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Salmonella Persistence in Tomatoes Requires a Distinct Set of Metabolic Functions Identified by Transposon Insertion Sequencing

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ABSTRACT Human enteric pathogens, such as *Salmonella* spp. and verotoxigenic *Escherichia coli*, are increasingly recognized as causes of gastroenteritis outbreaks associated with the consumption of fruits and vegetables. Persistence in plants represents an important part of the life cycle of these pathogens. The identification of the full complement of *Salmonella* genes involved in the colonization of the model plant (tomato) was carried out using transposon insertion sequencing analysis. With this approach, 230,000 transposon insertions were screened in tomato pericarps to identify loci with reduction in fitness, followed by validation of the screen results using competition assays of the isogenic mutants against the wild type. A comparison with studies in animals revealed a distinct plant-associated set of genes, which only partially overlaps with the genes required to elicit disease in animals. *De novo* biosynthesis of amino acids was critical to persistence within tomatoes, while amino acid scavenging was prevalent in animal infections. Fitness reduction of the *Salmonella* amino acid synthesis mutants was generally more severe in the tomato *rin* mutant, which hyperaccumulates certain amino acids, suggesting that these nutrients remain unavailable to *Salmonella* spp. within plants. *Salmonella* lipopolysaccharide (LPS) was required for persistence in both animals and plants, exemplifying some shared pathogenesis-related mechanisms in animal and plant hosts. Similarly to phytopathogens, *Salmonella* spp. required biosynthesis of amino acids, LPS, and nucleotides to colonize tomatoes. Overall, however, it appears that while *Salmonella* shares some strategies with phytopathogens and taps into its animal virulence-related functions, colonization of tomatoes represents a distinct strategy, highlighting this pathogen's flexible metabolism.

IMPORTANCE Outbreaks of gastroenteritis caused by human pathogens have been increasingly associated with foods of plant origin, with tomatoes being one of the common culprits. Recent studies also suggest that these human pathogens can use plants as alternate hosts as a part of their life cycle. While dual (animal/plant) lifestyles of other members of the *Enterobacteriaceae* family are well known, the strategies with which *Salmonella* colonizes plants are only partially understood. Therefore, we undertook a high-throughput characterization of the functions required for *Salmonella* persistence within tomatoes. The results of this study were compared with what is known about genes required for *Salmonella* virulence in animals and interactions of plant pathogens with their hosts to determine whether *Salmonella* repurposes its virulence repertoire inside plants or whether it behaves more as a phytopathogen during plant colonization. Even though *Salmonella* utilized some of its virulence-related genes in tomatoes, plant colonization required a distinct set of functions.

KEYWORDS *Salmonella*, foodborne pathogens, plant-microbe interactions

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Approximately 15% of all cases of human salmonellosis are thought to be associated with the consumption of fruits and vegetables (1). The ability of nontyphoidal strains of *Salmonella enterica* to colonize plants, including sprouts, alfalfa, lettuce, melons, and tomatoes, is highlighted by the rise in the number and severity of salmonellosis outbreaks linked to produce. The capacity of *S. enterica* (and other enteric pathogens) to readily multiply within plant tissues led to the hypothesis that persistence on plants is a part of the *Salmonella* life cycle, serving as reservoirs prior to reinfection of the preferred animal hosts (2–5). Several studies have dissected the molecular basis of plant-*Salmonella* interactions (6–16). However, the entire complement of genetic functions required for plant colonization by *Salmonella* is not yet known.

Previous studies established that colonization of plants by *Salmonella* is a complex process, dependent on both host and bacterial factors. It involves major changes in the bacterial transcriptome and requires genes involved in amino acid biosynthesis and transport, cellulose production, fimbriae, regulators, and surface structures (6–17). Some of the same mechanisms were involved in the colonization of both vegetative and reproductive tissues on different plant species. Colonization of the tomato pericarp involves differential regulation of at least 50 genes (9), some of which were also required for proliferation, including the gene *cysB*, encoding a cysteine metabolism regulator (9). Surface structures, like the O-antigen capsule and curli fimbriae, were also required for the colonization of tomatoes, as indicated by reduced fitness of the corresponding *Salmonella* mutants (11, 17). The O-antigen capsule also plays a role in the colonization of alfalfa sprouts by *Salmonella* spp., as do curli fimbriae, suggesting that these structures may be broadly required for the colonization and/or persistence within plants (7, 14). Iron acquisition was shown to play an important role during *Salmonella* colonization of vegetative and reproductive tissues. The siderophore transporter *fepDGC* was required for *Salmonella* proliferation in tomato pericarps (16), and siderophore synthesis was necessary for the colonization of lettuce and alfalfa (10). Biosynthesis of amino acids was also a likely factor required for *Salmonella* growth in alfalfa root exudates (15). Plant colonization by *Salmonella* is affected by host factors; the genetic background can influence *Salmonella* colonization of sprouts (18), proliferation in the tomato phyllosphere (19, 20), and pericarps (21).

Some of the previous work focused on the roles of *Salmonella* virulence factors during its interaction with plants. The term "virulence factor" was sometimes used to broadly define any gene (e.g., *rpoS*) required for full virulence in animal models. While the contribution of the *Salmonella* genes that generally impact animal virulence was characterized during the attachment and colonization of alfalfa sprouts (6), the involvement of the more dedicated virulence-related functions, such as type III secretion systems (T3SS), and their regulators encoded on the *Salmonella* pathogenicity islands (SPIs) remains controversial. Strains lacking SPI-1 and SPI-2 T3SSs had reduced proliferation in *Arabidopsis thaliana* leaves, failed to suppress the plant immune response against bacteria, and were required for the chlorotic appearance of plants (22). The effector *spvC* carried by the virulence plasmid suppressed inducible plant defenses when it was directly transformed into *Arabidopsis* protoplasts (22). *Salmonella* strains lacking SPI-1 T3SS induced a stronger response from plants, suggesting that this pathogenicity island represses plant immunity (23). However, studies linking the T3SSs encoded by SPIs to plant colonization by *Salmonella* required high bacterial titers, which are unlikely to happen naturally. Moreover, *Salmonella* spp. induced plant immune responses at lower levels than specialized plant pathogens, like *Pseudomonas syringae* (24). Some studies reported that *Salmonella* strains lacking SPI-1, SPI-2, SPI-3, SPI-4, and SPI-5 are not defective for the colonization of tomato and cantaloupe fruits (9, 25). Furthermore, there is no direct evidence that *Salmonella* can translocate effectors into plant cells via SPI-encoded T3SS, calling into question the requirement for the corresponding genes during plant colonization (unless the T3SS apparatus performs other noncanonical functions during plant colonization, a hypothesis that has not yet been ruled out).

Reverse genetics was previously employed to identify *Salmonella* virulence genes in animals. Mutants were created and individually screened to identify those defective in invasion and intracellular proliferation in cell cultures, and those with impaired virulence in intraperitoneal or oral infection models (26–33). Recently, the development of high-throughput technologies allowed coverage of the entire genome in these screens (34). Here, we used a similar approach to test the hypothesis that *Salmonella* genes required for the colonization of plants are different from those required for virulence in animals, and, furthermore, that the strategies that *Salmonella* uses to colonize plants are distinct from those used by phytopathogens. We used a transposon insertion sequencing approach to identify *Salmonella enterica* serovar Typhimurium loci required for persistence in tomatoes and subsequently confirmed the phenotypes using competition assays with isogenic mutants. We also compared the results obtained in this study with similar screens in animals, as well as with the data obtained using mutant screens of phytopathogens.

RESULTS

Transposon insertion library screening and sequencing. The tested transposon mutant library consisted of about 280,000 independent mutants with Tn*5* insertions, of which ~230,000 insertion sites were mapped onto the genome. The mapped insertions disrupted 88% of all features of the genome. The remaining 12% of the loci (1,263 features) are likely essential for *Salmonella* survival and proliferation under the culture conditions used. We defined 10,632 features in the *Salmonella enterica* serovar Typhimurium ATCC 14028 genome, including coding sequences, intergenic regions, and noncoding RNA.

Salmonella spp. can proliferate within red ripe tomato pericarps, reaching ~10^7 CFU per fruit (9, 19, 21, 25, 35). To represent the entire transposon insertion library, the screening needed to be done using an inoculum titer very close to the carrying capacity of tomatoes. Consequentially, bacterial growth appeared constrained, potentially imposing a limit on the possible changes in frequency of transposon insertions with differential fitness. We tested whether screening the mutant library under this condition would allow us to detect selection, using the *rcsA*::*kan* mutant strain, whose fitness is known to be modestly reduced in tomatoes (36). Because following the screen, recovered mutants were briefly grown out in LB to increase DNA output, we also confirmed in preliminary experiments with the *rcsA*::*kan* mutant that this outgrowth does not affect the recovery ratio of the mutant or its DNA (data not shown). These optimization experiments established the feasibility of a high-inoculum titer to identify *Salmonella* transposon insertions with differential fitness. Moreover, it suggested that even when the population is stagnant (death and reproduction were present at the same rate), the turnover of bacterial cells is sufficient to lead to changes in the abundance of genotypes with differential fitness.

The mutant library was screened in ripe Campari tomato pericarps using 12 biological replicates and three technical replicates each; the biological replicates were pooled, resulting in six final samples for library sequencing, with three input samples and three output samples. The screen identified 1,245 features with differential fitness (false-discovery rate [FDR] < 0.1); 1,112 of them were under negative selection, and only 132 features were under positive selection (Fig. 1 and Data Set S1). This represents 11.8% and 1.4% of all tested features, respectively. Among the loci with differential abundance, 886 loci were coding sequences. Loci with reduced fitness often appeared adjacent to each other, mostly due to operon organization.

***Salmonella* metabolic requirements during its interaction with tomatoes.** A high number of features identified in this screen were involved in metabolic pathways. To investigate the metabolic pathways required by *Salmonella* spp. during their interaction with the plant, we projected the KEGG Orthology terms for each locus against the *Salmonella* metabolic map using KEGG Mapper. Overall, the screen revealed that *Salmonella* proliferation within tomatoes is a complex process, requiring the combination of several catabolic and anabolic pathways, including biosynthesis of amino acids,

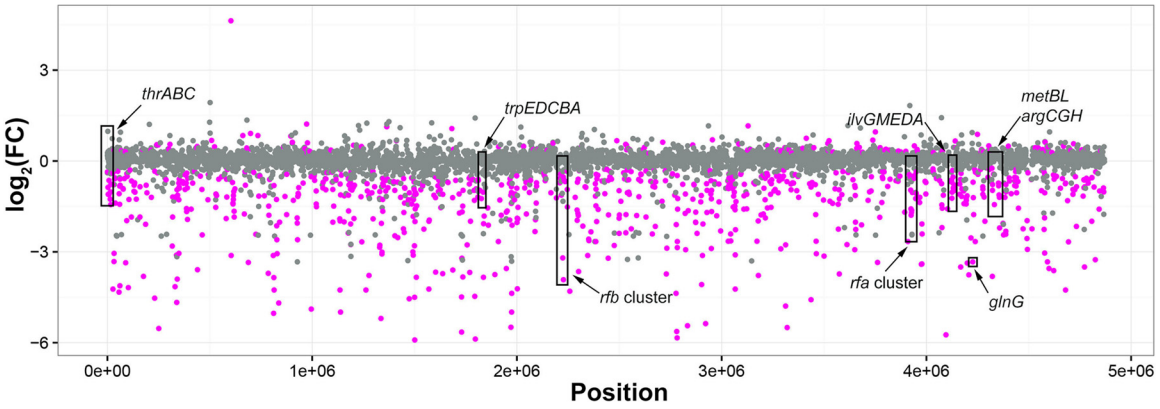

FIG 1 Change in abundance of *Salmonella* mutants after screening in tomatoes. The *Salmonella* transposon insertion library was inoculated into tomato pericarps (10^7 CFU per fruit) and incubated for 7 days at 22°C. Relative abundance of mutants after the incubation was compared to the initial inoculum. The position of each locus on the *y* axis represents the $\log_2$(fold change) of relative abundance, and on the *x* axis, each locus position represents a physical position on the *Salmonella* chromosome. Loci with significant changes in abundance (FDR $<$ 0.1) are shown in magenta, and loci with an FDR of $>$0.1 are shown in gray. Arrows point to loci and operons targeted for further experiments.

nucleotides, and lipids, as well as glycolysis (Fig. 2). The results indicate that carbohydrates that are metabolized to pyruvate via glycolysis are a major carbon source for *Salmonella* within tomatoes. In *Salmonella* spp. colonizing the tomato, pyruvate is likely fermented to acetate, as indicated by the reduced fitness of mutants in *pta* and *ackA* and the absence of phenotypes for the mutants of the genes involved in the tricarboxylic acid (TCA) cycle. The TCA cycle oxidizes acetyl-coenzyme A (acetyl-CoA) originating from pyruvate under aerobic conditions. The use of fermentation instead of the TCA cycle is consistent with the fact that the environment inside tomato fruits is microaerophilic or anaerobic.

The Gene Ontology enrichment analysis indicated that the methionine, arginine, and branched-chain amino acid synthesis pathways were overrepresented among the *Salmonella* functions required for tomato colonization. This is an indication that these nitrogen compounds are not readily available for the bacterium within tomatoes and that *Salmonella* has to synthesize them *de novo*. Since the data indicate that carbohydrates may provide the major carbon source for *Salmonella* within tomatoes, it is possible that these are providing the carbon backbone for bacterial amino acid and nucleotide synthesis in this environment. Four genes related to nitrogen uptake were identified in the screen: *lysP*, encoding the lysine-specific permease; STM14_1095, encoding a putative amino acid transporter; *potB*, encoding a high-affinity spermidine/putrescine ABC transporter; and STM14_1979, encoding a putative ABC polar amino acid transporter. None of these genes are involved in the transport of the most abundant nitrogen compounds available in tomatoes (glutamine, gamma-amino butyric acid [GABA], and inorganic nitrogen). *Salmonella* spp. may be exploiting several nitrogen sources and then synthesize the complement of the needed amino acids.

Fatty acid biosynthesis and degradation were required by *Salmonella* spp. within tomatoes, perhaps because functional cellular membrane synthesis is necessary for bacterial proliferation and maintenance. Genes in fatty acid metabolism that contributed to *Salmonella* fitness included the 3-oxoacyl-[acyl-carrier-protein] synthase gene *fabF*, and the acyl-CoA dehydrogenase gene *fadE*. Besides these two well-known genes, we also identified STM14_1005, another putative acyl-CoA dehydrogenase. Of interest is that *fadH* (2,4-dienoyl-CoA reductase) was among the genes differentially regulated in tomatoes, and its expression was shown to be dependent on the availability of linoleic acid, which in turn differentially accumulates in tomatoes as they mature (9).

***Salmonella* metabolic requirements for proliferation within tomatoes differ from the requirements for systemic infection of mice.** We explored whether *Salmonella* metabolic requirements for the systemic infection of mice were comparable with

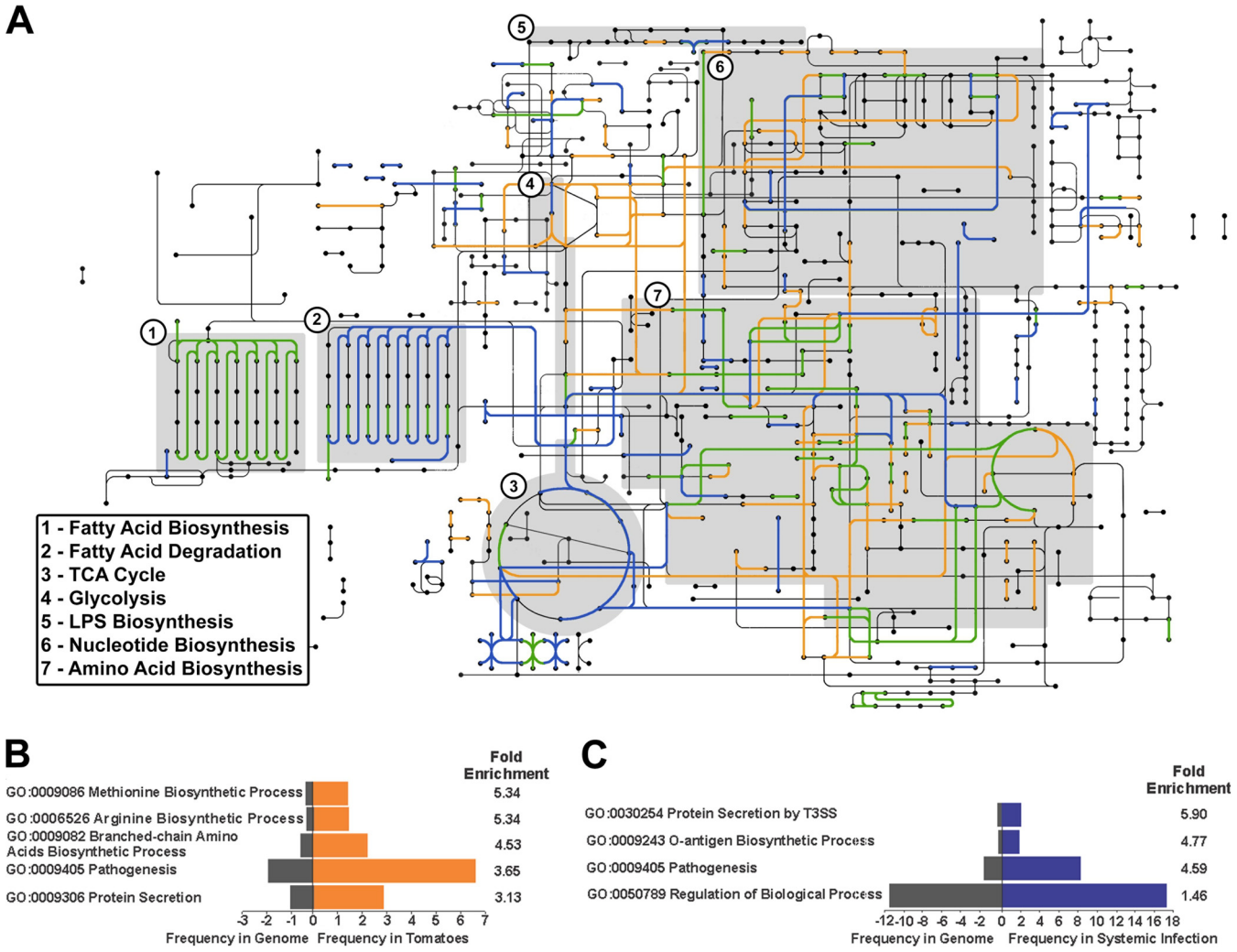

FIG 2 Comparison of functions required for colonization of tomatoes and systemic infection in mice. (A) *Salmonella* metabolic pathways involved in tomato colonization are shown in orange, pathways involved in the systemic infection of mice are shown in blue, and pathways involved in both tomato colonization and mouse infection are shown in green. Pathways were identified as required for tomato colonization if the corresponding mutants were less represented in the output pool [$\log_2(FC) < -0.5$, $P < 0.1$]; data on murine infection are from previously published reports (37, 38, 51). Key metabolic steps are shown as shaded boxes. KEGG Orthology terms for the protein sequences corresponding to the underrepresented mutants were retrieved using BlastKOALA and mapped using KEGG Mapper. False color overlays were imposed in Adobe Photoshop 2014. (B) Gene Ontology (GO) term enrichment analysis for loci required for tomato colonization [$\log_2(FC) < -0.5$, $P < 0.1$], showing frequency (%) in genome versus screening. Frequency of the GO terms in the genome is shown in gray, and frequency of the GO terms required for tomato colonization is shown in orange. (C) GO term enrichment analysis for those required for systemic infection in mice, showing frequency (%) in the genome and in screenings (37, 38, 51). Only significant terms (Bonferroni correction, $P < 0.05$) are shown.

the metabolic requirements for fitness within tomatoes. We retrieved data from previous work using transposon insertion libraries in *Salmonella enterica* serovar Typhimurium (37, 38) that had been applied in a mouse model for systemic infection and compared them with our results from the *Salmonella*-tomato interaction. A total of 327 KEGG Orthology (KO) identifiers were mapped for the genes required for persistence in tomatoes, and 505 KO identifiers required for systemic infection of mice were mapped. Only 125 KO identifiers were shared by the two conditions, showing that the *Salmonella* metabolic requirements under these conditions are different (Fig. 2A).

While amino acid biosynthesis was required for *Salmonella* spp. in the interaction with tomatoes, scavenging of amino acids was required in the systemic mouse infection. Transposon disruptions in just a few pathways (e.g., the urea cycle) impaired *Salmonella* fitness during systemic infection (37, 38). Besides being involved in the synthesis of arginine, the urea cycle is also involved in the incorporation of excess nitrogen.

Carbohydrates were likely a major carbon source for *Salmonella* spp. during systemic animal infection and during tomato colonization. However, *Salmonella* spp. may use the less-energy-efficient pathway of acetate fermentation in tomatoes. During systemic infection, *Salmonella* oxidizes carbohydrates using the TCA cycle, as indicated by phenotypes of the mutants that represent this pathway. Phenotypes observed for the genes encoding 2-ketoglutarate dehydrogenase and succinate dehydrogenase, which are only fully expressed under aerobic conditions, support the role of oxidation in the bacterial metabolism in animals.

Nucleotide biosynthesis is required by *Salmonella* spp. in murine systemic infection and in proliferation within tomatoes, as evidenced by the reduction in fitness when mutations occurred in the genes involved in the biosynthesis of pyrimidines and purines in either model of infection. While most *Salmonella* metabolic requirements diverge for colonization of plant and animal hosts, nucleotide biosynthesis is a point of convergence.

Enrichment analysis also showed that genes related to pathogenesis (GO: 0009405) contribute to fitness during systemic infection and proliferation within tomatoes (Fig. 2B and C). This GO term is heterogeneous and includes regulators, effectors, and surface proteins. The global regulator *phoPQ*, for example, belongs to this group of genes and displayed a phenotype in both tomatoes and mice. The *phoPQ* regulator activates the expression of other pathogenesis-associated genes that code for effectors and have a role in adaptation to low Mg^{2+} and resistance to antibacterial peptides. Several effectors and the *mtgC* virulence factor required for survival in low Mg^{2+} had phenotypes under both conditions, indicating that some *Salmonella* adaptations for virulence in animals are also at play during colonization of tomato fruits.

Few metabolic parallels between fitness of *Salmonella* in tomatoes and behavior of phytopathogens. After finding that *Salmonella* metabolic requirements for proliferation within tomatoes differed from the requirements for the systemic infection of mice, we explored whether these requirements are similar to the metabolic requirements of bacterial phytopathogens during plant infection. To test the hypothesis that *Salmonella* behavior in tomatoes is similar to the behavior of plant pathogens, we extracted the information identified in mutant screens of *Pectobacterium carotovorum* in Chinese cabbage (39), *Ralstonia solanacearum* in *Arabidopsis thaliana* and tomatoes (40), *Xanthomonas citri* in grapefruit (41), *Xanthomonas oryzae* pv. oryzicola in rice and tobacco (42), *Pseudomonas tolaasii* in *A. thaliana* (43), *Pseudomonas syringae* pv. maculicola in *A. thaliana*, and *Xanthomonas campestris* pv. campestris in cabbage (44). We adopted the strategy of grouping the results of different screens to increase the coverage of KO identifiers, since most of these studies did not have the same depth used in transposon sequencing methods, and to obtain a broader comparison with the different lifestyles that phytopathogens may assume. There appears to be a limited overlap of metabolic functions between *Salmonella* requirements for colonization of tomatoes and those of phytopathogens (Fig. 3), indicating that the mechanisms employed by *Salmonella* to colonize tomatoes require a distinct combination of functions. Lipopolysaccharide (LPS) and nucleotide biosynthesis were among the functions shared by phytopathogens and *Salmonella* in tomatoes and were also involved in systemic infection of mice, suggesting that there is a small set of genes required for pathogenesis, regardless of the host. These functions are likely involved in resistance to host defense and metabolic limitations that bacteria experience within eukaryotes. Amino acid biosynthesis was a requirement shared by *Salmonella* during colonization of tomatoes and by phytopathogens but absent from *Salmonella* during systemic infection of mice. This divergence between colonization of animal and plant hosts could be associated with the nutrient availability. Fatty acid biosynthesis and degradation were required for *Salmonella* infection of various hosts, but this requirement was absent from phytopathogens.

***Salmonella* requires biosynthesis of arginine, glutamine, glutamate, branched amino acids, methionine, tryptophan, and threonine to colonize tomatoes.** Nitrogen metabolism, including amino acid biosynthesis and the global nitrogen regulator

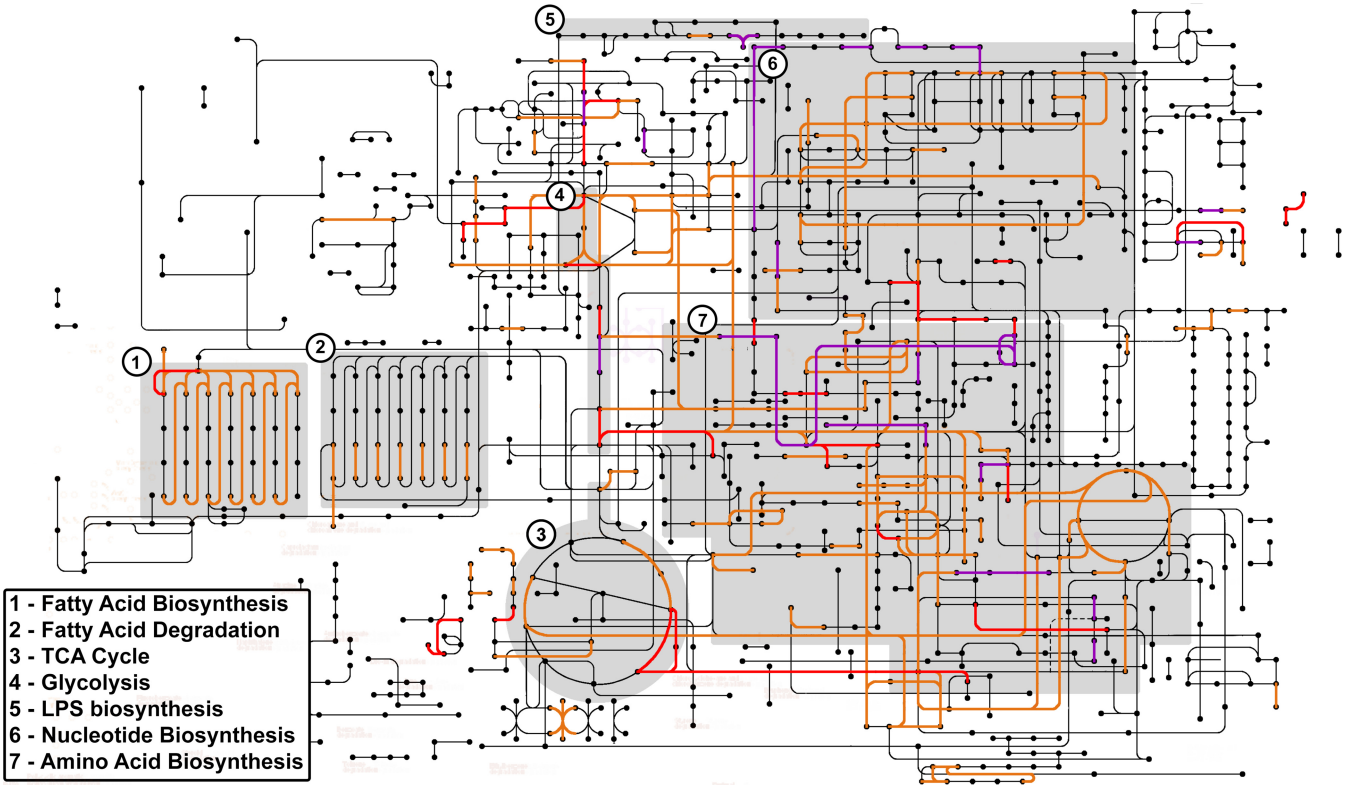

FIG 3 Comparison of functions required by *Salmonella* for colonization of tomatoes and phytopathogens to elicit disease. *Salmonella* metabolic pathways involved in tomato colonization are shown in orange, pathways required by phytopathogens to elicit disease are shown in red, and pathways involved in both are shown in purple. Pathways were identified as required for tomato colonization if the corresponding mutants were less represented in the output pool [$\log_2$(FC) < -0.5, $P < 0.1$]; data from phytopathogens are from previously published reports (39–42, 44, 60). Key metabolic steps are shown as shaded boxes. KEGG Orthology terms for the protein sequences corresponding to the underrepresented mutants were retrieved using BlastKOALA and mapped using KEGG Mapper. False color overlays were imposed in Adobe Photoshop 2014.

glnG, contributed to *Salmonella* fitness during its colonization of tomatoes. We explored this role further using competition assays with isogenic mutants. A lower bacterial inoculum was used in these assays to more realistically approximate natural interactions.

We identified the amino acid biosynthetic pathways required by the bacterium for proliferation within tomatoes. Transposon insertion sequencing revealed the synthesis of tryptophan, arginine, branched amino acids (leucine, isoleucine, and valine), glutamine, glutamate, threonine, methionine, and proline to be required for complete bacterial fitness (Fig. 4A). Mutants were created in the genes responsible for the key steps of each biosynthetic pathway, and their auxotrophic phenotype was confirmed in minimal medium. However, because there are at least two enzymes that can catalyze the last step in the formation of methionine and glutamate, *metA* and *gltB* were not auxotrophs. The competition assays confirmed the transposon insertion sequencing results for all but one of these 10 metabolic pathways (Fig. 4B). Only the auxotrophic mutant for proline (*proA*::*kan*) deviated from the transposon insertion sequencing results and did not appear to have a reduced fitness compared to the wild type. A deletion of the global nitrogen regulator *glnG* also led to a reduction in fitness of *Salmonella* in tomatoes, consistent with its function in bacterial responses to nitrogen assimilation.

To verify that the observed reduction in fitness caused by the disruption in *trpC*, *argA*, *ilvD*, *glnA*, *serA*, *thrC*, *gltB*, *metA*, and *glnG* was not due to a reduction in overall fitness, we compared the growth kinetics of these isogenic mutants in LB. We observed that the growth kinetics of these mutants did not differ from the wild-type strain; consequently, the reduced fitness observed in tomatoes is not caused by a reduction in overall fitness (Fig. S1).

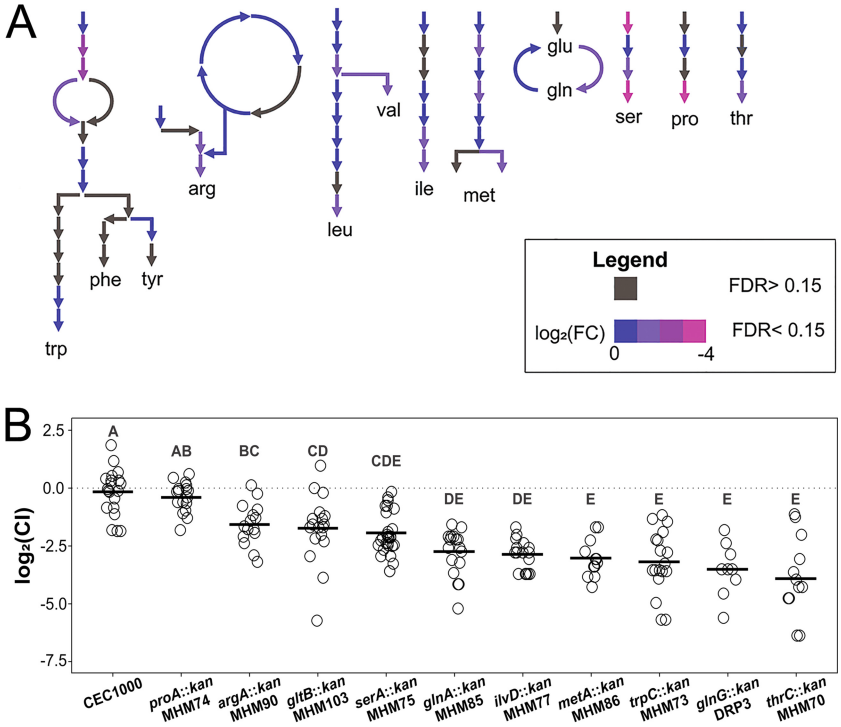

FIG 4 Fitness of *Salmonella* amino acid synthesis mutants in tomatoes. (A) Representation of the amino acid biosynthetic pathways in *Salmonella* using data extracted from transposon insertion sequencing screening. Each arrow represents an enzyme involved in the amino acid synthesis. Biosynthetic pathways: trp, tryptophan; phe, phenylalanine; tyr, tyrosine; arg, arginine; leu, leucine; val, valine; ile, isoleucine; met, methionine; glu, glutamate; gln, glutamine; ser, serine; pro, proline; thr, threonine. The color of the arrow indicates fold change and FDR [the gradient of color from blue to magenta corresponds to 0 to −4 $\log_2$(FC) and FDR less than 0.15, and dark gray corresponds to FDR of >0.15 regardless of the fold change]. (B) Competitive fitness of isogenic mutants involved in the amino acid biosynthetic pathways. Approximately 10^3 CFU of a mix of the wild type and an isogenic mutant (1:1) was inoculated into each tomato, followed by a 7-day incubation at 22°C. Competitive index was calculated using the formula $\log_2[(MUT_{out}/WT_{out})/(MUT_{in}/WT_{in})]$. The replicates were obtained from six tomato fruits with three inoculation sites per fruit. Each dot represents the CI from a single replicate, and the black bar represents the mean CI of all replicates. Statistical significance was established by comparing CIs using ANOVA and Tukey's post hoc test. Groups with the same letter had values that were not statistically different ($P >$ 0.05). CIs of mutants were considered different when they did not group with the neutral mutant CEC1000 ($P <$ 0.05).

We tested whether *trpC*, *argA*, *ilvD*, *glnA*, *serA*, *thrC*, *gltB*, and *metA* are expressed within tomato fruits using resolvase-*in vivo* expression technology (RIVET) reporters that allow quantification of gene expression of the targets *in vivo*. One day after inoculation, the RIVET resolution for the reporters of *trpC*, *argA*, *ilvD*, *glnA*, *serA*, *thrC*, *gltB*, and *metA* was between 80 and 100%, and 3 and 7 days after inoculation, the resolution reached almost 100% for all reporters (Fig. 5). When tested in LB, the reporters were not resolved, while in M9 medium with low concentration of amino acids, the resolution reached high levels (Fig. 5). The results of the RIVET experiments are consistent with the fitness phenotypes of the corresponding mutants: amino acid biosynthesis genes are strongly expressed in tomatoes and are required for proliferation within the fruit.

Tomato genotype affects *Salmonella* fitness. Tomatoes carrying a ripening inhibitor (*rin*) mutation do not present the characteristic ripening phenotype, they do not accumulate lycopene, and their fruits do not soften. Fruits of *rin* mutant tomatoes accumulate many amino acids in different (generally larger) amounts during their development compared to the wild type (45). Because amino acids accumulate at higher levels within *rin* mutant tomatoes, we tested the hypothesis that the phenotypes of the amino acid biosynthesis mutants will be less severe than in the wild-type

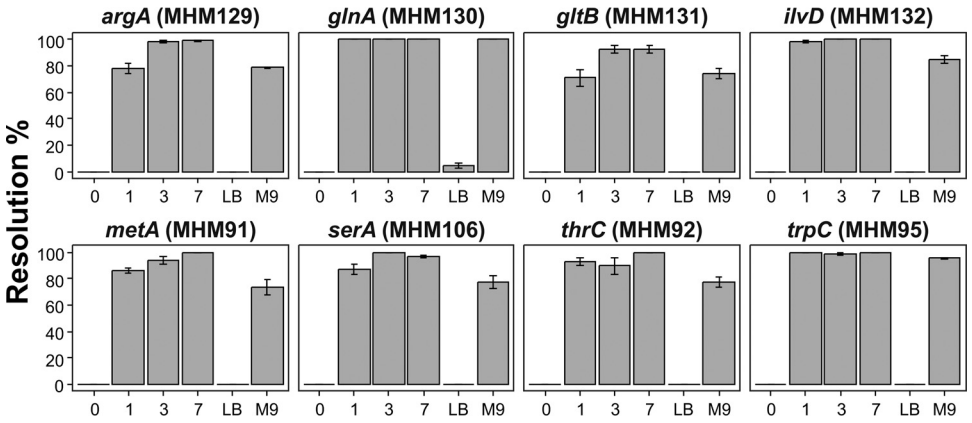

FIG 5 Expression of *Salmonella* genes involved in amino acid biosynthesis in tomatoes and in culture medium. Recombinase-*in vivo* expression technology (RIVET) reporters were used to determine gene expression within tomatoes on days 0, 1, 3, and 7, and in LB or M9 soft agar (0.7%). M9 medium was supplemented with 0.2% glucose and with the amino acid specific for the isogenic mutant (leucine, isoleucine, and valine for *ilvD*, arginine for *argA*, serine for *serA*, glutamine for *glnA*, glutamate for *gltB*, methionine for *metA*, tryptophan for *trpC*, and threonine for *thrC*). After incubation, bacteria were streaked onto XLD and then patched onto LB with tetracycline (20 μg/ml). Resolution is reported as a percentage of the total number of cells that lost tetracycline resistance. Bars are means, and error bars are standard errors. Three tomato fruits with three inoculation sites per fruit were used.

tomatoes. To this end, we performed competition assays using isogenic mutants in *trpC*, *argA*, *ilvD*, *glnA*, *serA*, *thrC*, *gltB*, and *metA* in the tomato cultivar Ailsa Craig and its *rin* homozygous mutant.

The competitive indices of *argA*, *gltB*, *thrC*, and *trpC* mutants were not affected by the tomato genotype, while isogenic mutants for *glnA*, *ilvD*, *metA*, and *serA* presented a reduction in their fitness within *rin* mutant tomatoes compared to wild-type tomatoes. The *metA* mutant exhibited the highest difference: in wild-type tomatoes, the CI indicated a 2-fold reduction compared to wild-type *Salmonella* spp., whereas in *rin* mutant tomatoes, the reduction was 32-fold (Fig. 6).

LPS biosynthesis is required for *Salmonella* proliferation in tomatoes. Most of the data presented so far underscore the differences in *Salmonella* requirements for animal and plant colonization. However, biosynthesis of LPS was needed under both conditions. Genes involved in LPS core and O-antigen synthesis, contained by the *rfa*

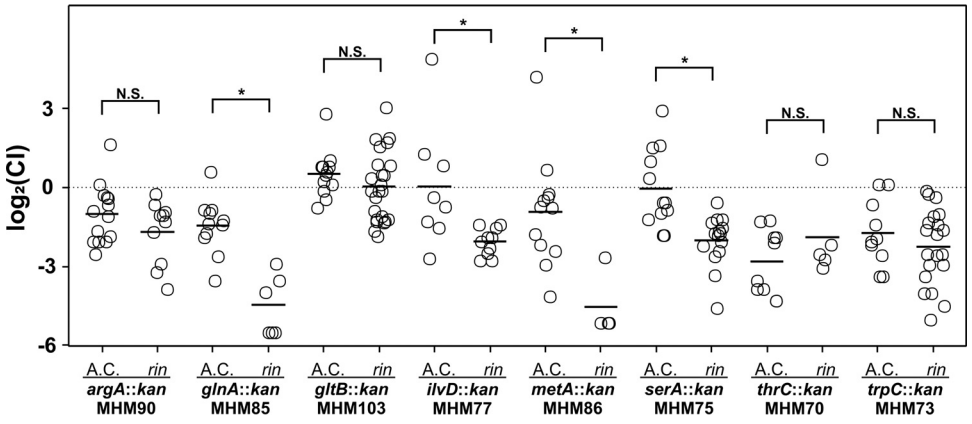

FIG 6 Fitness of *Salmonella* amino acid synthesis mutants in *rin* mutant tomatoes. Fruits of the greenhouse-grown tomatoes (cv. Ailsa Craig and the isogenic *rin* mutant) were harvested 34 days postanthesis and inoculated with 10^3 CFU of a 1:1 mixture of the *Salmonella* wild-type and isogenic mutants, followed by a 7-day incubation at 22°C. Bacterial cells were recovered on XLD agar, and the competitive index was calculated using the formula $\log_2[(MUT_{out}/WT_{out})/(MUT_{in}/WT_{in})]$. Each dot represents a replicate, and the black bar is the mean CI of all replicates. Statistical significance was established using a two-tailed *t* test between the CI of isogenic mutants in Ailsa Craig (wild-type) and *rin* mutant tomatoes. Asterisks mark groups that are statistically significant ($P < 0.05$), and N.S. marks groups that are not statistically significant ($P > 0.05$).

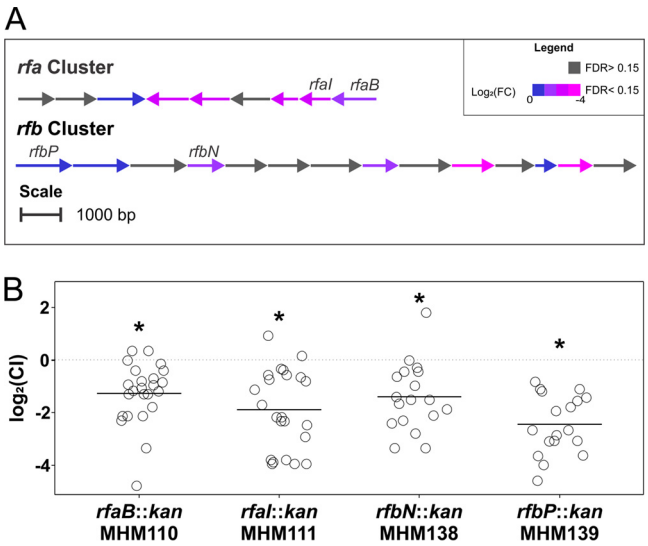

FIG 7 Fitness of the *Salmonella* LPS mutants in tomatoes. (A) A diagram of the *rfa and rfb* clusters in *Salmonella* using data extracted from the transposon insertion sequencing screening. Each arrow represents a gene in the cluster, and they are arranged according to their physical position and scaled to size. The arrows are color-coded for the fold change and FDR. (B) Competitive fitness of isogenic mutants involved in LPS biosynthetic pathways. Approximately 10^3 CFU of a mixture of the wild-type and an isogenic mutant was inoculated into each tomato. Competitive index (CI) was calculated using the formula $\log_2[(MUT_{out}/WT_{out})/(MUT_{in}/WT_{in})]$. The replicates were obtained from six tomato fruits with three inoculation sites per fruit. Each dot represents a replicate, and the black bar is the mean CI of all replicates. Statistical significance was established comparing CIs using ANOVA and Tukey's post hoc test. The CIs of isogenic mutants were considered different when they did not group with the neutral mutant CEC1000 ($P < 0.05$), and they are indicated with an asterisk.

and *rfb* clusters, respectively, were identified as required for tomato colonization in our screen, as well as in transposon insertion sequencing analyses of mouse colonization (Fig. 2 and 7A).

The competition assays using isogenic mutants for *rfaB*, *rfaI*, *rfbN*, and *rfbP* confirmed the transposon insertion sequencing data (Fig. 7B). All mutants had an ~4-fold fitness reduction. The similar CI for the mutants involved in the same biological process strongly suggests that the observed phenotype is related to the defective LPS biosynthesis and not to a secondary mutation.

The role of LPS during disease in animals has been extensively studied for several pathogens. LPS protects bacterial cells from antibacterial peptides produced by host organisms (reviewed in reference 46). *Salmonella* LPS mutants are severely attenuated during systemic infection and are incapable of developing disease. In bacterial plant pathogens, LPS is also a major virulence factor, protecting the pathogen against plant defenses. We hypothesize that the immunity mechanisms in plants and animals target the same cellular structures in bacterial cells, and *Salmonella*'s countermeasures may be similar in animal and plant hosts.

***Salmonella* inoculation titer affects fitness of SPI and nucleotide synthesis mutants.** The transposon insertion sequencing experiments suggested that genes involved in nucleotide biosynthesis and those that are a part of the *Salmonella* pathogenicity islands (SPI) 1, 2, and 3 were required for *Salmonella* full fitness during proliferation in tomatoes (Data Set S1). Auxotrophic mutants for purines and pyrimidines exhibited strong attenuation (Data Set S1). SPI-1, -2, and -3 are the major *Salmonella* virulence determinants required for the invasion of epithelial cells, macrophages, and survival during the intracellular phase; strains lacking any of these SPIs are avirulent in vertebrate animal models. However, the observed phenotypes of the SPI mutants were in direct contradiction to the previous study (9). It is of note that the inoculum doses used in the transposon insertion sequencing screen were ~4 to 5 orders of magnitude higher than in the study of infections in tomatoes performed by

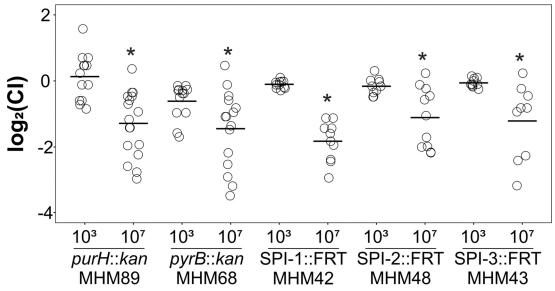

FIG 8 Fitness of SPIs and nucleotide biosynthesis mutants in tomatoes. Tomatoes were inoculated with 10^3 CFU or 10^7 CFU of isogenic mutants and the wild-type *Salmonella* (1:1). Competitive index (CI) was calculated using the formula $\log_2[(MUT_{out}/WT_{out})/(MUT_{in}/WT_{in})]$. The replicates were obtained from six tomato fruits with three inoculation sites per fruit. Statistical significance was established comparing CIs using ANOVA and Tukey's post hoc test. Isogenic mutant CIs were considered different when they did not group with the neutral mutant strain CEC1000 ($P < 0.05$), and they are marked with an asterisk.

Noel et al. (9). Therefore, we tested a hypothesis that the phenotypes of the SPI (and nucleotide synthesis) mutants were dependent on the inoculum dose.

Competition assay for the *pyrB*, *purH*, SPI-1, SPI-2, and SPI-3 mutants and the wild type were carried out with an inoculum of 10^3 and 10^7 CFU per tomato. The results of these competition assays showed that the reduction in fitness for all mutants tested was inoculum dependent, with phenotypes apparent only at the high inoculum doses (Fig. 8).

For *pyrB* and *purH* mutants, which are purine and pyrimidine auxotrophs, respectively, the competition to scavenge the nucleotides available in the environment will increase at higher titers, and the mutants unable to synthesize purines or pyrimidines *de novo* will have a stronger deficit for these compounds that will negatively affect their growth. Proteins encoded on SPI-1, -2, and -3 are not known to contribute to population density-dependent functions, and we do not have a ready explanation for the mutants' phenotype that was dependent on the inoculum density. We hypothesize that for the SPI-3 mutant, an increasing Mg^{2+} deficit could lead to the observed phenotype at higher concentrations. One of the genes present in SPI-3, *mgtC*, encodes a virulence factor required for intracellular proliferation under low-Mg^{2+} conditions. The screening data suggest that *Salmonella* in tomatoes has a deficit in Mg^{2+}, as indicated by the loss of fitness for mutants in the *phoPQ* system that is activated by low Mg^{2+}, and by the loss of fitness for *corA* mutants, which lack a Mg^{2+} channel.

DISCUSSION

The ability of human enteric pathogens, such as *Salmonella*, to robustly colonize plant tissues likely helps this pathogen build up populations that are high enough to then reinfect herbivorous hosts. This ability of *Salmonella* to exploit plants as a part of its life cycle and use them as vehicles to reach preferred animal hosts was recently supported by multitrophic experiments (47). *Salmonella* and enteropathogenic *E. coli* are not the only members of the *Enterobacteriaceae* family capable of forming successful relationships with both plants and animals. *Klebsiella pneumoniae* is both a nitrogen-fixing plant endophyte and an opportunistic pathogen in animals and humans (48). *Pantoea stewartii*, the causal organism of Stewart's wilt in sweet corn, spends a portion of its life within the gut of the corn beetle (49). *Dickeya dadantii* is a pathogen causing soft rots in plants and is an aphid pathogen (50). It is possible that these bacteria "repurpose" their metabolic and virulence genes to adapt to different hosts, or they may have evolved two distinct strategies for the infections of plants and animals. The hypothesis of distinct strategies is supported by the discoveries of two distinct type III secretion systems in *P. stewartii*, one used in plants and another in beetles (49).

To gain insight into the interactions of *Salmonella* spp. with their different hosts and to compare *Salmonella* behavior with that of the phytopathogens, we carried out high-throughput screens, followed by the comparative analyses. It appears that ~40%

of the genes required for tomato colonization by *Salmonella* are also involved in a systemic infection in mice, and 35% of the genes required for murine systemic infection are required for colonization of tomato fruits. The same pattern was found in the metabolic requirements for phytopathogens, where 42% of the mapped KO identifiers were shared with *Salmonella* colonization of tomatoes. This partial overlap suggests a certain degree of repurposing of the *Salmonella* metabolism to adapt to different hosts but also reveals metabolic versatility of this bacterium.

Salmonella mutants in amino acid biosynthesis genes had the most pronounced phenotype in tomatoes but were not required for murine systemic infection, as determined in high-throughput screenings (37, 38, 51) and competition assays with isogenic mutants (52). We focused on this group of genes to explore the differences between the requirements for interactions with animals and plants. Competition assays confirmed that the biosynthesis of tryptophan, arginine, branched amino acids, glutamine, serine, threonine, glutamate, and threonine is required for colonization of tomatoes. In this regard, *Salmonella* behavior closely paralleled that of phytobacteria: amino acid biosynthesis was required for plant colonization by the pathogens *Pseudomonas tolaasii* (43) and *X. campestris* pv. campestris (44) and by the rhizosphere colonizer *Pseudomonas fluorescens* WCS365 (53). The amino acid metabolism requirements for plant-associated bacteria could be a result of unbalanced amino acid concentrations in this environment. Amino acids appear to be a relatively minor component of the exudates of mature fruits, leaves, and roots of tomatoes (20), which is consistent with the phenotypes of the auxotrophic mutants.

Because tomatoes carrying the *rin* mutation are known to accumulate higher levels of certain amino acids (45), we tested the hypothesis that the auxotrophy of the *Salmonella* mutants will be at least partially complemented within *rin* mutant tomatoes. Surprisingly, this did not prove to be the case, and *Salmonella* auxotrophic *glnA*, *ilvD*, *metA*, and *serA* mutants were even less fit within *rin* mutant tomatoes than in the wild type. Differences between *Salmonella* proliferation in Campari and Ailsa Craig cultivars or between the wild type and the *rin* mutant could influence these results. Levels of *Salmonella* growth in Ailsa Craig (wild-type) and Campari tomatoes are not significantly different (35), but *Salmonella* growth is reduced in Ailsa Craig *rin* mutants (35). This reduction in growth might indicate that the reduction in fitness of amino acid biosynthesis mutants in *rin* mutant tomatoes could be even bigger and was masked by a limited number of cell doubling. Our observations suggest that either plant amino acids are not accessible to *Salmonella* spp. (due to their compartmentalization or chemical modification) or that *Salmonella* spp. can make better use of inorganic nitrogen sources during plant colonization.

LPS biosynthesis was a point of convergence between the requirements for *Salmonella* colonization of plants and animals, as indicated by our screening and confirmed for the competition assays with *rfaB*, *rfaI*, *rfbN*, and *rfbP* mutants. The LPS role for *Salmonella* virulence in animals is well established; mutants with a truncated LPS are attenuated, and *Salmonella* uses *phoPQ* and *pmrAB* to modify its LPS to evade the innate immunity through resistance against antibacterial peptides (46). Plants have antibacterial peptides that are not homologous to the ones found in animals, but they share the same mechanisms of action (reviewed in references 54 and 55). These plant-based antibacterial peptides are small cationic molecules that target the cell membranes, leading to destabilization and cell death. LPS is required by plant bacterial pathogens to avoid being targeted by the plants' antibacterial peptides. *Salmonella* LPS may similarly support bacterial proliferation in this environment.

Another point of convergence was the biosynthesis of nucleotides and the requirement for the *Salmonella* pathogenicity island 1 (SPI-1), SPI-2, and SPI-3. Competition assays confirmed phenotypes of these mutants only for high, but not low, titers. SPI-1 and SPI-2 T3SSs were shown to be important factors for plant colonization from high-inoculum (10^8 CFU/plant) experiments with *Arabidopsis* (24). It is possible that the phenotypes observed are related to increased intraspecies competition due to a relative scarcity of available resources within the plant. SPI-1 and SPI-2 may also be

needed to subvert immunity, which may become more important as more bacterial cell numbers are invading and therefore activating the plant's immune response. While *Salmonella* effectors were shown to be functional when directly transformed into cell wall-less plant protoplasts (22), it is not clear how they are delivered across the plant cell wall.

This study demonstrated that while *Salmonella* is capable of repurposing some of the genes it uses during animal infections to colonize alternate hosts (such as plants), the overlap between the sets of genes required for animal and plant infection is only ~40 to 50%. Conversely, only similarly partial overlap was observed when *Salmonella* genes required for tomato colonization were compared with the genes required for virulence of phytopathogens. Considering that 520 features required for fitness of *Salmonella* in tomatoes have no known functions, there remains much to be learned about the ecology of this human pathogen inside alternate hosts, such as plants.

MATERIALS AND METHODS

Bacterial strains and DNA manipulation. Bacterial strains were grown in LB broth at either 30°C or 37°C (as indicated in the text), and antibiotics were added at the following concentrations: 100 μg/ml ampicillin, 50 μg/ml kanamycin, and 20 μg/ml tetracycline, unless otherwise indicated. A full list of the strains and plasmids used in this study is in Table 1. *Salmonella* isogenic mutants used to confirm screening data were constructed using Datsenko-Wanner mutagenesis (56) by replacing a specific open reading frame (ORF) with the kanamycin resistance cassette from pKD4, followed by P22-mediated transduction into *S. enterica* serovar Typhimurium ATCC 14028. RIVET reporters to evaluate *in planta* gene expression were constructed as described previously (57); the kanamycin cassette from isogenic mutants was removed with FLP recombinase using pCP20, followed by the insertion of *tnpR-lacZ*, from pCE70 or PCE71 in the remaining FLP recombination target (FRT) site. The *tnpR-lacZ* cassette, which replaced almost the entire ORF of the deleted gene, was inserted immediately after the start codon. The tetracycline resistance gene *tetA*, flanked by the *res1* sites in a neutral site of the genome, was transduced using P22 to the final construct from the donor strain JS246. Primers used for cassette construction and deletions confirmation are listed in Table S1 in the supplemental material.

Plant material. Tomatoes of the cultivar (cv.) Campari were purchased at the local supermarket. Tomatoes of the cv. Ailsa Craig and its isogenic derivative *rin* mutant line were grown in a rooftop greenhouse. Tomatoes were tagged at anthesis and were harvested 34 days later to ensure that all tomatoes were in the same developmental stage, as described before (35).

Transposon insertion library construction. A library of *S. enterica* serovar Typhimurium 14028 Tn*5* insertion mutants was constructed using the Epicentre EZ-Tn*5* <T7/Kan2> promoter insertion kit. Briefly, primers Right_Tn_T7_Kan2 and Kan2_right_code were used in a standard PCR to add N_{18} barcodes to both sides of the EZ-Tn*5* <T7/KAN-2> transposon. The PCR product was gel purified using the QIAquick gel extraction kit (Qiagen), according to the manufacturer's recommendations. About 200 ng of the purified PCR product was used in an 8-μl transposase reaction mixture in 0.17% glycerol that also contained 1 μl of TypeOne restriction inhibitor and 2 U of EZ-Tn*5* transposase. The reaction mixture was incubated at room temperature for 3 h and subsequently dialyzed against water for 30 min before electroporation into fresh electrocompetent cells of *S. enterica* serovar Typhimurium 14028 (MZ1597). Transformed cells were isolated on LB agar plates with kanamycin (60 μg/ml) after overnight growth at 37°C, enumerated, and collected. The final library consisted of a pool of about 280,000 independent colonies.

Mapping of library barcodes to the *S. enterica* serovar Typhimurium ATCC 14028 genome. Genomic DNA of the obtained library of *S. enterica* serovar Typhimurium ATCC 14028 Tn*5* insertion mutants was prepared using the GenElute bacterial genomic DNA kit (Sigma-Aldrich). The DNA was fragmented and ligated to Illumina primers using standard methods. Approximately 150 ng of this material was then used to PCR amplify the regions flanking the transposon insertion sites, in a stepwise PCR regimen. In the first PCR, primer Illumina_P5_Read1 was paired with a primer that aligned with a region close to either the left end (primer Tn5_Left_CGTACA_Read2) or the right end (primer Tn5_Right_ACATGC_Read2) of the inserted transposon. PCR proceeded for 20 cycles, using 1.25 U of *Taq* polymerase and under standard conditions. Exactly 1/10 of the 1st PCR product was subsequently used in a 2nd PCR that engaged Illumina primers Illumina_P5_Read1 and Illumina_P7 and proceeded for 10 cycles. Products were subjected to QIAquick PCR product purification (Qiagen), according to the manufacturer's recommendations. The material was subsequently Illumina sequenced for 150-bp reads at both ends. Sequence analysis to map the barcode to the exact location on the genome is described further below.

Screen optimization. To ensure that the inoculum representative of the transposon insertion library was seeded into tomatoes and that a mutant's fitness could be assessed by changes in its relative abundance, competitive fitness assays with mutants known to have a phenotype in tomatoes (*hns*::*kan*, Δ*bcsA* Δ*lpfA* Δ*fadH cysB*::*kan*, *phoN*::*kan*, and *rcsA*::*kan*) were carried out. Although not all combinations of these mutants were tested in all preliminary experiments, we compared the impact of the different inoculum doses (10^4 and 10^7) and various ratios of the mutants to the wild type. Prior to the infections into tomatoes, strains were grown for 16 h in LB broth at 37°C 250 rpm. The cultures were pelleted, washed in phosphate-buffered saline (PBS) twice, and diluted 1:10, reaching a final density of approxi-

TABLE 1 Strains used

Strain name	Genotype	Reference/source or construction
MHM80	ATCC 14028 *glnA15*::FRT-*kan*-FRT	Constructed using Datsenko and Wanner mutagenesis with primers MHM197 and MHM198, confirmed with primers MHM199 and MHM200
MHM85	ATCC 14028 *glnA15*::FRT-*kan*-FRT	*S. enterica* serovar Typhimurium ATCC 14028 × P22/MHM80
MHM101	JS246 *glnA*15::FRT-*kan*-FRT	JS246 × P22/MHM85
MHM130	JS246 *glnA*15::*tnpR-lacZY*	JS246 *glnA*15::FRT electroporated with pCE71
MHM84	ATCC 14028 *argA*16::FRT-*kan*-FRT	Constructed using Datsenko and Wanner mutagenesis with primers MHm201 and MHM202, confirmed with primers MHM276 and MHM277
MHM90	ATCC 14028 *argA*16::FRT-*kan*-FRT	*S. enterica* serovar Typhimurium ATCC 14028 × P22/MHM84
MHM97	JS246 *argA*16::FRT-*kan*-FRT	JS246 × P22/MHM90
MHM129	JS246 *argA*16::*tnpR-lacZY*	JS246 *argA*16::FRT electroporated with pCE70
MHM63	ATCC 14028 *proA*17::FRT-*kan*-FRT	Constructed using Datsenko and Wanner mutagenesis with primers MHM205 and MHM206, confirmed with primers MHM207 and MHM208
MHM74	ATCC 14028 *proA*17::::FRT-*kan*-FRT	*S. enterica* serovar Typhimurium ATCC 14028 × P22/MHM63
MHM65	ATCC 14028 *metA*18::FRT-*kan*-FRT	Constructed using Datsenko and Wanner mutagenesis with primers MHM209 and MHM210, confirmed with primers MHM211and MHM212
MHM69	ATCC 14028 *metA*18::FRT-*kan*-FRT	*S. enterica* serovar Typhimurium ATCC 14028 × P22/MHM65
MHM86	JS246 *metA*18::FRT-*kan*-FRT	JS246 × P22/MHM65
MHM91	JS246 *metA*18:: *tnpR-lacZY*	JS246 *metA*::FRT electroporated with pCE70
MHM62	ATCC 14028 *serA*19::FRT-*kan*-FRT	Constructed using Datsenko and Wanner mutagenesis with primers MHM213 and MHM214, confirmed with primers MHM215 and MHM216
MHM75	ATCC 14028 *serA*19::FRT-*kan*-FRT	*S. enterica* serovar Typhimurium ATCC 14028 × P22/MHM62
MHM98	JS246 *serA*19::FRT-*kan*-FRT	JS246 × P22/MHM62
MHM106	JS246 *serA*19::*tnpR-lacZY*	JS246 *serA*19::FRT electroporated with pCE71
MHM64	ATCC 14028 *thrC*20::FRT-*kan*-FRT	Constructed using Datsenko and Wanner mutagenesis with primers MHM217 and MHM218, confirmed with primers MHM219 and MHM220
MHM70	ATCC 14028 *thrC*20::FRT-*kan*-FRT	*S. enterica* serovar Typhimurium ATCC 14028 × P22/MHM64
MHM87	JS246 *thrC*20::FRT-*kan*-FRT	JS246 × P22/MHM64
MHM92	JS246 *thrC*20::*tnpR-lacZY*	JS246 *thrC*20::FRT electroporated with pCE70
MHM66	ATCC 14028 *trpC*21::FRT-*kan*-FRT	Constructed using Datsenko and Wanner mutagenesis with primers MHM221 and MHM222, confirmed with primers MHM223 and MHM224
MHM73	ATCC 14028 *trpC*21::FRT-*kan*-FRT	*S. enterica* serovar Typhimurium ATCC 14028 × P22/MHM66
MHM88	JS246 *trpC*21::FRT-*kan*-FRT	JS246 × P22/MHM66
MHM95	JS246 *trpC*21::*tnpR-lacZY*	JS246 *thrC*21::FRT electroporated with pCE70
MHM72	ATCC 14028 *ilvD*22::FRT-*kan*-FRT	Constructed using Datsenko and Wanner mutagenesis with primers MHM225 and MHM226, confirmed with primers MHM270 and MHM270
MHM77	ATCC 14028 *ilvD*22::FRT-*kan*-FRT	*S. enterica* serovar Typhimurium ATCC 14028 × P22/MHM72
MHM96	JS246 *ilvD*22::FRT-*kan*-FRT	JS246 × P22/MHM72
MHM132	JS246 *ilvD*22::*tnpR-lacZY*	JS246 *ilvD*22::FRT electroporated with pCE70
MHM83	ATCC 14028 *purH*23::FRT-*kan*-FRT	Constructed using Datsenko and Wanner mutagenesis with primers MHM233 and MHM234, confirmed with primers MHM270 and MHM271
MHM89	ATCC 14028 *purH*23::FRT-*kan*-FRT	*S. enterica* serovar Typhimurium ATCC 14028 × P22/MHM83
MHM67	ATCC 14028 *pyrB*24::FRT-*kan*-FRT	Constructed using Datsenko and Wanner mutagenesis with primers MHM237 and MHM238, confirmed with primers MHM239 and MHM240
MHM68	ATCC 14028 *pyrB*24::FRT-*kan*-FRT	*S. enterica* serovar Typhimurium ATCC 14028 × P22/MHM67
MHM94	ATCC 14028 *gltB*25::FRT-*kan*-FRT	Constructed using Datsenko and Wanner mutagenesis with primers MHM282 and MHM283, confirmed with primers MHM284, MHM285 and MHM286
MHM103	ATCC 14028 *gltB*25::FRT-*kan*-FRT	*S. enterica* serovar Typhimurium ATCC 14028 × P22/MHM94
MHM126	JS246 *gltB*25::FRT-*kan*-FRT	JS246 × P22/MHM94
MHM131	JS246 *gltB*25::*tnpR-lacZY*	JS246 *gltB*25::FRT electroporated with pCE70
MM_015_H02 (MHM110)	ATCC 14028 *rfbA*26::FRT-*kan*-FRT	38
MM_015_G02 (MHM111)	ATCC 14028 *rfaI*27::FRT-*kan*-FRT	38
MM_011_G12 (MHM138)	ATCC 14028 *rfbN*28::FRT-*kan*-FRT	38
MM_011_D12 (MHM139)	ATCC 14028 *rfbP*30::FRT-*kan*-FRT	38
MHM42	ATCC 14028 (*invH-avrA*)::FRT	*S. enterica* serovar Typhimurium ΔSPI-1 (23)
MHM43	ATCC 14028 (*sugR-mtgC*)::FRT	*S. enterica* serovar Typhimurium ΔSPI-3 (23)
MHM48	ATCC 14028 (*ssrB-ssaU*)::FRT	*S. enterica* serovar Typhimurium ΔSPI-2 (23)
MHM53	ATCC 14028 *phoN*::FRT-*kan*-FR	23

mately 10^8 CFU/ml. When 10^7 CFU of *Salmonella* was inoculated into tomatoes, we were able to reliably observe even modest decreases in fitness. Therefore, this inoculation dose was selected for the library screen.

Library screening. The MZ1597 library was screened in Campari tomatoes using the tomato wound model to identify loci that affect *Salmonella* fitness in this environment. We chose this model based on the FDA assessment that wounds and punctures are a potential route for internalization of human pathogens (such as *Salmonella*) in fresh fruits and vegetables, and specifically in tomatoes

(http://www.fda.gov/Food/GuidanceRegulation/HACCP/ucm082063.htm). MZ1597 cultures were grown (with shaking at 250 rpm) for 16 h in LB broth supplemented with kanamycin at 37°C. The cultures were pelleted, washed in PBS twice, and diluted 1:10, reaching a final density of approximately 10^8 CFU/ml; 3 μl of this suspension was inoculated into three shallow (2- to 3-mm deep, 1 mm in diameter) wounds in tomato pericarps ($\sim 10^6$ CFU per tomato) using a small pipette tip insertion under the epidermis of the tomato fruits. Tomatoes were incubated at 22°C and a relative humidity of ~60% for 7 days, a period of time that allows *Salmonella* to reach the carrying capacity of tomato fruits and approximates the time period between tomato harvest and consumption. *Salmonella* was recovered by collecting ~1-g samples of the pericarp around the inoculation site; samples from the same fruit were combined and homogenized in a stomacher (Sevard). *Salmonella* cells were recovered by centrifugation and were then resuspended in 50 ml of LB broth, followed by 6 h growth at 37°C and 250 rpm, reaching $\sim 10^8$ CFU/ml. One milliliter of culture was recovered and used for library preparation.

Library preparation for sequencing. Aliquots of around 5×10^7 CFU from input and output libraries were subjected to three washes in water, followed by proteinase K (100 μg) digestion for 1 h at 55°C in lysis buffer (10 mM Tris [pH 8.0], 1 mM EDTA, 0.1% Triton X-100). After inactivation of the enzyme for 10 min at 95°C, a nested PCR regimen was performed to specifically amplify the DNA regions adjacent to the inserted transposons. Primers 1st_PCR_Tn5_EZ_left_reverse/Left_Forward_201 and Right_Reverse_fixed/Right_Forward_983 were used to amplify the left and right flanks, respectively, in a 20-cycle PCR in 50 μl of 1× Kapa HiFi reaction mixture. One microliter of the successful PCR product was then utilized as the template in a second rate-limited PCR amplification using 1.25 U of *Taq* (Invitrogen) and 0.1 mM dinucleoside triphosphates (dNTPs), with primers 2nd_PCR_Tn5_EZ_Right_Forward and Tn_SetYY_XXXXXXXX. The second PCR amplified the (already enriched) right flank of the transposon insertion site. Products of the second PCR were pooled and subjected to QIAquick PCR product purification (Qiagen), according to the manufacturer's recommendation. Illumina sequencing proceeded with custom primers Tn5_EZ_Right_Seq_fixed and Tn5_EZ_Index_Seq_new for a single indexed run, with a read length of 25 bases.

Sequence analysis. For mapping of the barcodes introduced into the mutants of the transposon insertion library, the Tn*5* primers and the corresponding N_{18} sequence flanking the Tn*5* priming sites were trimmed from the raw sequence reads using custom Perl scripts. The trimmed reads were mapped to the *S.* Typhimurium 14028 reference genome using Bowtie2. PCR duplicate reads were removed using Picard tools (https://github.com/broadinstitute/picard). Only reads mapped in proper pairs were considered for further downstream analysis. The left-most position of read 2 for each mapping was extracted from the SAM alignments using custom scripts, and this was identified as the Tn*5* insertion site in a strand-specific manner. The N_{18} barcode tag for each mapped read was then identified from the raw untrimmed reads using custom Perl scripts. The above-mentioned analysis identified the N_{18} barcode tag flanked by conserved priming sites for each Tn*5* insertion mutant.

For the identification of barcoded mutants, raw sequencing data consisted of single-end 25-bp reads. The first 18 bases, which represented the unique N_{18} tag for each Tn*5* mutant, were extracted, and the abundance of all unique 18-mers was calculated using custom Perl scripts. The abundances of all N_{18} barcodes mapped within each annotated genome feature were summed in a strand-specific manner. This represented the aggregated abundance for each feature in the coding strand and the noncoding strand. The aggregated abundances for the input and output libraries were statistically analyzed using edgeR, and the $\log_2$ fold changes and FDRs were reported.

Competition assays. Competition assays were used to estimate the fitness of specific mutants. Overnight cultures of the wild type and an isogenic mutant were adjusted based on optical density at 600 nm (OD_{600}) to the same population density and combined in a 1:1 ratio. The combined cultures were washed three times in PBS and diluted 10,000-fold, and 3 μl of the diluted mixture was inoculated into the tomato pericarp in three separate shallow wounds ($\sim 10^3$ CFU per tomato). An aliquot of the inoculum was plated onto xylose lysine deoxycholate (XLD) plates to enumerate CFU. The inoculated tomatoes were incubated at 22°C and relative humidity ~60% for 7 days, and *Salmonella* cells were recovered by inserting a loop into the wound and streaking the material onto XLD plates. The competition index was calculated as previously described (9) using the formula $\log_2([MUT_{out}/WT_{out}]/[MUT_{in}/WT_{in}])$, where MUT is the number of mutant colonies recovered, WT is the number of wild-type colonies recovered, "in" represents the cultures inoculated in tomatoes, and "out" represents the colonies recovered after the experiment. Competition index (CI) statistical significance values of isogenic mutants were compared against the CI for the neutral mutant CEC1000 using analysis of variance (ANOVA). The CI significance for the *rcsA*::*kan* mutant against CEC1000, the CI for isogenic mutants in the wild type against *rin* mutant tomatoes, and the comparison of different inoculation titers were done using a pairwise *t* test. The software JMP version 12 was used for all CI analyses.

RIVET assays. RIVET reporters were used to evaluate *Salmonella* gene expression *in planta*. In this system, gene expression is quantified as the percentage of cells that lost tetracycline resistance [which ultimately results from the activation of the promoter of interest that leads to the transcription of recombinase *tnpR*, and TnpR-catalyzed excision of *tetA* flanked by *res1* sites, causing tetracycline sensitivity (57)]. For RIVET assays in tomatoes, *Salmonella* strains were grown in LB broth with tetracycline for 16 h at 37°C. Cultures were then washed extensively in PBS to remove traces of the medium and the antibiotic, and inoculum suspensions in PBS were seeded into tomato pericarps ($\sim 10^3$ cells per tomato) in three separate shallow wounds. Cells were recovered 1, 3, and 7 days after inoculation using a sterile wire loop, and the material was streaked onto XLD plates. For the positive control of RIVET activation, cultures were grown in M9 agar (0.7%) supplemented with the appropriate amino acids (leucine, isoleucine, and valine for *ilvD*, arginine for *argA*, serine for *serA*, glutamine for *glnA*, glutamate for *gltB*,

methionine for *metA*, tryptophan for *trpC*, and threonine for *thrC*). The tested amino acid concentrations ranged from 2 mg/ml to 8 μg/ml in a 2-fold dilution series, and cells were recovered from the lowest concentration that allowed for growth of the reporter. Cultures were then streaked onto XLD. The percentage of *Salmonella* cells sensitive to tetracycline recovered from tomatoes and M9 with the amino acid was estimated by plating the colonies from XLD plates into LB tetracycline plates. Similar control experiments were done in LB agar (0.7%), only without the supplementation with amino acids.

Metabolic mapping and functional characterization. Genes required for *Salmonella* systemic infection in mice were retrieved from data sets deposited with the original publications using parameters established by the authors of the original papers. For the study by Chaudhuri et al. (37), a *P* value of <0.05 and $\log_2$(fold change) of less than -1 were used; for the study by Santiviago et al. (38), a *P* value of <0.0005 and $\log_2$(fold change) of less than -0.75 were used; and for the study by Silva et al. (51), a *P* value of <0.001 and $\log_2$(fold change) of less than -0.75 were used. BlastKOALA (58) was used to assign KEGG Orthology (KO) terms for *Salmonella enterica* ATCC 14028 coding sequences, and the KEGG Mapper Web interface was used to visualize metabolic pathways. Gene Ontology (GO) term enrichment was performed with Panther (59), with Bonferroni's correction.

Genes required for bacterial phytopathogens to elicit disease in their preferred plant host were extracted from previously published studies (39–42, 44, 60), and the KO terms for them were also retrieved with BlastKOALA. It should be noted that the results of mutant screens in phytopathogens were less comprehensive than the high-density transposon screens. We also note that, generally, screens in phytopathogens identified fewer (10 to 200) functions required for virulence in plants, which may also represent more stringent screening conditions than those of the studies with high-density transposon insertion libraries.

Growth in LB. To evaluate potential growth defects of isogenic mutants, we compared their growth kinetics with the wild-type strain. Cultures grown for 16 h at 250 rpm and 37°C in LB broth were diluted to an OD_{600} of ~0.02 in 50 ml of LB broth. Three replicate cultures of each strain were incubated at 37°C and 250 rpm. Samples were withdrawn hourly, and the OD_{600} was measured with a BioSpec-mini spectrophotometer (Shimadzu).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.03028-16.

TEXT S1, PDF file, 0.3 MB.

DATA SET S1, XLSX file, 0.8 MB.

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