates later characterized as a sugar-like carbon (30). This sugar-like carbon was the first plant-derived chemoattractant of a human bacterial pathogen. In a mixed plant environment, root exudates may be chemoattractant cues to facilitate colonization of preferential plant hosts (4). Roots of spinach plants directly seeded into *S. enterica*-contaminated soil were more likely to be contaminated as the plants grew and their roots developed (1). Plant-derived chemoattractants of human bacterial pathogens are not limited to root exudates. Photosynthates produced in leaves attract *S. enterica* to enter stomates (31). Plant nutrients not only play a role in the survival and growth of human-pathogenic bacteria, but also function as signals for these bacteria to locate and exploit preferential colonization niches (1, 30, 31).

*S. enterica* can use plants as vectors to complete its life cycle and return to animal hosts. To successfully exploit these vectors, *S. enterica* explores plant niches for nutrient acquisition (30, 31); however, the metabolic networks necessary to colonize plants remain to be determined. In this study, we documented the neces-

Received 14 December 2011 Accepted 9 April 2012
Published ahead of print 20 April 2012
Address correspondence to Jeri D. Barak, barak@plantpath.wisc.edu.
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doi:10.1128/AEM.07867-11



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

| Strain or plasmid | Genotype or relevant characteristics | Source or reference |
|---|---|---|
| Strains | | |
| 14028s | Wild type | ATCC |
| LT2 | Wild type | ATCC |
| JDB812 | 14028s Δ*aroA*::*kan* | This study |
| JDB890 | 14028s Δ*aroA*::*kan* complemented with pJDB5 | This study |
| JDB785 | 14028s Δ*entB*::*kan* | This study |
| DM10438 | LT2 Δ*entB*::MudJ | Diana Downs |
| JDB859 | 14028s Δ*iroB*::*kan* | This study |
| SA2438 | LT2 Δ*trpB76*::*kan* | Diana Downs |
| JDB818 | 14028s Δ*trpB*::*kan* | This study |
| DM1041 | LT2 *phe*::MudJ | Diana Downs |
| DM11000 | LT2 KTHLT2 | Diana Downs |
| Plasmids | | |
| pJDB5 | pGEM::*aroA*; 1,747-bp fragment containing *aroA* cloned from 14028s using primers *aroA* For and *aroA* Rev, inserted into pDrive | This study |
| pKD3 | *bla* FRT *cat* FRT PS1 PS2 ori6K | 14 |
| pKD4 | *bla* FRT *aph* FRT PS1 PS2 ori6K | 14 |

sity of *S. enterica* replication for plant colonization in leaves and on roots. We discovered that chorismic acid biosynthesis and downstream metabolic products, such as aromatic amino acids and enterobactin, are fitness factors for *S. enterica* plant colonization. Additionally, we characterized the roles of salmochelin and enterobactin, siderophores produced by *S. enterica*. Understanding the nutritional requirements of *Salmonella in planta* may reveal specific strategies utilized by this enteric pathogen to persist outside its animal hosts.

## MATERIALS AND METHODS

**Bacterial strains, media, and culture conditions.** *S. enterica* serovar Typhimurium strain 14028s and the isogenic mutant derivatives used in this study are described in Table 1. All strains were routinely grown on LB plates, and the following antibiotics were added if needed: kanamycin (50 μg/ml) and ampicillin (100 μg/ml). *Salmonella-Shigella* (SS) agar (Difco), a *Salmonella* semiselective indicator medium, was used to enumerate *S. enterica* populations. M9 minimal medium with glucose was prepared as described previously (48). Swarming motility plates were composed of LB with 0.5% (wt/vol) glucose (LBG) and 0.5% (wt/vol) agar, and swimming motility plates were composed of LB with 0.3% (wt/vol) agar. Swarm induction was verified by the presence of hyperflagellated cells. The number of flagella per swarm cell was determined following flagellar staining as described previously (2). Tryptophan and ferrous sulfate were added to final concentrations of 100 μM and 10 μM, respectively.

**Generation of deletion mutants and complementing plasmids.** The *aroA* mutant originally screened for plant colonization was from a deletion library generated by the λ Red recombinase method in *S. enterica* serotype Typhimurium ATCC 14028 (49). The *iroB* mutant listed in Table 1 was constructed with the gene of interest replaced by a FLP recombination target (FRT)-flanked kanamycin resistance cassette in an *S.* Typhimurium strain LT2 background (14). To obtain isogenic strains and reduce the possibility of secondary mutations, each mutation was transduced with phage P22 into the 14028s background (22). The *entB*, *phe*, and *trpB* deletions were transduced from the LT2 strains listed in Table 1 with phage P22 into the 14028s background. All transductants and mutants were confirmed by PCR analysis using gene-flanking primer sets (Table 2). To confirm the nutritional phenotypes of the *entB*, *phe*, and *trpB* mutants, ferrous sulfate, phenylalanine, or tryptophan, respectively, was added to the surface of an M9-glucose minimal medium plate. Each mutant was inoculated onto the appropriate plate and grew only when the limiting nutrient was present. To construct the *aroA* complementing plasmid, the *aroA* gene was PCR amplified from *S.* Typhimurium strain

**TABLE 2** Primers used in this study

| Primer | Sequence (5′–3′) | | Efficiency[a] |
|---|---|---|---|
| | Forward | Reverse | |
| Mutational analysis | | | |
| *aroA* | TACGCCGCCGACTTTCGCCT | CCGTCGACTGGCGCAACAGA | |
| *entB* | CAGCGGTTGGCGTCACGTTC | GATCGAAGCCGATCACCCGCG | |
| *trpB* | CTGGGTCTGAAATGCCGTAT | ACCACCAGCAGTTGCTCTTT | |
| *iroB* | ACGCGCGTAAATAAGCCTGAAACC | CACGCGGGCAAGGCGAACTAT | |
| T7 | TAATACGACTCACTATAGGG | | |
| SP6 | ATTTAGGTGACACTATAGAA | | |
| *iroB*-d1 | ATGCGTATTCTGTTTGTCGGTCCACCACTGTATGGAC TGCGTGTAGGCTGGAGCTGCTTC | | |
| *iroB*-d2 | TTAGCCGTGTTGCAGCATGGCGATCAGTTTTTTTGC CACCCATATGAATATCCTCCTTAG | | |
| RT-qPCR | | | |
| *iroB* | GTCAGGAGGCATTACGAA | CGGGTCAGTCATCTCTTC | 95.4 |
| *entA* | TTTGTTTCTTGCCTCCGAT | GGCTCCCAATGTTGAACC | 83.2 |
| *entF* | CATCATACCGCTTACATCATT | TTGACTATCGCCGTTTGT | 94.9 |
| *gapA* | GAAACTGGTCTCCTGGTA | TTGGAGATGTGAGCAATC | 96.4 |
| *dnaN* | GATTTCCCGAATCTTGAC | AACAGCATACCGTTTAAG | 102.0 |
| *gyrB* | CGTTTGTTGAATATCTGAA | GGAATGTTGTTGGTAAAG | 89.3 |
| *rplU* | GAGTTCGCTGAAGTTCTGA | TTTGATTACGCCGCCATC | 98.5 |
| *rpoD* | GTGGATACCGTCTTATGG | GATAGCCTTGCTCCTTAC | 91.4 |
| *rpsL* | GCGTATGTACTCGTGTATA | TTCAAAACCGTTAGTCAGA | 97.1 |

[a] Primer efficiencies were determined with the MyiQ Cycler software from a standard dilution curve of *S.* Typhimurium chromosomal DNA as described in Materials and Methods.

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14028s genomic DNA using the PCR Master Mix (Promega) and primers *aroA* For and *aroA* Rev (Table 2). The product was ligated into the pGEM-T easy cloning vector (Promega) according to the manufacturer's instructions and sequenced by using primers T7 and Sp6 to verify the cloning of *aroA* (Table. 2). Sequences were compared to the published sequences of *S.* Typhimurium (GenBank accession no. NC_003197) by blastn.

**Alfalfa root colonization assays.** Alfalfa seeds were surface sterilized with 3% calcium hypochlorite as previously described (11) and used to test the alfalfa attachment and colonization phenotypes of *S.* Typhimurium wild-type (WT) and isogenic mutant strains. Bacteria were grown on solid media, suspended in sterile water with a sterile swab to an optical density at 600 nm ($OD_{600}$) of 0.2, and diluted to a concentration of $\sim 10^5$ CFU/ml. The titers of cultures used to inoculate plants were verified by quantitative plating on SS agar. For individual inoculation experiments, 20 ml of the bacterial inoculum was added to 0.3 g of alfalfa seeds in a petri plate. Each bacterial suspension was replaced with 20 ml of sterile water after 1 h, and the water was changed again at 24 h postinoculation (p.i.). Tryptophan and ferrous sulfate were added each time the water was changed. Seeds were germinated at 25°C with constant shaking at 40 rpm for 48 h. For coinoculation assays, two different bacterial strains ($\sim 10^5$ CFU/ml of each) were inoculated into a single petri dish at equal volumes (10 ml each). To determine bacterial populations, 10 alfalfa seeds with seed coats were sampled 1, 4, or 24 h p.i., or 10 seedlings without seed coats were sampled at 48 h p.i. Each seed or seedling was rinsed individually in 1 ml of sterile water for 30 s and then homogenized with a 4.8-V rotary tool (Dremel, Mount Prospect, IL) in 500 μl of sterile water. The homogenate of each sample was dilution plated on SS agar, and the plates were incubated at 37°C overnight. For coinoculation assays, the homogenate for each seedling was dilution plated on both SS agar, to determine the total populations of both bacterial strains, and SS agar with kanamycin, to determine the population of the mutant. All experiments were performed on three separate occasions.

***In planta* growth assay.** Bacterial growth in alfalfa root exudates was determined by quantification of the planktonic- cell population, i.e., those cells found in the seedling irrigation water that were not attached to any plant surface. Surface-sterilized alfalfa seeds were incubated with a bacterial suspension of $\sim 10^3$ CFU/ml as described above for 36 h without water change at room temperature ($\sim$25°C) with constant shaking. Samples were taken at 0, 12, 24, and 36 h p.i. and dilution plated on SS agar for a kinetic growth curve or at 40 h p.i. for an endpoint growth curve. Three replicates were included for each strain, and each replicate was sampled twice. All experiments were repeated three times.

**CAS assay.** Chrome azurol S (CAS) agar plates were used to detect siderophore production of *S.* Typhimurium strains (52). Bacteria were streaked for single colonies on CAS agar and incubated at 37°C overnight. Siderophore-producing strains were confirmed based on the observation of a color change of CAS from blue to orange resulting from removal of iron from the dye (39).

**Motility assays.** The motility of *S.* Typhimurium strains was tested using swarming or swimming plates, as described previously, with modification (2). Overnight bacterial cultures grown on LB agar were suspended in sterile water to reach an $OD_{600}$ of $\sim$1.5 or $\sim$0.5 for swarming and swimming assays, respectively. Twenty microliters of the bacterial suspension was spotted in the middle of a swarming plate, or 10 μl on a swimming plate, and the spot was allowed to dry for 1 h at room temperature. All plates were incubated at 28°C for 15 to 20 h for swarming assays or 25 h for swimming assays. The swarming or swimming distance was measured from the center of the original inoculation spot to the furthest distance that bacteria were visible. To determine if swarming deficiencies were related to a lack of iron or surfactant, 10-, 50-, or 90-μM filter-sterilized ferrous sulfate or *Bacillus subtilis* surfactin (2.5 mg/ml) was added to the LBG plates, and the swarming distance was measured as described above. Five replicates were included for each strain for the swarming assay and 10 replicates for the swimming assay. All experiments were repeated on at least two separate occasions.

**RNA purification and real-time qPCR.** Real-time reverse transcriptase quantitative PCR (real-time RT-qPCR) was performed following the MIQE guidelines (9). RNA was isolated from mid-exponential-phase cells ($OD_{600}$ = 0.4 to 0.5) of *S.* Typhimurium strain 14028s cultured in M9 glucose minimal medium at 28°C, in alfalfa root exudates at room temperature ($\sim$28°C), or in the two above-mentioned environments supplemented with ferrous sulfate at a final concentration of 10 μM. RNAprotect (Qiagen) was added to each sample. RNA was purified using the RNeasy Mini Kit (Qiagen), and contaminating DNA was removed with Turbo DNA-*free* using the stringent protocol (Ambion, Inc.). We synthesized first-strand cDNA from 1 μg of total RNA using the iScript cDNA synthesis kit and following the manufacturer's protocol (Bio-Rad Laboratories, Inc.). Total RNA was quantified using microspectrophotometry (Nano-Drop Technologies, Inc.). RNA integrity was evaluated by microfluidic capillary electrophoresis using the Experion automated electrophoresis system (Bio-Rad Laboratories). Removal of DNA from the RNA samples was confirmed by performing real-time qPCR on 100 ng of total RNA using the *rplU* primer set (Table 2), but without a reverse transcriptase step (no-cDNA control). Those RNA samples found to yield quantification cycle ($C_q$) values larger than 32 were judged to be sufficiently free of contaminating DNA for further analysis. Purified RNA was converted to cDNA immediately or stored frozen at −80°C.

Primers were designed using Beacon Designer software (Premier Biosoft International), a program designed to generate primer pairs suitable for real-time PCR. The SYBR green module with the "avoid template structure" setting was chosen to limit primer sequences to regions with little secondary structure. Primers were obtained from Integrated DNA Technologies (IDT) and are listed in Table 2. Primer pair amplification efficiencies were determined using a serial dilution of *S.* Typhimurium genomic DNA with the percent efficiency (Table 2) determined by the MyiQ software (Bio-Rad Laboratories, Inc.). Suitable internal reference gene primers were chosen on the basis of two criteria: (i) primer efficiencies close to 100% and (ii) stable expression within wild-type and mutant samples. The stability of reference transcripts was validated using the BestKeeper program (44). We evaluated 6 *S.* Typhimurium genes (*gapA*, *dnaN*, *rplU*, *rpoD*, *rpsL*, and *gyrB*) for production of stable reference transcripts under our growth conditions. All transcripts except *gapA* were found to be stably expressed. We used 2 of these 5 transcripts, *rpsL* and *rplU*, to normalize expression of our target transcripts.

Real-time RT-qPCR experiments were performed using a 1:10 dilution of cDNA synthesized from 1 μg of total RNA, 300 nM primers (Table 2), and SsoFast EvaGreen Supermix (Bio-Rad Laboratories, Inc.). Real-time PCR was performed using the MyiQ Real-Time PCR Detection System, and the results were analyzed using the MyiQ software package (Bio-Rad Laboratories, Inc.). In all experiments, cDNAs from two independent iScript cDNA reactions (Bio-Rad Laboratories, Inc.) were analyzed, giving a total of four 20-μl replicate reaction mixtures for each RNA sample. We analyzed total RNAs from 4 independent biological replicates for each growth condition. The mean $C_q$ of each target transcript was normalized to the $C_q$ of the reference transcripts *rpsL* and *rplU* using the following formula: $2^{Cq\ \text{internal standard}\ -\ Cq\ \text{target}}$. To determine the relative expression ratio (RER) of the target gene, the normalized target RNA value was divided by the average of the normalized values of four control samples (growth on alfalfa roots plus iron). This average value was designated the "calibrator," since the variation of all samples, including the individual control samples, was determined relative to this value. This method of calculating the RER was derived from the previously published $2^{-\Delta\Delta Cq}$ formula (33, 43), as previously described (47). Statistical analysis of RER values was performed with GraphPad Prism software using the unpaired two-tailed *t* test function (GraphPad Software, Inc.).

**Growth in lettuce leaf lysate.** Bagged romaine lettuce was obtained from a local grocery store. Lettuce leaf lysate was extracted as described previously, with some modification (32). Lettuce leaves were homoge-

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nized by hand, using a mortar and pestle within 5 min and immediately collected into 50-ml conical tubes and centrifuged at 9,000 $\times$ *g* for 4 min. The liquid was transferred into new 50-ml conical tubes and centrifuged again at 9,000 $\times$ *g* for 3 min. The supernatant was filter sterilized, first with a 0.8-μm filter (grade 40; Ashless; Whatman) and then with a 0.2-μm filter. One milliliter of leaf lysate was inoculated with $\sim 10^3$ CFU/ml bacteria of each strain, and samples were serially diluted, plated, grown at 24 h p.i. on SS agar plates, and incubated at 37°C overnight. Three replicates were included for each strain, and each replicate was sampled three times. Freshly made lysates were used for each experiment. All experiments were repeated on two separate occasions.

**Lettuce inoculation assays.** To determine the survival and colonization of *S.* Typhimurium in lettuce leaves in different environments, bagged romaine lettuce leaves were purchased from a local grocery store and inoculated with *S. enterica*. For inoculation, 2 μl of a $10^5$-CFU/ml bacterial suspension in sterile water was applied, using a 10-μl pipette, to the middle of the stem of each lettuce leaf at one strain per leaf. Three leaves were wrapped in a plastic bag and put in GasPak 100 System jars containing GasPak EZ Anaerobe Container System Sachets and incubated at 24°C. According to the manufacturer, anaerobic conditions ($\leq$1% oxygen) are achieved within 2.5 h at 35°C, and within 24 h, the carbon dioxide concentration is $\geq$13%. These conditions are similar to those reported for lettuce under modified-atmosphere packaging (MAP) (5). At 72 h p.i., leaves were sampled from the inoculation sites by excising tissue discs with a 0.5-cm radius, and one sample was taken per leaf. Plant samples were weighed and homogenized in sterile water equal to five times the tissue weight. Nine replicates were included for each strain, and all experiments were repeated on two separate occasions.

Leaves from 3- to 4-week-old lettuce plants, *Lactuca sativa* cv. Black Seeded Simpson, were used for infiltration assays. For infiltration, 100 μl of a $10^5$-CFU/ml bacterial suspension in sterile water was infiltrated with a needleless 15-ml syringe between the major veins of each lettuce leaf. The plants were kept in transparent plastic boxes at 25°C with covers on top to maintain $\sim$90% humidity. Two 1-cm-diameter leaf discs from the infiltrated site were taken at 48 h p.i. from each inoculated leaf. All samples were homogenized in 500 μl of sterile water, dilution plated on SS agar, and incubated at 37°C overnight for bacterial-population enumeration. Five leaves each from a total of three plants were used for each strain, and all experiments were repeated three times.

**Alfalfa root exudate analysis.** To determine the mineral concentrations in alfalfa root exudates, 20 ml of the 24- and 48-h-postgermination exudates were produced as described above. Sterile water used for seed germination was used as a control. All samples were collected into 50-ml conical tubes and sent to the Department of Soil Sciences, University of Wisconsin—Madison, for mineral analysis. Three samples were analyzed for each time point.

**Statistical analysis.** Student *t* tests were performed on log-transformed data to determine the significance between mean populations of strains per unit. The α level was adjusted using a Bonferroni adjustment procedure for multiple tests of significance.

## RESULTS

**Replication of *S. enterica* determines successful colonization of roots.** To identify metabolic networks essential for *S. enterica* plant colonization, we screened mutants on alfalfa roots at 24 (with seed coats) or 48 (without seed coats) h p.i. and growth of planktonic cells in irrigation water over time. An *aroA* mutant, disrupted in chorismic acid biosynthesis, was found to have a significantly reduced population size compared to the WT at both 24 and 48 h p.i. (Fig. 1A). Initial attachments to seeds were similar in the WT and the *aroA* mutant (data not shown). To determine if the colonization defects of the *aroA* mutant were due to an inability to grow, we measured the growth of the planktonic bacterial cells in the alfalfa seedling environment. While the WT strain was able to grow to above $10^9$ CFU/ml, the *aroA* mutant had approximately 3-log-unit (CFU/ml) reduction after 48 h p.i. (Fig. 1B). Complementation of *aroA in trans* restored both the growth and colonization defects of the mutant to WT levels (Fig. 1A and B). We also measured the growth of the *aroA* mutant and complement in M9-glucose and LB. Similar to previous reports (25, 40), the *aroA* mutant failed to grow in M9-glucose, although the *aroA* complement grew to the same extent as the WT (data not shown). Both the *aroA* mutant and *aroA* complement grew similarly to the WT in LB (data not shown).

**Aromatic amino acid biosynthesis is not involved in the root colonization defect of an *aroA* mutant.** Because chorismic acid biosynthesis is a critical metabolic node leading to the biosynthesis of different essential metabolites, such as aromatic amino acids and siderophores (Fig. 1C), we examined the several metabolic pathways downstream of *aroA* for a role in *S. enterica* plant colonization. Plants are notoriously low in available tryptophan (23). We found that the addition of neither tryptophan nor phenylalanine was able to restore either the root colonization (Fig. 2A) or the growth defect (data not shown) of the *aroA* mutant. Thus, the *aroA* mutant's growth defect is independent of its inability to produce tryptophan or phenylalanine.

Since tryptophan biosynthesis is a fitness factor for plant-associated bacteria (23), we examined the growth of both a tryptophan and a phenylalanine auxotroph in alfalfa root exudates, as well as root colonization. At 48 h p.i., the *trpB* mutant had less than 0.5-log-unit reduction in the bacterial population compared to the WT (Fig. 2B). At 48 h p.i., the phenylalanine auxotroph had a 1-log-unit reduction in the bacterial population colonizing the root compared to the WT (data not shown). These results suggest that tryptophan is available at nearly sufficient levels in alfalfa root exudates, while phenylalanine is more limited.

We were interested in whether aromatic amino acids were also limiting in other plant tissues. We examined lettuce leaf lysate as a growth medium and quantified bacterial population growth 24 h p.i. Growth rates of the WT and of phenylalanine and tryptophan auxotrophs in leaf lysate were similar (data not shown). These results suggest that aromatic amino acid availability to bacteria can differ between the rhizosphere and the leaf apoplast.

**Siderophore production is required for *S. enterica* alfalfa root colonization.** Next, we examined siderophore production, another pathway downstream of chorismic acid biosynthesis. *Salmonella* produces siderophores, iron chelators, in low-iron environments. The *aroA* mutant did not produce an orange halo on chrome azurol agar, confirming that iron was not removed from the agar, a sign that siderophores were not produced by the mutant (data not shown). We supplemented germinating alfalfa seeds with ferrous sulfate and found that the addition of iron fully restored both the *aroA* mutant root colonization and *in planta* growth defects to WT levels (Fig. 2C and D). To confirm that siderophore production is required for *S. enterica* plant colonization, we examined growth in root exudates and root colonization of an *entB* mutant that is unable to synthesize the siderophore enterobactin. At 48 h p.i., the *entB* mutant had an approximately 15-fold reduction in population size associated with alfalfa roots (Fig. 2C) and more than 100,000-fold reduction in the planktonic-cell population compared to the WT (Fig. 2D). The ability of the *entB* mutants to grow, both attached to alfalfa roots and as planktonic cells in root exudates, was fully restored to the WT level by the addition of ferrous sulfate (Fig. 2C and D). Analysis of mineral

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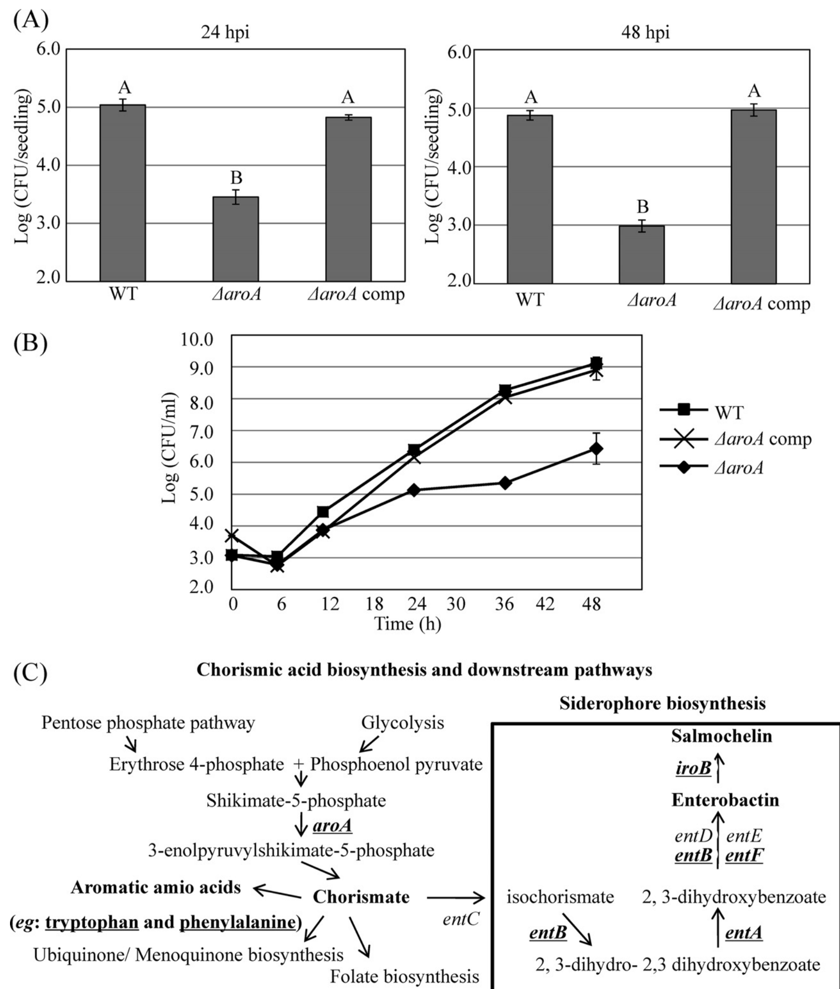


**FIG 1** An *aroA* mutant neither grew in alfalfa root exudates nor colonized alfalfa roots to WT levels. Shown is the mean log population of *S. enterica* in association with alfalfa. (A) Colonization of roots at 24 or 48 h p.i. (B) Growth of planktonic cells in alfalfa exudates over time. (C) Diagram of the chorismate metabolic network. The data represent the means of three experiments completed on different days, and the error bars represent the standard errors. Different letters represent $P$ values of $<0.01$ between mean log *S. enterica* population sizes using a two-tailed Student $t$ test. The α level was adjusted using a Bonferroni adjustment procedure for multiple tests of significance.

concentrations in alfalfa root exudates at 24 and 48 h postgermination confirmed that alfalfa root exudates are iron-poor environments (Table 3).

**Siderophore production-deficient mutants display motility defects *in vitro*.** Previously, swarming was shown to be important for *S. enterica* to colonize alfalfa roots (2). Swarming facilitates *Salmonella* movement through the viscous root exudates to reach preferred colonization sites. We examined swarming of the *aroA* mutant by quantification of the bacterial colony radius on semisolid LBG plates over time. The *aroA* mutant had a significant reduction in the travel distance compared to the WT. While the colony radius of the WT reached ~20 mm at 20 h p.i., the radius of the *aroA* mutant was ~7 mm (Fig. 3A). To determine whether the inability to produce siderophore inhibited swarming, we examined the swarming of the *entB* mutant. The *entB* mutant also displayed significant reduction in the travel distance compared to the WT, similar to the *aroA* mutant (Fig. 3A). However, addition of 10, 50, or 90 μM ferrous sulfate to the LBG plates failed to restore either mutant's swarming defect (data not shown). It is unclear whether addition of iron in this manner would make it available to swarming cells. Addition of surfactant also failed to restore swarming of the *aroA* and *entB* mutants, although WT cells responded by increasing their motility compared with untreated cells (data not shown). These results suggest that failure to acquire iron hinders *S. enterica* swarming motility, but not swarm cell induction, e.g., hyperflagellated cells (data not shown). In addition, the *aroA* mutant also had significant reduction in swimming distance compared to the WT. While the colony radius of the WT reached ~35 mm at 25 h p.i., the radius of the *aroA* mutant was ~25 mm (Fig. 3B).

***S. enterica* siderophore biosynthesis genes are induced in alfalfa root exudates.** *S. enterica* produces two different siderophores, enterobactin and salmochelin, which are synthesized by the *ent* and *iro* gene clusters, respectively (13). Since salmochelin is required for colonization of animals and we found that enterobactin biosynthesis is required for root colonization, we were interested in whether *ent* and *iro* genes were both induced by root exudates. We examined the induction of both siderophore pro-

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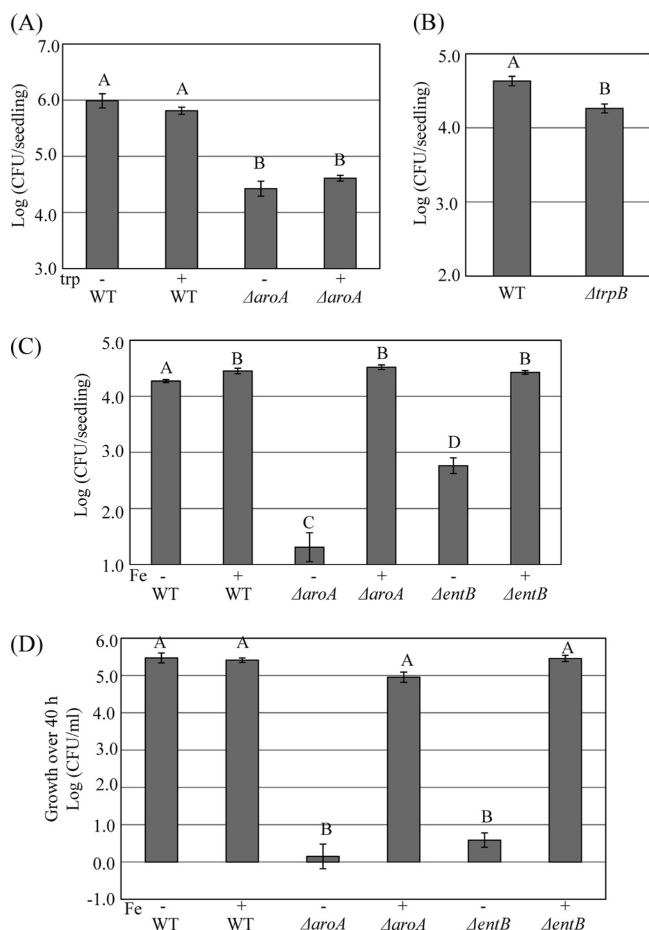


**FIG 2** Iron, but not tryptophan, deficiency accounts for the growth and colonization defects of the *aroA* mutant. Shown is the mean log population of *S. enterica* mutants in association with alfalfa. (A) Alfalfa root colonization 48 h p.i. with or without addition of tryptophan. (B) Alfalfa root (with seed coats attached to seedlings) colonization 24 h p.i. (C) Alfalfa root colonization 48 h p.i., with or without addition of ferrous sulfate. The pluses and minuses indicate the presence and absence, respectively, of treatment additions. (D) Growth of planktonic cells from 0 to 40 h p.i. with addition of ferrous sulfate. Population sizes were determined by subtracting the starting population from the population at 40 h p.i. The data represent the means of three experiments completed on different days, and the error bars represent standard errors. Different letters represent $P$ values of $<0.01$ between mean log *S. enterica* population sizes using a two-tailed Student $t$ test. The α level was adjusted using a Bonferroni adjustment procedure for multiple tests of significance.

duction pathways by determining the expression of three genes. *entF* produces the enterobactin synthetase component F; *entA* produces 2,3-dihydro-2,3-dihydroxybenzoate dehydrogenase, which catalyzes conversion of chorismic acid to isochorismate; and *iroB* produces a glycosyltransferase to transfer glucose groups to enterobactin. These genes are located in three separate transcriptional units. The *entA* and *entF* genes are in operons (promoter-*entC*-*entE*-*entB*-*entA*-*ybdB* and promoter-*fes*-*ybdZ*-*entF*), while *iroB* is transcribed as a single transcript. Since our previous results confirmed that alfalfa root exudates are iron poor and thus a siderophore-inducing environment, we compared the expression levels of genes encoding siderophores in root exudates with and without the addition of ferrous sulfate. In alfalfa root exudates, *entF* was induced 25-fold, while *entA* and *iroB* were both

**TABLE 3** Alfalfa seedling exudate analysis

| Mineral | Water (ppm) | 24-h alfalfa root exudates (ppm) | 48-h alfalfa root exudates (ppm) |
|---|---|---|---|
| P | <0.05 | 44.31 | 8.41 |
| K | <0.03 | 83.34 | 15.90 |
| Ca | <0.02 | 1.14 | 0.13 |
| Mg | <0.003 | 2.011378 | 0.081345 |
| S | <0.05 | 2.537115 | 0.416393 |
| Zn | <0.001 | 0.030159 | 0.002796 |
| B | <0.02 | 0.022722 | <0.02 |
| Mn | < 0.001 | 0.018237 | 0.001564 |
| Fe | < 0.001 | 0.004688 | 0.001171 |
| Cu | <0.005 | <0.005 | <0.005 |
| Al | <0.05 | <0.05 | <0.05 |
| Na | 0.1199 | 8.260459 | 1.207958 |

induced 5-fold, compared to the root exudates supplemented with iron (Fig. 4). When grown in M9-glucose, the increase in expression of *entA* and *entF* under low-iron conditions was comparable to the differences seen in root exudates (data not shown). The mean expression of *iroB* in M9-glucose was considerably higher than in M9-glucose with iron (data not shown). However, the sample-to-sample variation in *iroB* expression using M9-glucose was large, resulting in no statistically significant difference between the two growth conditions using M9-glucose (data not shown). We have previously encountered large sample-to-sample variation within a subset of the genes analyzed by real-time RT-qPCR, and in our experience, this type of selective variation seems to be a relatively common occurrence in bacterial gene expression (27).

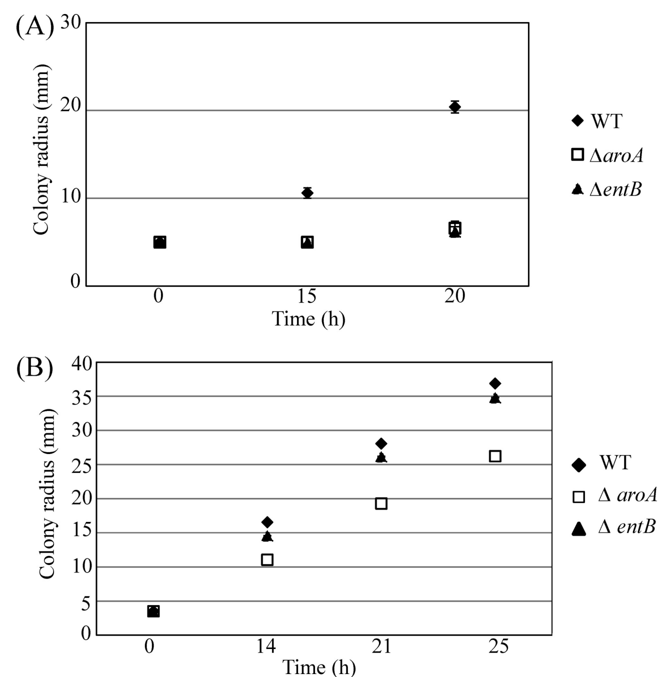


**FIG 3** An *aroA* mutant fails to swim or swarm while an *entB* mutant is only reduced in swarming. Shown are mean swarming or swimming distances of *S. enterica* on LBG plates. (A) Swarming distance over time. (B) Swimming distance over time. The data represent the means of two experiments completed on different days, and the error bars represent standard errors.

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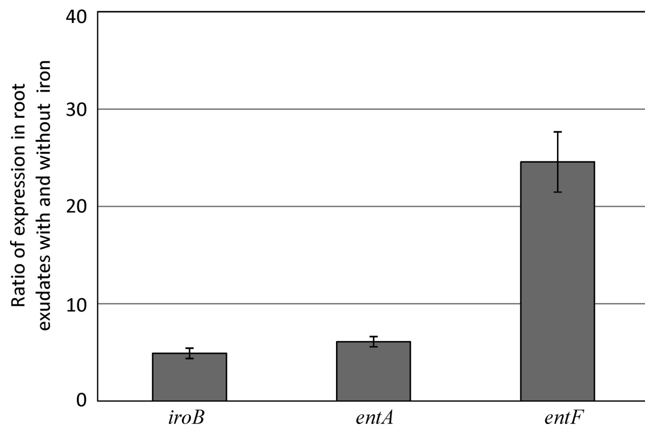


**FIG 4** Enterobactin and salmochelin biosynthetic genes are expressed in root exudates. Shown are the mean induction levels of *iroB*, *entA*, and *entF* in 48-h alfalfa root exudates compared to root exudates plus iron. The error bars represent the standard errors. All differences relative to the control were statistically significant at a 95% confidence internal, as determined by two-tailed *t* tests.

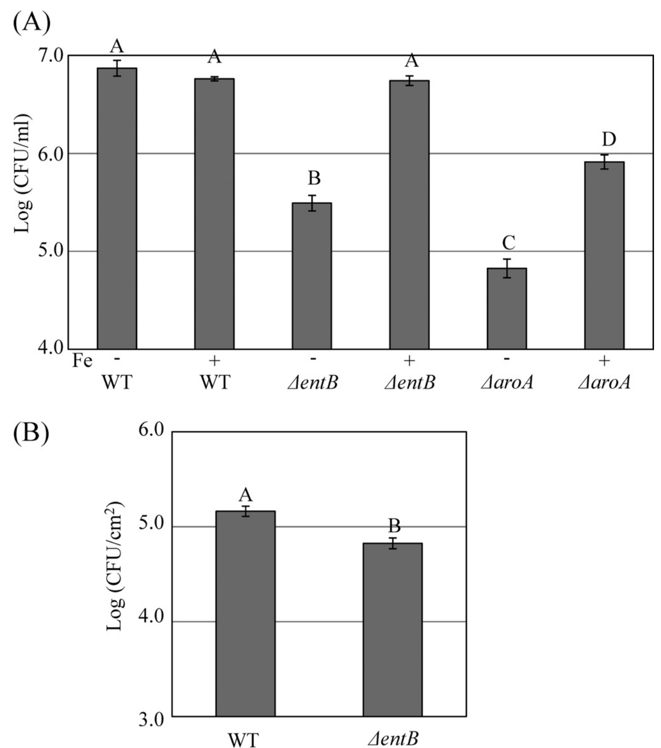


**FIG 5** Siderophore production is required for *S. enterica* in leaves. Shown are mean log populations of *S. enterica* in association with lettuce leaves. (A) Growth in leaf lysate 24 h p.i. with or without addition of ferrous sulfate. (B) Survival in leaves of 3- to 4-week-old lettuce plants 48 h postinfiltration. The pluses and minuses indicate the presence and absence, respectively, of ferrous sulfate additions. The data represent the means of two experiments completed on different days, and the error bars represent standard errors. Different letters represent *P* values of $<0.01$ between mean log *S. enterica* population sizes using a two-tailed Student *t* test. The α level was adjusted using a Bonferroni adjustment procedure for multiple tests of significance.

**Salmochelin is not required for *S. enterica* root colonization.** With the addition of two glucose groups, enterobactin is converted into salmochelin. In mammalian pathosystems, enterobactin is sequestered by host siderochalin, but salmochelin evades host recognition, providing *Salmonella* with an effective iron-scavenging system. To determine the role of salmochelin in *S. enterica* plant colonization, we measured growth in root exudates and root colonization of the *iroB* mutant. Planktonic cells of the WT and the *iroB* mutant grown in alfalfa root exudates replicated at similar rates over 48 h (data not shown). At 48 h p.i., the *iroB* mutant had no significant difference from the WT in root colonization at room temperature or 37°C (data not shown). Competition assays between the *iroB* mutant and the WT also revealed no fitness defect of the mutant at either temperature (data not shown).

**Siderophore production is a universal mechanism required for *S. enterica* survival in different plant environments.** To determine whether siderophore production is required for *S. enterica* to survive in other plant environments, we examined lettuce leaf lysate as a growth medium and quantified bacterial population growth 24 h p.i. Both the *aroA* and *entB* mutant populations were significantly reduced compared to the WT in the leaf lysate after 24 h (Fig. 5A). Addition of iron failed to induce a growth response in the WT; however, iron addition restored the *entB* mutant population to WT levels (Fig. 5A). Growth of the *aroA* mutant increased by more than 10-fold with the addition of iron but failed to attain WT levels.

To determine the extent of the *S. enterica* requirement for siderophore production during plant colonization, lettuce leaves were infiltrated and incubated aerobically or under modified atmosphere. Bagged lettuce is packaged under MAP to reduce spoilage organisms, reduce cut surface browning, and maintain leaf vigor. Lettuce under MAP represents half of the lettuce consumed in the United States (USDA Economical Research Service). We were interested in whether *S. enterica* siderophore biosynthesis is common in plant environments and if it is a fitness factor in modern production of fresh produce under MAP, where many damaged plant cells can provide ample carbon and nitrogen sources. The *entB* mutant endophytic population was significantly reduced compared to the WT in lettuce leaves at 48 h p.i. under aerobic conditions (Fig. 5B). The *entB* mutant population was nearly 0.5-log-unit lower than the WT population. These data suggest that iron is limited in lettuce leaves, as well as leaf lysate and root exudates. No differences between bacterial populations of the WT and the *entB* mutant were observed on lettuce leaves under microaerophilic conditions, although both populations grew extensively after inoculation (data not shown). These data suggest that neither *S. enterica* colonization nor replication is limited by iron acquisition within leaves under MAP.

## DISCUSSION

Accumulating evidence has shown that *Salmonella* can survive on and in plants following contact with either the plant root or the phyllosphere (55). In this study, we investigated metabolic networks essential for *S. enterica* to colonize plants. We used alfalfa seedlings as a model root system because the young plants are presently the most common vehicle for salmonellosis outbreaks (10) and seedling roots are fully developed. We found that replication of *S. enterica* cells in the rhizosphere dictates the success of alfalfa root colonization. Mutations in genes required for biosynthesis of environmentally limited amino acids or siderophore biosynthesis caused replication defects in cells in the rhizosphere, and

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subsequently, fewer cells were available to attach and form aggregates on roots. Although growth is required for colonization in a particular environment, its necessity appears to have been overlooked in recent literature studying human pathogen association with plants. Screening deletion mutants for growth deficiencies revealed limiting nutritional resources in the environment. While identification of available nutrients in environmental samples, e.g., root exudates or leaves, is cost prohibitive or unattainable, mutant analysis of bacteria that inhabit these environments can reveal biologically relevant nutritional statuses.

We found an *aroA* mutant deficient in colonization of alfalfa roots. AroA is involved in the biosynthesis of chorismic acid, a common precursor for the biosynthesis of multiple important metabolites, including aromatic amino acids, such as phenylalanine, tyrosine, and tryptophan; folate cofactors; benzoid and naphthoid coenzymes; and siderophores (17). Previously, an *aroA* mutant was reported to be much less virulent in animal infection models, providing the possibility of using this mutant as a vaccine strain (51). In our study, close examination of individual steps in plant colonization—initial attachment, growth, colonization, and persistence—revealed a significant growth defect of the *aroA* mutant in the alfalfa rhizosphere, where root exudates are the sole nutrient source. This result suggested alfalfa root exudates are a nutrient-poor environment, requiring one or multiple pathways downstream of chorismic acid biosynthesis for the pathogen to grow.

Tryptophan biosynthesis is a biologically expensive process using chorismic acid as a precursor and requiring the subsequent participation of several other compounds, such as glutamine and L-serine (45). Due to the low level of soluble tryptophan present in plants (23), tryptophan biosynthesis is a fitness and virulence factor for some phytopathogenic bacteria. Tryptophan auxotrophs of *Ralstonia solanacearum* failed to cause systemic infection in tobacco and tomato (8, 12). Tryptophan biosynthesis-defective mutants of *Pseudomonas fluorescens* failed to colonize tomato roots, though addition of tryptophan to the rhizosphere restored the mutant's colonization ability (53). These findings suggest that plants fail to release enough tryptophan in xylem fluid or root exudates to support these pathogens. Without bacterial biosynthesis of tryptophan, plant-associated bacteria poorly colonize plants. In our study, we found minor colonization defects in *S. enterica* tryptophan auxotrophs. Since a tryptophan biosynthesis mutation would be lethal to cells growing in the absence of tryptophan, our results suggest that a suboptimal tryptophan concentration is available in the alfalfa rhizosphere for *Salmonella*. Thus, *Salmonella* is required to offset the alfalfa root exudate's tryptophan deficiency by synthesizing more to successfully persist in this environment. Tryptophan biosynthesis may become more important as plants age. In that case, *Salmonella* tryptophan auxotrophs' replication defect would be similar to that of other plant-associated bacteria (e.g., *P. fluorescens*).

Aromatic amino acid availability differs between the phyllosphere and rhizosphere. *S. enterica* tryptophan and phenylalanine auxotrophs had restricted replication in the rhizosphere, similar to other plant-associated bacteria, e.g., *P. fluorescens* (34). However, neither *S. enterica* (this study) nor other plant-associated bacteria appear to be limited by tryptophan or phenylalanine concentrations in the phyllosphere, excluding the plant vasculature. Thus, bacterial mutant analysis can reveal the biologically relevant nutritional status of environments.

Iron is essential for bacteria, as it is involved in a wide variety of important metabolic processes, such as energy production and enzymatic reactions. Eukaryotes and prokaryotes often battle over the limited availability of iron. In mammals, a fine-tuned system of iron homeostasis is required to limit iron availability for pathogen survival and growth (50). Not surprisingly, iron is required for bacteria to colonize plants. *Pseudomonas putida* fails to colonize corn seeds following mutation of its TonB iron uptake system or addition of iron chelators to the corn spermosphere (37). Iron acquisition is a pathogenicity factor for both plant and animal bacterial pathogens (19, 38). Iron limitation by plants is a basal defense mechanism against successful phytobacterial infection (16). Low levels of soluble iron were found in both 24- and 48-h alfalfa root exudates. Addition of ferrous sulfate to the seedling environment fully restored both *in planta* growth and root colonization of the *aroA* and siderophore mutants. We found in this study that alfalfa root exudates are poor in iron and that siderophore production is an important root colonization fitness factor for human pathogens to thrive in the rhizosphere.

In aerobic environments, surface iron is usually converted into hydroxyl polymers, and the concentration of free soluble ferric iron can be as low as $10^{-18}$ M (6). Thus, microorganisms produce siderophores, effective ferric iron chelators, to scavenge iron from the environment (39). To determine the role of siderophore production in *S. enterica* plant colonization, we examined root colonization of an *entB* mutant unable to produce any siderophores and found a reduction similar to that of the *aroA* mutant in populations associated with both root colonization and planktonic-cell growth in root exudates. The growth and colonization defects of the *entB* and *aroA* mutants were restored with the addition of exogenous ferrous sulfate. The expression of genes that are required for the biosynthesis of each *Salmonella* siderophore, enterobactin and salmochelin, was downregulated by the addition of iron to alfalfa root exudates. Our results support the conclusion that *S. enterica* encounters an iron-poor environment requiring siderophore production during alfalfa root colonization, similar to other plant-associated bacteria (15).

We also examined whether *S. enterica* is iron limited in a second crop, lettuce, and an additional environment, the phyllosphere. The *aroA* and siderophore mutants were defective in growth in both leaf lysate and intact leaves. Leaf lysate is a medium that models the nutrients available to *S. enterica* in precut bagged lettuce or leaves damaged by biotic or abiotic mechanisms. Although the addition of exogenous iron did not fully restore the growth of the *aroA* mutant, we conclude that both leaves and rhizosphere due to root exudates are iron limiting. This is supported by the finding that addition of iron did restore the *entB* mutant population to WT levels. Subsequent leaf infiltration assays confirmed the lack of iron available to endophytic *S. enterica* populations. However, no such difference was observed under minimal oxygen, although the *S. enterica* cells were actively replicating. This suggests that under modified-atmosphere packing environments, e.g., bagged lettuce, available iron remains as Fe(II), and thus, siderophore production by *Salmonella* is unnecessary.

In an aerobic environment, iron mainly exists in the oxidized state as Fe(III) that cannot be directly utilized by *Salmonella*. Thus, a functional siderophore system or another mechanism to scavenge iron from the environment is required. *S. enterica* requires siderophore biosynthesis for replication at WT rates inside lettuce leaves, as the *entB* mutant had a significantly reduced population

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size. This is in sharp contrast to the phytobacterial pathogens *Pseudomonas syringae* pv. tomato (28) and *Xanthomonas oryzae* pv. oryzae (41). Deletion of the *X. oryaze* pv. oryzae siderophore biosynthesis genes caused no defect; however, deletion of the Feo (ferrous iron transporter) system caused virulence deficiency on rice. Deletion of three siderophores produced by *P. syringae* pv. tomato caused no growth reduction in tomato leaves. Either these phytobacterial pathogens can pirate plant iron compounds, such as iron-nicotianamine, to fulfill their iron requirements, or the leaf apoplast is iron replete. Our data suggest that the lettuce leaf apoplast is iron poor, since the *S. enterica entB* mutant populations were unable to replicate to the extent of the WT. The rice apoplast appears iron poor as well, since the *X. oryzae* pv. oryzae iron transporter is actively expressed during *in planta* growth (41). It seems more likely that phytopathogenic bacteria can steal plant iron compounds when necessary. Phytopathogenic pseudomonads, such as *P. syringae* and *P. aeruginosa*, can import either plant- or bacterium-derived iron(III) dicitrate through FecA (28, 35); however, unlike *Pseudomonas* or *Escherichia coli*, *Salmonella* is devoid of a citrate-dependent iron transport system (56). *S. enterica* relies on its own siderophore biosynthesis during root colonization, similar to plant-associated pseudomonads (15). However, *Salmonella*'s dependency on its own siderophore biosynthesis during leaf colonization differs from phytobacterial pathogens, such as *Pseudomonas* and *Xanthomonas*, but may be similar to the enteric *Dickyea dadantii* (21).

Both swimming and swarming are required for *Salmonella* colonization of the phyllosphere and the roots of plants (2, 30, 31). Swarming confers increased antibiotic resistance and helps cells to avoid unfavorable environments and locate preferential niches (29). Alfalfa root exudates mainly consist of mucilage and proteins, an inducible environment for the swarming of *S. enterica* (2). Disruption in *S. enterica* genes involved in swarming results in defects in root attachment or colonization. In our study, we found that both the *aroA* and *entB* mutants failed to move very far once the swarm state was induced. This suggests that an additional mechanism necessary for plant colonization was compromised in the *aroA* and *entB* mutants, besides growth. Compared to swimming cells, *Salmonella* swarm cells upregulate iron metabolism (57). In *E. coli*, iron acquisition genes are also found to be involved in swarming (26). Thus, iron acquisition plays a dual role for *Salmonella* during plant colonization of direct growth and optimal-niche discovery.

Using chorismic acid as a precursor, *S. enterica* produces the first siderophore, enterobactin, for iron acquisition. However, the mammalian hosts of the bacterium are able to produce an antimicrobial, siderocalin, that specifically binds enterobactin, preventing its utility in iron acquisition (46). *S. enterica* produces a second siderophore, salmochelin, that is able to evade the host immune system; thus, salmochelin is an important virulence factor during infection of mammalian hosts. Since plants do not produce siderocalin to bind *Salmonella*'s primary siderophore, enterobactin, we were not surprised to observe no difference in plant colonization of the *iroB* mutant compared to the WT. However, *iroB* had gene induction similar to that of *entA* in alfalfa root exudates, suggesting *S. enterica* produces both siderophores. Enterobactin and salmochelin biosynthesis, export, and uptake are finely regulated (13). In an environment lacking the antagonism of siderocalin, such as plants, production of enterobactin alone would be sufficient to acquire the necessary iron. In an *iroB* mutant unable to synthesize salmochelin, a compensatory increase in enterobactin secretion occurs in an iron-limiting environment (13). The lack of reduction in the *iroB* mutant's plant colonization suggests that enterobactin production may be increased in the *iroB* mutant and thus eliminate the need for salmochelin production during plant colonization.

Although the role of siderophore production in the interaction between *S. enterica* and animal hosts has long been recognized, its role in the pathogen's survival and persistence outside the animal host has not previously been reported. Our study is the first to show the importance of siderophore production for *S. enterica* to survive and grow in association with plants. This result may have significant implications in agricultural practices. Fertilizer containing iron may increase *in planta* iron availability or iron release to the surrounding environment and thus increase the populations of human pathogens on plants (20). On the other hand, potential iron chelators might be explored as effective pathogen sanitation strategies, since *Salmonella* fails to utilize plant iron compounds. Identifying the metabolic pathways required for *S. enterica* persistence and growth on plants, such as the chorismic acid biosynthetic pathway, is necessary for discovery of novel control strategies.

## ACKNOWLEDGMENTS

We thank Diana Downs for providing bacterial strains and Jessica Lund for assistance with the assay of bacterial growth in lettuce lysate.

Funding was provided by the USDA-NIFA (grant no. 2011-670137-30166).

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