COVER SHEET

TITLE: Identification of Salmonella enterica genes that influence plant host association

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Identification of *Salmonella enterica* genes that influence plant host association

Human enteric pathogens, such as *Salmonella enterica*, spend a significant portion of their life cycle associated with plant hosts. However, the specific genes required for *S. enterica* plant colonization have only begun to be examined. The goal of this study was to rapidly identify genes not previously known to influence *S. enterica* colonization of plants. To do so, a mutant library containing 40,000 unique transposon insertions was used to screen the entire *S. enterica* genome for fitness in germinating alfalfa sprouts over time. Transposon insertion sequence analysis revealed a number of genes related to metabolism, amino acid biosynthesis, virulence, transcriptional regulation, hypothetical and putative proteins, and non-coding intergenic regions that are required for *S. enterica* colonization of plants over time. The results of this study put us closer to creating novel strategies that prevent human enteric pathogens from colonizing fresh produce, leading to an overall improvement in food safety.

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ABSTRACT
Human enteric pathogens, such as *Salmonella enterica*, spend a significant portion of their life cycle associated with plant hosts. However, the specific genes required for *S. enterica* plant colonization have only begun to be examined. The goal of this study was to rapidly identify genes not previously known to influence *S. enterica* colonization of plants. To do so, a mutant library containing 40,000 unique transposon insertions was used to screen the entire *Salmonella* genome for fitness in germinating alfalfa sprouts over time. Transposon insertion sequence analysis revealed a number of genes related to metabolism, amino acid biosynthesis, virulence, transcriptional regulation, as well as hypothetical and putative proteins that are required for *S. enterica* colonization of plants over time. Interestingly, many non-coding intergenic regions were also shown to be important for *S. enterica* colonization of plants. The mutant screen results were validated by co-inoculation assays with single deletion mutants in genes of interest representing each of the major gene categories the mutant library screen showed important for plant colonization. The co-inoculation assays confirmed that biosynthesis of both mannose-6-P and serine are required for plant colonization. Surprisingly, genes on the *Salmonella* Pathogenicity Island-2, previously only shown as important for animal host infection, are also important for successful plant colonization. Finally, the transcriptional regulator *yncC* becomes increasingly important for colonization over time. The results of this study shed light on novel genes related to *S. enterica* plant host colonization, which put us closer to creating novel strategies that prevent human enteric pathogens from colonizing fresh produce, leading to an overall improvement in food safety.

INTRODUCTION

Salmonella enterica contamination of fresh produce is the leading cause of salmonellosis annually in the United States (Scallan *et al.,* 2011). Though animals are significant reservoirs of *S. enterica* (Hutchison *et al.,* 2005), they no longer represent the most common route of transmission to humans. Though *S. enterica* is considered a human pathogen, plants are a recognized alternative host (Jablasone *et al.,* 2005). Animal feces containing enteric pathogens such as *S. enterica* are generally spread to agricultural fields directly as fertilizer or through irrigation or floodwater (Lewis *et al.,* 2005). Consumption of contaminated produce is the link

that will lead to human foodborne illness. Interestingly, the least understood portion of the *S. enterica* lifestyle is its association with plant hosts. Knowledge gained from this research will highlight innovative approaches to achieving greater food safety.

S. enterica has been found to colonize the roots, stems, leaves, and fruit of many fresh produce crops such as sprouted seeds, leafy greens, and tomatoes (Barak and Schroeder, 2012; Jablasone *et al.,* 2005). Sprouted seeds are the most common raw produce products that have been linked to outbreaks of foodborne illness (Barak and Schroeder, 2012; Martinez-Vaz *et al.,* 2014). They provide a very conducive environment for the growth of human enteric pathogens. Exudates released by germinating seeds are rich in nutrients; *S. enterica* can rapidly multiply from cell densities of 10^3 to 10^9 CFU/ml in only 48 hours (Hao *et al,* 2012). *S. enterica* preferentially colonizes the spermosphere, the highly dynamic zone of soil surrounding germinating seeds, and early rhizosphere of plants due to the abundance of nutrients exuded by imbibing seeds and subsequent roots (Barak and Schroeder, 2012; Nelson, 2004). The microbial composition in the rhizosphere of any given plant is largely determined by its root exudates. These exudates contain sugars, amino acids, and many other organic molecules that support microbial growth (Badri and Vivanco, 2009; Bais *et al.,* 2006). Currently, little is known as to how the chemical makeup of exudates varies from one plant to another. Even less is understood about how the differences in exudates may influence the metabolic activity of microbes in the rhizosphere (Bais *et al.,* 2006; Kwan *et al.,* 2015 and Roberts *et al.,* 2009). This presents a food safety issue because the infectious dose of *S. enterica* to cause salmonellosis in humans is only 10 to 100 cells; higher pathogen cell density increases the likelihood an infective dose will be consumed (Harris *et al,* 2003).

The intimate interactions between *Salmonella* and its plant hosts are both elaborate and nuanced. Previously characterized *S. enterica* factors important for successful plant growth and colonization include amino acid transport and biosynthesis (Kwan *et al.,* 2015), cellulose and curli production (Barak *et al.,* 2007; Cowles *et al.,* 2016), O-antigen capsule formation (Barak *et al.,* 2007; Marvasi *et al.,* 2013), as well as iron acquisition (Nugent *et al.,* 2015) and siderophore biosynthesis (Hao *et al.,* 2012). Taken together, it is clear that *S. enterica* has evolved a wide range of adaptations allowing it to be a successful member of the plant microbiome. This study set out to discover additional *S. enterica* genes that influence plant host association.

Until now, techniques for exploring specific *S. enterica* genes and pathways important for plant colonization have been relatively slow. The process of creating single or multiple deletion mutants is time consuming and labor intensive, creating a need for more efficient high-throughput assays. In recent years, transposon mutant libraries and sequence analysis have become extremely effective methods to study bacteria-host interactions. Many transposon mutant libraries for various *S. enterica* serovars have been generated and used for screens in animal hosts (Carnell *et al.,* 2007; Hensel *et al.,* 1996), and are just now being used for plant host screens (de Moraes *et al.,* 2017). The transposon mutant library in this study contained a pool of 40,000 (40K) independent mutants, constructed to be highly saturated with Tn5 insertions in both the coding and noncoding regions (Canals et al., 2012). Each mutant in the 40K pool contains a single transposon insertion that is uniquely labeled, making it possible to determine exactly which genes or genomic regions are disrupted. In this way, genes that influence *S. enterica* growth and colonization in germinating alfalfa sprouts were rapidly identified when input and output library pools were compared. Transposon sequence analysis revealed a large number of genes differentially recovered in the output pool compared to the input pool related to

metabolism, attachment, motility, animal virulence, and two-component signaling systems that had not yet been studied in the context of a plant host. Subsequently, site-directed mutants were created for genes of interest and were individually tested in co-inoculation assays to confirm the results of the mutant library screen. The results of this study help to shed additional light on the robust molecular mechanisms that makes *Salmonella* such a ubiquitous and flourishing member of the plant microbiome.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The bacterial strains and plasmids used in this study are listed in Table 1. Strains were cultured in lysogeny broth (LB) and grown at 37°C with shaking at 200 rpm for 8 h. If necessary, antibiotics were added at the following concentrations: chloramphenicol (Cm), 30 µg ml^{-1}; kanamycin (Kan), 50 µg ml^{-1}; and nalidixic acid (Nal), 30 µg ml^{-1}.

Transposon mutant library screen. In order to prevent a sequencing bottleneck and ensure mutants in the screen were actually influenced by selection and not by chance, ten times the number of CFU/ml present in the input pool needed to be recovered at each time point (output pools). A bottleneck in this study means not enough CFU/ml were recovered in an output pool to provide a representation of the entire 40K library. Optimization revealed 10^5 CFU/ml was a sufficient inoculum load to recover enough bacterial cells for accurate assessment of mutant fitness. Mutant library cultures were normalized to 10^8 CFU/ml at optical density at 600 nm (OD_{600}), which was verified by dilution plating using a spiral plater. Three aliquots of this inoculum (input pool) were taken and frozen at -80°C to await sequencing. Alfalfa seeds were surface sterilized (Cowles *et al.*, 2016). Seeds were then germinated in 20 ml of 10^5 CFU/ml mutant library suspension (input pool) and incubated at 24°C in full light with gentle swirling at

~40rpm for three days. Ever 24 h, aliquots of the library growing in the alfalfa irrigation water were sampled and placed in 5 ml LB broth for an 8 h grow-out (to reach a cell density of ~10^8CFU/ml) at 37°C with shaking at 200 rpm; CFU/ml were verified by dilution plating using a spiral plater. Additionally, alfalfa seeds with seed coats (at 24 hpi) or without seed coats (at 48 hpi and 72 hpi) were sampled. Individual seeds or sprouts were placed in a 50 ml conical tube with 35 ml sterile water and vortexed for 30 s to remove any loosely attached bacterial cells. Washed seeds or sprouts were then homogenized in 500 µl sterile water and CFUs/ml were enumerated. Aliquots of seed homogenate were placed in 5 ml LB broth for an 8 h grow-out at 37°C with shaking at 200 rpm. Samples from both the irrigation water and seed homogenate grow-out were pelleted and frozen at -80°C to await sequencing. Transposon sequencing was performed in the lab of Dr. Michael McClelland at UC-Irvine as described by de Moraes *et al.*, 2017.

Mutant construction, confirmation, and complementation. All mutant strains were constructed in *S. enterica* serovar Typhimurium strain 14028s. A mutation in *serA* was generated using the λ-Red recombination method (Datsenko & Warner, 2000). The *manA* mutant was generated with the same protocol for a separate project by Dr. Grace Kwan. All other mutants were obtained from a mutant library collection (Santiviago *et al.*, 2009). All deletion-insertion mutations were confirmed by PCR. Genetic complementation of Δ*serA::*Cm and Δ*manA*::Cm was done by cloning *serA* or *manA* into pEVS141. Cloned vectors were then transformed into respective mutant strains. Plasmid DNA was extracted, confirmed by PCR, and sequenced to ensure there were no errors present in the cloned gene.

Co-inoculation assays in germinating alfalfa. Broth cultures for each strain were suspended in deionized sterile water. Each bacterial suspension was normalized to ~10^8 CFU/ml by

determining OD_{600}. Dilution plating using a spiral plater was used to verify that there was a 1:1 ratio (±10%) of wild type to each mutant. It became clear that at OD_{600}=0.200, not all strains were at a 1:1 ratio with wild type. The mutant OD_{600} values were adjusted to ensure that the seed assays were started with equal populations of all mutant strains and wild type. Co-inoculation assays were performed using the same protocol as for the 40K mutant library screen above. Due to technical variability between experiments, inoculum was mixed in ratios of 0.9:1.1, 1:1, and 1.1:0.9, to ensure a true 1:1 ratio of wild type to mutant. Seed and irrigation water were sampled in triplicate at each time point, and three biological replicates were performed per mutant unless otherwise noted.

Statistical analysis. Statistical analyses for transposon mutant library assays were performed by Dr. Michael McClelland at UC-Irvine. Statistical analyses for co-inoculation assays were performed using R software as previously described (Kwan *et al.,* 2015). For co-inoculation assays, percent total population values for each biological replicate were calculated using CFU/ml data for both mutant and wild type at all time points. The average mutant population percentages were compared to $\mu=50$ in one sample t-tests (significant if $p<0.1$). Because strains were inoculated in a 1:1 ratio, mutants with no competitive defect would comprise 50% of the total population.

RESULTS

Many *S. enterica* genes and intergenic regions influence plant colonization. To rapidly identify loci in the *S. enterica* genome that are important for plant colonization, a transposon mutant library was used to screen the entire genome for fitness, by taking advantage of high-throughput sequence analysis. The transposon mutant library employed in this study contained a pool of 40,000 (40K) independent Tn5 insertion mutants (Figure 1). The transposon mutations

randomly disrupt coding and intergenic regions of the genome. The mutant library assay was designed to screen for *S. enterica* fitness in two environments: colonizing seedlings and growth in root exudates over time. Three input pool aliquots from 0 hpi were averaged and compared to the mean of six seed colonization samples and three exudate growth samples every 24 h for three days. Upon sequencing and analysis, it was noted that the 48 hpi output pool sample populations experienced a bottleneck, which prevented reliable assessment of fitness for genes at that time point. To determine patterns of fitness over time, fold change from the input pool was calculated at both 24 hpi (Table 2) and 72 hpi (Table 3).

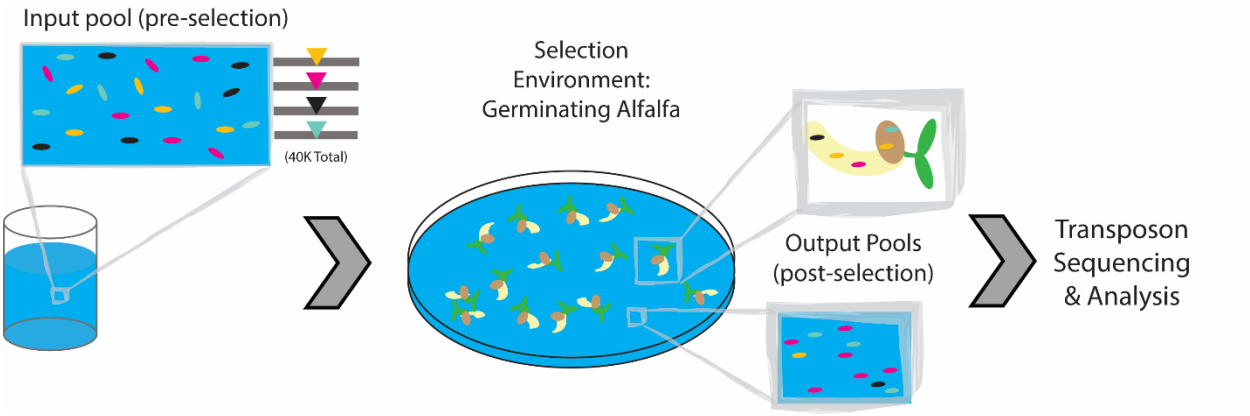

Figure 1. Using a 40K random insertion transposon mutant library, *S. enterica* genes contributing to plant colonization were identified by comparing the input (control) and output (experimental) pools after being subjected to selection for plant colonization. The mutants that were lost (selected against) were considered important for survival in this environment.

The mutant library screen revealed genes that reduce *S. enterica* fitness in a plant host environment. Mutations in these genes result in increased fitness. At 24 hpi, a mutation in *yjcC*, an EAL-containing phosphodiesterase (PDE) resulted in a greater than 10-fold increase in seedling colonization compared to the input pool (Table 2). At 72 hpi, mutations in genes encoding additional EAL-containing PDEs, flagellar genes, and *bcsA*, the gene encoding cellulose synthase, conferred fitness advantages in both exudate growth and seedling

colonization (Table 3). The screen also revealed many genes that are required for *S. enterica* colonization of plants. Mutations in these genes resulted in reduced fitness. At both 24 (Table 2) and 72 hpi (Table 3), a substantial number of genes related to amino acid transport, biosynthesis and degradation, carbon metabolism, virulence, and transcriptional regulation are important for both growth and colonization. Mannose-6-P biosynthesis is required at both 24 and 72 hpi, as a *manA* mutant exhibits a 20- and 1000-fold decrease respectively in seed colonization compared to the input pool. Mutants for *de novo* serine biosynthesis also exhibited colonization defects (Tables 2 and 3). Surprisingly, the *Salmonella* Pathogenicity Island-2 (SPI-2) type-3 secretion system (T3SS) apparatus, secreted effector proteins, and regulatory genes also appear important for plant colonization (Table 4 & Figure 2C). In addition, YncC, a transcriptional regulator, becomes important for colonization by 72 hpi (Table 3). Many putative or hypothetical proteins that have not yet been characterized are also important for both growth and colonization (Tables 2 and 3). Additionally, loci at many intergenic regions, which include could include small regulatory RNAs and regulatory elements, are important for *S. enterica* in plant host association (Tables 2 and 3).

Due to variability in abundance of recovered mutants between seed colonization samples at 24 hpi (output pools, n=6), conventional statistics could not be used to effectively assess fitness of all mutants in the library. Instead, a method was devised to hypothesize how genes influence colonization based on trends in raw data abundance. Mutants with a two-fold or greater decrease in seed colonization in at least 4 out of 6 samples are hypothesized to be important for plant colonization. Mutants with a two-fold or greater decrease in seed colonization in less than 4 out of 6 samples are hypothesized to not be involved in plant colonization. A few mutants could not be detected in the input pool, making their role in seed colonization undetermined by this

screen (Table 4). We then generated a list of genes of interest to be tested in competition against their isogenic wild type.

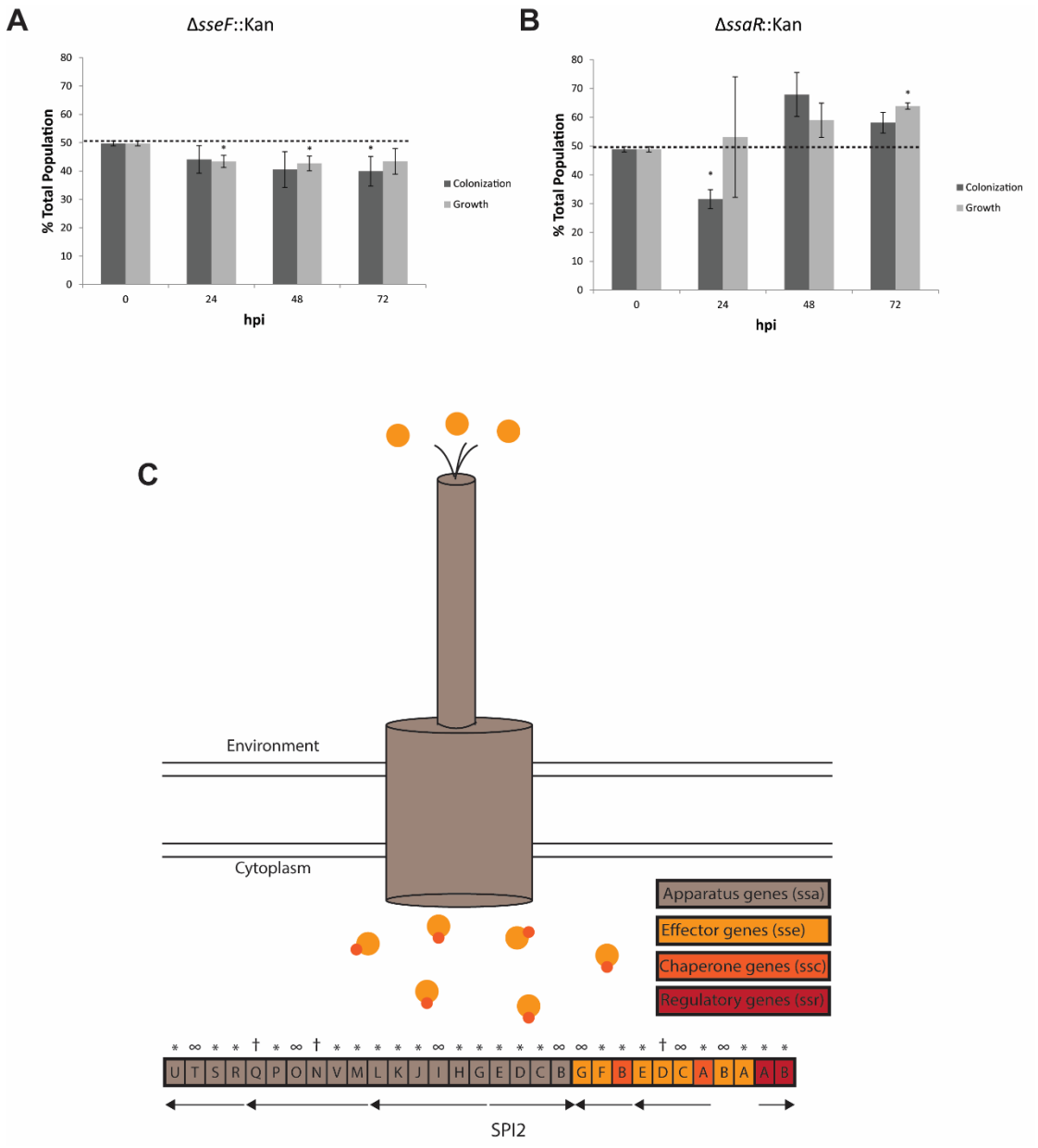

Figure 2. The 40K mutant library screen revealed a wide variety of *Salmonella* Pathogenicity Island-2 genes that may be important for *S. enterica* colonization of plants. (A) 14028s Δ*sseF*::Kan exhibited a growth defect at 24 and 48 hpi and a colonization defect at 72 hpi. (B) 14028s Δ*ssaR*::Kan exhibited a colonization defect on germinating alfalfa seedlings at 24hpi. Bars show the mean percent total population of mutants following a 1:1 co-inoculation with the isogenic WT. *, statistically different from 50% (One-sample t-test, Δ*sseF*::Kan n=3; Δ*ssaR*::Kan n=2, $p<0.1$). Error bars represent standard deviation. (C) Summary of SPI-2 genes and their hypothesized role in seedling colonization based on the 40K mutant library screen. *Denotes mutant with a two-fold or greater decrease in seed colonization in at least 4 out of 6 or †less than 4 out of 6 samples. ∞Role in seed colonization undetermined due to absence from input pool.

Metabolism and animal virulence genes are involved in plant colonization. To confirm the results of the mutant library screen were due to selection and not just chance, a series of co-inoculation assays in germinating alfalfa were carried out. Genes of interest included *serA, manA, sseF, ssaR,* and *yncC,* because they are representatives of the major groups that were shown to be important for *S. enterica* plant colonization across multiple time points in the library screen: carbon metabolism, amino acid metabolism, virulence, and transcriptional regulation, respectively. Serine is required for both *S. enterica* growth and colonization on plants. The *serA* mutant was non-competitive in both growth and colonization at 24 hpi, 48 hpi, and 72 hpi (Figure 3A-B). These defects were fully rescued by genetic complementation with a plasmid containing a wild type copy of *serA* (Figure 3A-B). Mannose is required early on for *S. enterica* plant colonization. The *manA* mutant was at a competitive disadvantage compared to wild type at 24 hpi, but not 48 hpi or 72 hpi (Figure 4A). This colonization defect was fully rescued by genetic complementation with a plasmid containing a wild type copy of *manA* (Figure 4A). The mutant library screen results led us to hypothesize that SPI-2 influences *S. enterica* plant colonization. To test this, Δ*ssaR*::Kan and Δ*sseF*::Kan were constructed and screened in competition with their isogenic wild type. The *ssaR* mutant had a colonization defect at 24 hpi, but growth advantage by 72 hpi (Figure 2B). Additionally, the *sseF* mutant was non-competitive for growth at both 24 hpi and 48 hpi, and colonization-defective at 72 hpi (Figure 2A). Finally, the transcriptional regulator YncC is required for *S. enterica* plant colonization over time. The *yncC* mutant became increasingly non-competitive for seedling colonization at 48 hpi and 72 hpi (Figure 5). These co-inoculation assays both validate the 40K mutant library results as well as shed light on genes previously unknown to influence *S. enterica* colonization of plants.

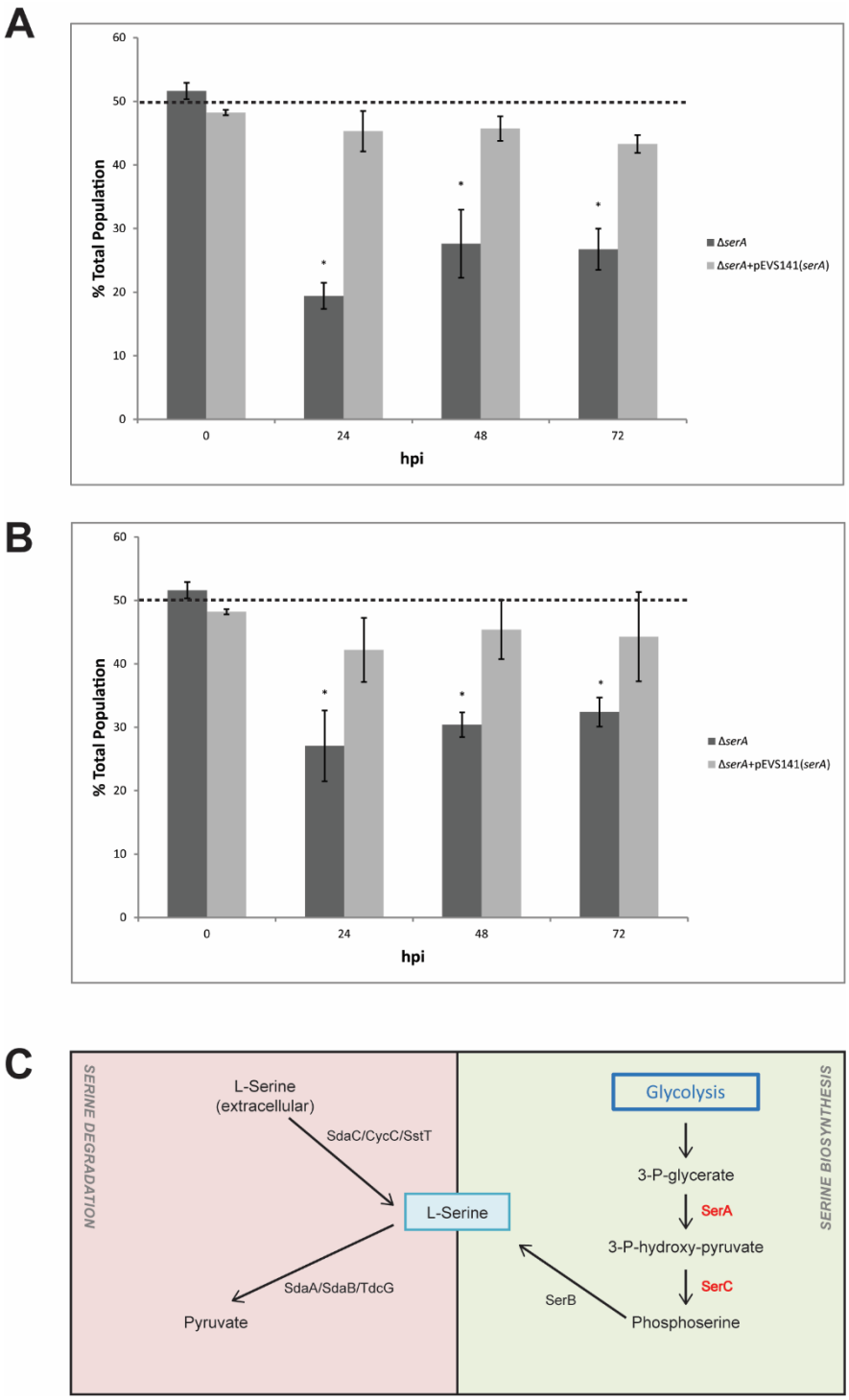

Figure 3. Serine is required for *S. enterica* growth and colonization on plants. 14028s Δ*serA*::Cm exhibited a substantial colonization (A) and growth (B) defect on germinating alfalfa seedlings and in the exudates. The defects were fully complemented by transforming 14028S Δ*serA*::Cm with a *serA*-containing plasmid. Bars show the mean percent total population of Δ*serA* following a 1:1 co-inoculation with the isogenic WT. *, statistically different from 50% (One-sample t-test, n=2, p<0.1). Error bars represent standard deviation. (C) Model of the serine metabolism network of *S. enterica*. Names in red indicate proteins produced in abundance by *S. enterica* during plant colonization (Kwan *et al.*, 2015).

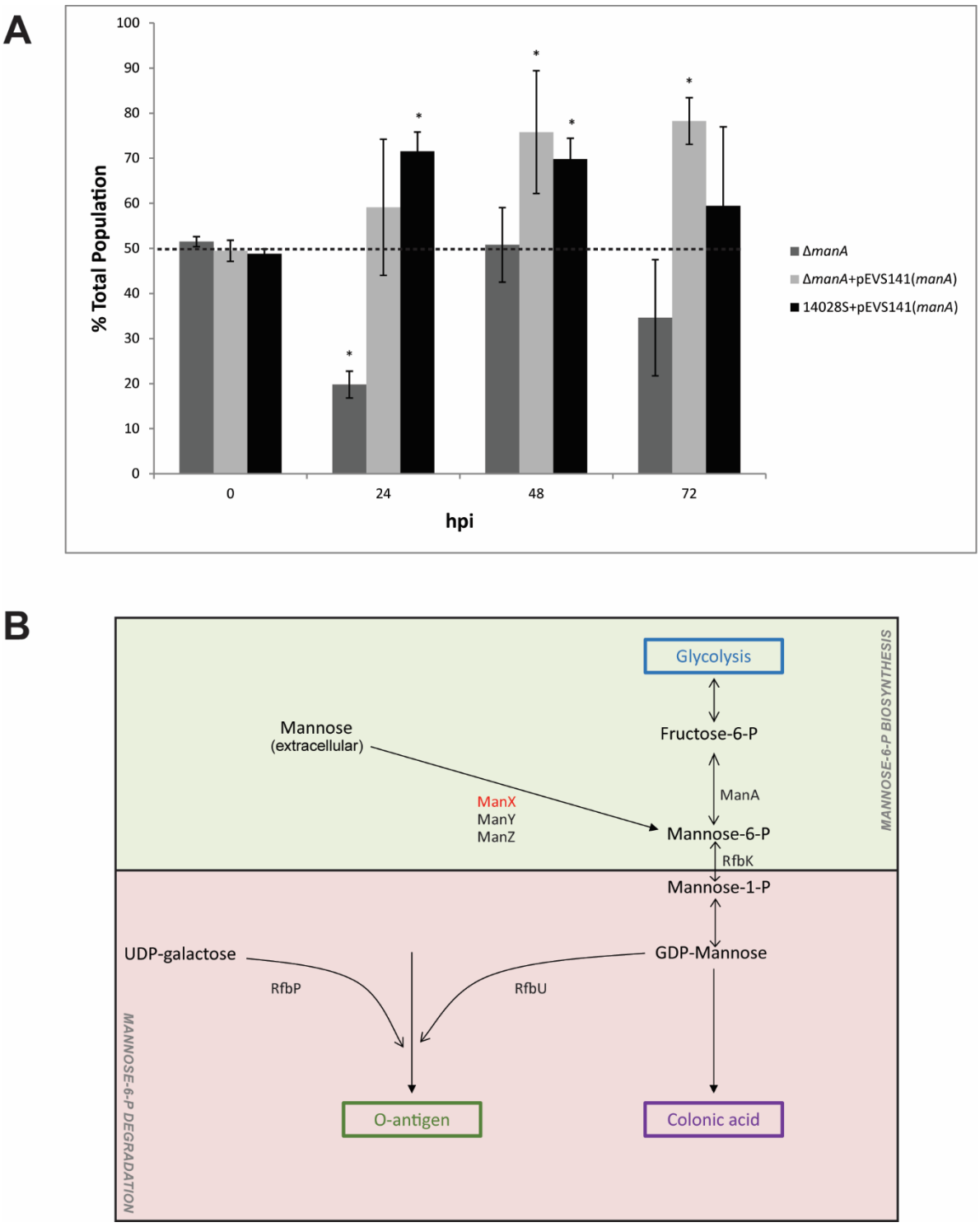

Figure 4. Mannose is important for *S. enterica* colonization on plants. (A) 14028s Δ*manA*::Cm exhibited a substantial colonization defect on germinating alfalfa seedlings, but recovered by 48 hpi. The colonization defect was fully complemented by transforming 14028s Δ*manA*::Cm with a *manA*-containing plasmid. Bars show the mean percent total population of Δ*manA* following a 1:1 co-inoculation with the isogenic WT. *, statistically different from 50% (One-sample t-test, n=3, $p<0.1$). Error bars represent standard deviation. (B) Model of the mannose metabolism network of *S. enterica*. Names in red indicate proteins produced in abundance by *S. enterica* during plant colonization (Kwan *et al.*, 2015).

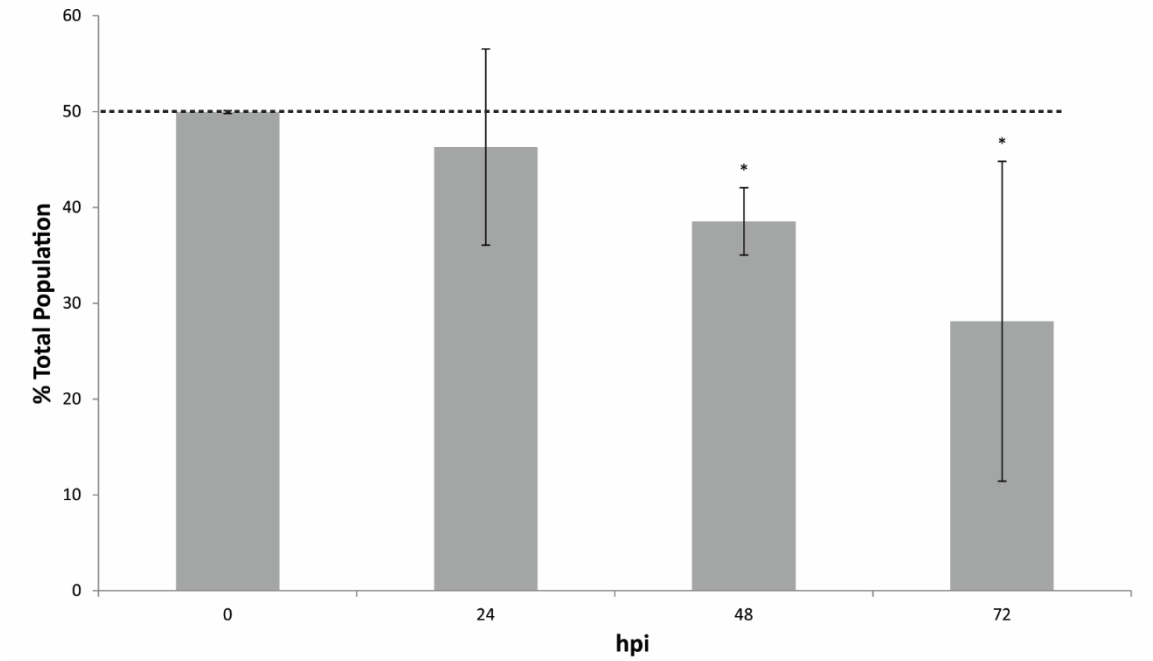

Figure 5. YncC, a transcriptional regulator in the GntR family, is required for *S. enterica* colonization of plants. 14028s Δ*yncC*::Kan exhibited a progressive colonization defect on germinating alfalfa seedlings over time. Bars show the mean percent total population of the *yncC* mutant following a 1:1 co-inoculation with the isogenic WT. *, statistically different from 50% (One-sample t-test, n=3, $p<0.05$). Error bars represent standard deviation.

DISCUSSION

In recent years, consumption of *S. enterica*-contaminated fresh agricultural crops has become the leading cause of food borne illness outbreaks. Recently, research efforts have been focused on understanding the genetic requirements and molecular mechanisms used by *S. enterica* to colonize plants. The microbiology of the plant rhizosphere is incredibly complex. Many of the plant-microbe interactions that occur in the rhizosphere are driven by plant exudates. It is important to understand the interactions that occur in the spermosphere and early rhizosphere because the highest levels of exudation occur in the hours immediately following seed imbibition (Nelson, 2004). Not surprisingly, the most rapid levels of microbial growth occur during this phase. The first 24 hours following seed imbibition bring about the most rapid replication of *S. enterica* (Hao *et al.*, 2012). Furthermore, it is expected that the variation of

exudates between different plant species is largely responsible for the dynamic microbiology of each individual rhizosphere (Roberts *et al.,* 1996). However, the specific metabolic needs of most members of the plant microbiome, including *S. enterica*, are largely unknown in this dynamic environment. In this study, we screened the entire *S. enterica* genome to maximize the number of genes identified that could play a role in plant colonization and that were previously unknown.

The robust metabolism of *S. enterica* allows it to successfully colonize not only animal but plant hosts as well. Transport of mannose and biosynthesis of mannose-6-P is required for *S. enterica* colonization of plants at 24 h. The mannose transport protein ManX is produced in abundance at 24 hpi, suggesting transport from the environment is preferential to biosynthesis *de novo* (Kwan *et al.,* 2015). The 40K mutant library screen showed a *manA* mutation resulted in 18.43 and 988.00-fold decreases from the input pool at 24 hpi and 72 hpi respectively (Tables 2 and 3). Co-inoculation assays confirm a Δ*manA*::Cm colonization defect at 24 hpi that can be rescued by genetic complementation (Figure 4A). These data suggest that while mannose is present in alfalfa root exudates, *S. enterica* must biosynthesize it *de novo* to successfully colonize at 24 h. Mannose-6-P, produced by ManA from Fructuse-6-P, is required to produce the nucleotide sugar GDP-Mannose, which in turn is required for colanic acid and O-antigen biosynthesis (Figure 4B). Additional genes in the GDP-Mannose biosynthetic pathway include *rfbK*, which the 40K mutant library screen also showed as important for colonization at 24 hpi (Table 2). O-antigen regions of lipopolysaccharides (LPSs) are responsible for membrane integrity as well as antibody recognition in animal hosts and colanic acid is an important component of the *S. enterica* extracellular matrix. However, colonic acid has been previously shown as unimportant for *S. enterica* colonization of roots (Barak *et al.,* 2007; Cowles *et al.,*

2016). On the other hand, O-antigen capsule synthesis is important for *S. enterica* colonization of alfalfa sprouts (Barak *et al.,* 2007). Thus, we hypothesize that a decrease in the intracellular pool of GDP-Mannose influences O-antigen production, thereby disrupting plant colonization. Future experimentation will be required to shed light on the molecular mechanisms underlying this hypothesis.

We also showed that serine biosynthesis is required for both growth and colonization in alfalfa. SerA and SerC, two *S. enterica* enzymes involved in serine biosynthesis via the glycolysis intermediate 3-phosphoglycerate, are produced in abundance at 24 hpi during growth in germinating alfalfa exudates (Kwan *et al.,* 2015). The 40K mutant library screen revealed that a mutation in *serC* resulted in a 1,180-fold decrease in alfalfa colonization at 24 hpi (Table 2). By 72 hpi, the same mutant had a 538-fold decrease in colonization paired with a 226-fold decrease in growth compared to the input pool (Table 3). Thus, these genes are not only expressed, but are required for colonization of alfalfa. Additionally, the 40K mutant library analysis led us to hypothesize that *serA* influenced both growth and colonization in germinating alfalfa (Table 4). We chose to screen a *serA* mutant (as opposed to *serC*) in the co-inoculation assays because this strain was readily available in our lab's collection from another mutant library (Santiviago *et al.,* 2009). The *serA* mutant was non-competitive for growth or colonization of alfalfa (Figure 3). The fact that environmental levels of serine from root exudates are not sufficient to support optimal growth (Kwan, personal communication) and colonization of *S. enterica* suggests that *de novo* biosynthesis is required on plants. Expression of both SerA and SerC were also detected in a murine enteritis model of infection with *S. enterica* (Becker *et al.,* 2006), suggesting that serine availability is limiting in animals as well. Taken together with

our results, we suggest that serine biosynthesis is essential in both animal and plant hosts, for successful infection and colonization, respectively.

A surprising avenue pursued in this study was the effect of SPI-2 genes on plant colonization. SPI-2 genes are essential for infection of animal hosts, by facilitating intracellular replication and formation of the *Salmonella*-containing vacuole (SCV) (Figueira and Holden, 2012). Our 40K mutant library screen revealed that 21 of the 31 genes located on SPI-2 are expected to be important for plant colonization (Table 4). These include genes for the T3SS apparatus, effector proteins, chaperone proteins, and regulators. Furthermore, entire transcriptional units of SPI-2 genes, or the majority of genes on certain transcriptional units, are hypothesized to be important for plant colonization at 24 h (Figure 2C). To test our hypothesis, we performed a co-inoculation assay with an *ssaR* mutant unable to form a functioning T3SS apparatus. It was expected that if the apparatus mutant showed no colonization defect, we should be skeptical of effector proteins influencing plant colonization, since no effectors could be secreted in this mutant. However, the *ssaR* was defective for colonization at 24 hpi (Figure 2B), suggesting that either the T3SS needle complex itself, or effectors secreted through it, are important for plant colonization. To examine whether T3SS effectors play a role, an effector mutant, Δ*sseF*::Kan, was screened in competition with isogenic wild type. This mutant was non-competitive for growth at 24 hpi and 48 hpi, and for colonization at 72 hpi (Figure 2A). If SseF was the effector causing the phenotype of the Δ*ssaR*::Kan mutant, we would expect the Δ*sseF*::Kan mutant to phenocopy Δ*ssaR*::Kan at 24 hpi. However, there are still six additional secreted effectors on the SPI-2 that have yet to be screened for a role in plant colonization. Additionally, genetic complementation will be necessary to rescue the defect for both the *ssaR* and *sseF* mutants. While more experimentation is required to make definitive conclusions on the

role of SPI-2 in plant colonization, including the mechanisms causing the phenotypes described here, we posit that that the SPI-2 is at least important, if not essential, for plant host colonization.

Finally, we discovered that YncC, a putative DNA-binding transcriptional regulator, becomes increasingly important to *S. enterica* plant colonization over time. The 40K mutant library screen did not register a fold decrease until 72 hpi (Table 3), however co-inoculation assays with Δ*yncC*::Kan reveal colonization defects at 48 hpi and 72 hpi (Figure 5). BLAST analysis suggests that *yncC* plays a role in biofilm regulation as a part of the GntR superfamily of transcriptional regulators. Mutations in genes known to be required for biofilm formation, such as *bcsA* (Barak *et al.,* 2007), decrease the fitness of *S. enterica* in germinating alfalfa seedlings. We found that a *bcsA* mutant had a >10-fold increase in seedling colonization at 72 hpi (Table 3). Our previous work showed that BcsA is essential for *S. enterica* colonization of and perhaps attachment at 24 hpi but by 48 hpi is was dispensable (Cowles *et al.,* 2016). Taken together, we hypothesize that *yncC* is not involved in cellulose regulation as the pattern of colonization defect is distinct between a *yncC* and *bcsA* mutant. The *yncC* mutant was less fit in germinating alfalfa seedlings. We hypothesize its function is to inhibit biofilm formation at the transcriptional level. However, genetic complementation with a wild type copy of *yncC* is still necessary to show this mutation is responsible for the demonstrated phenotype. High resolution chromatin immunoprecipitation (ChIP) assays may be necessary to discover exactly which genes YncC targets. Microarrays or RNA-sequencing can then be used to correlate expression levels of YncC-controlled genes.

Collectively, this study sought to uncover previously uncharacterized genes used by *S. enterica* to colonize plant hosts. Until recently, more emphasis has been placed on studying the animal host portion of the life cycle of *Salmonella*. Understanding the genes and mechanisms

essential for the plant environment will provide fundamental information about the unique biology and challenges this bacterium faces there. One direction for future research could be on characterizing the intergenic regions that the 40K mutant library screen uncovered. Finding genes and loci required for plant colonization is not enough. Characterizing the molecular mechanisms underlying the colonization defects will allow for the system to be manipulated, preventing *S. enterica* colonization of agricultural crops. The results of this study may also be applicable to other human enteric pathogens such as Shiga-toxin producing *Escherichia coli*, another food safety concern in fresh produce. As we characterize more of the mechanisms that contribute to the fitness of *S. enterica* in various plant environments, we move closer to creating a foundation for effective method development to prevent outbreaks of *S. enterica* on fresh produce, lowering food-borne illness and improving public health.

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Table 1. Strains used in this study.

***S. enterica* strain**	**Genotype**	**Reference**
JDB1034	*S. enterica* serovar Typhimurium 14028S; Nal^r	Cowles *et al.*, 2016
JDB1338	14028S Δ*manA::*Cm	Dr. Grace Kwan
JDB1349	14028S Δ*serA*::Cm	This work
JDB1354	14028S Δ*serA*::Cm + pEVS141(*serA*)	This work
JDB1388	14028S Δ*manA*::Cm + pEVS141(*manA*)	This work
JDB1370	14082S Δ*yncC*::Kan	This work
JDB1372	14028S Δ*ssaR*::Kan	This work
JDB1389	14028S + pEVS141(*manA*)	This work
JDB1366	14028S Δ*sseF*::Kan	This work

Table 2. Results of the 40K random insertion transposon mutant library screen shows *S. enterica* genes that influence colonization in germinating alfalfa by 24hpi. Numbers represent fold change compared to input pool at 0 hpi. Color of cells correspond to continuum of fold decrease (red) to fold increase (green) compared to input pool. Statistical cutoff for data shown is p<0.002, with a fold change cutoff of greater than +4 or less than -4 compared to the input pool.

Gene	Fold Change
apbA	-3337.00
hlpA	-1769.00
bioF	-1419.00
bioH	-1199.00
serC	-1180.00
Intergen_3203	-1144.00
STM14_1104	-928.00
cysH	-904.00
serC	-806.75
bioC	-806.00
panB	-775.33
tuf_1	-731.00
dcrB [D]	-697.00
wecE	-697.00
wzxE	-690.00
pstB	-657.50
minE	-646.00
cysQ	-626.50
Intergen_2217	-599.00
bioD_1	-592.00
rstB	-539.00
nlpI	-522.00
wecF [D]	-423.80
wecD	-407.00
dmsC_1	-365.00
STM14_4149	-355.80
bioB	-328.00
yhbE	-299.50
yjeK	-299.00
Intergen_2839	-299.00
Intergen_2894	-273.00
Intergen_1208	-271.00
Intergen_1988	-263.00
Intergen_1988	-243.00
pcm	-232.00

Gene	Fold Change
cysJ	-229.75
Intergen_1000	-224.33
bioA	-215.86
dcrB [D]	-199.29
pnp	-195.30
Intergen_1000	-157.00
hisG	-157.00
pfkA	-156.00
bioH	-137.96
Intergen_3994	-108.40
STM14_5573	-108.40
panD	-97.22
ompA	-96.29
yjgA	-94.74
rluC	-82.80
yjgA	-82.00
cysI	-76.48
ygiM	-72.25
exbB	-61.18
cysW	-57.14
hemK	-56.15
deaD	-51.46
Intergen_2114	-46.43
pfkA	-44.40
Intergen_2512	-40.78
STM14_4872	-39.09
STM14_4746	-38.92
dsbA_2	-38.80
yhhK	-38.13
panB	-38.12
fis	-37.04
Intergen_0106	-37.00
Intergen_3018	-36.48
dsbA_2	-35.88
hisF	-33.11
cysJ	-32.34
metJ	-32.00

Gene	Fold Change
STM3841	-31.67
Intergen_3460	-31.25
Intergen_3020	-30.18
mdoH	-29.65
asmA	-26.64
panC	-26.09
prc	-25.39
Intergen_3529	-24.84
STM14_1000	-24.83
queA [J]	-24.71
Intergen_2530	-24.00
mdoG	-23.92
sirB1	-23.40
folC	-23.30
rnfD	-21.74
sapA_2	-21.55
ugtL	-21.31
Usg	-20.83
betB [J]	-20.78
Intergen_0887	-20.76
STM3785	-20.14
rfbK	-19.82
samB	-19.50
STM4030	-18.70
thrV	-18.67
STM4610	-18.55
typA	-18.20
manA	-18.13
secG	-17.65
Intergen_3536	-17.21
Intergen_2147	-17.13
damX	-16.88
STM14_5573	-16.24
mdfA	-16.21
STM2593	-15.88
pmrL [R]	-15.71

Gene	Fold Change
mrcB	-15.59
cysD	-15.37
dedD	-15.29
ddl_2	-14.84
Intergen_3492	-14.52
STM14_4883.P	-14.52
STM14_5125	-14.41
symE [D]	-14.38
STM14_0812	-14.04
gor	-13.98
STM0080	-13.71
trkA	-13.56
orf245	-13.41
pitA	-13.17
fes	-13.09
ibpA	-12.81
ompR	-12.77
chaA	-12.26
mdoH	-12.21
proC	-12.21
STM14_0457	-12.21
glf	-12.14
STM14_4946	-12.11
Intergen_0155	-12.00
recG	-11.56
STM14_5526	-11.55
STM14_4017	-11.48
STM14_3170	-11.46
rcK [J]_1	-11.27
gntR	-11.00
STM14_4779	-10.98
serB_2	-10.91
tatC	-10.88
*ccmC*_1	-10.58
STM3131	-10.54
STM3602	-10.53
dam	-10.35
STM0028.1n	-10.33
STM14_0242	-10.27

Gene	Fold Change
Intergen_3017	-10.24
STM14_4196	-10.24
srmB	-10.15
tatC	-9.95
wecB	-9.95
mdoG	-9.92
STM2761	-9.84
bcsC [J]	-9.66
Intergen_2842	-9.66
erfK [J]	-9.60
yjiS	-9.50
Intergen_2695	-9.46
STM14_3588	-9.45
Intergen_3024	-9.30
phoE	-9.20
cysK	-8.97
STM14_2433	-8.93
ndh	-8.91
Intergen_1395	-8.91
rluD	-8.87
STM4417	-8.86
Intergen_0012	-8.81
aroE_2	-8.63
tktA	-8.39
wecB	-8.38
STM14_0340	-8.38
Intergen_0253	-8.38
cysN	-8.32
Intergen_1299	-8.27
STM1001	-8.23
STM14_2225	-8.21
yobG	-8.21
cysG	-8.17
STM4478	-8.13
phnU	-7.90
Intergen_3900	-7.73
cysA	-7.62
STM1987	-7.54
Intergen_0175	-7.48
pduL	-7.37

Gene	Fold Change
STM14_5560	-7.35
cysC	-7.32
gnd	-7.29
rlmI [R]	-7.23
cdsA	-7.22
STM0345	-7.16
STM14_0099	-7.04
metC	-6.99
Intergen_0328	-6.98
dam	-6.95
cysU	-6.81
envZ	-6.77
ddl_1	-6.73
proA	-6.73
traM	-6.68
acnB	-6.67
aroA	-6.66
ompR	-6.60
Intergen_3150	-6.59
Intergen_3851	-6.41
corA	-6.39
Intergen_0298	-6.33
Intergen_1240	-6.31
Intergen_1825	-6.30
mraW	-6.29
recG	-6.27
gshB	-6.23
ioll [J]	-6.22
Intergen_1778	-6.21
potB	-6.17
yqcC	-6.17
orf5	-6.13
nqr4 [J]	-6.02
Intergen_3728	-6.02
smtA	-5.98
deaD	-5.98
rfaB	-5.93
ptsJ	-5.91
Intergen_0578	-5.71
sroC [P]	-5.71

Gene	Fold Change
yhbY	-5.70
spoU	-5.68
STM14_5350	-5.63
yjgF	-5.63
Intergen_3688	-5.61
Intergen_2017	-5.54
ypeA [R]	-5.51
gogB	-5.49
Intergen_0299	-5.47
mrcB	-5.41
srlR	-5.38
STM14_5116	-5.38
znuB	-5.33
tlpA	-5.32
yceG	-5.30
tatB	-5.29
trpC	-5.23
rfe	-5.20
STM2908	-5.19
speA	-5.18
metA	-5.18
STM2336	-5.17
pmrL [R]	-5.14
Intergen_0038	-5.12
carA	-5.08
STM14_0046	-5.06
STM14_4746	-5.02
yifL	-5.02
STM14_4061	-4.98
ftsN	-4.95
slyD	-4.95
srfK [D]_1	-4.91
cysK	-4.88
STM4203	-4.88
mug	-4.85
gntK	-4.84
pstC	-4.79
Intergen_3349	-4.79
pncB	-4.77

Gene	Fold Change
STM2759	-4.76
yjcO	-4.75
Intergen_2854	-4.69
rhaR	-4.66
STM14_4768	-4.60
lysP	-4.56
STM14_3628	-4.52
ychJ	-4.50
gppA	-4.47
Intergen_3320	-4.44
STnc400 [P]	-4.44
STM14_1631	-4.44
Intergen_1183	-4.44
STM1055	-4.43
upp	-4.42
STM14_5225	-4.41
yjjA	-4.39
fklB	-4.38
pabB	-4.35
cysU	-4.34
sbp	-4.33
recF	-4.33
yhhQ	-4.33
yjiN	-4.31
yqjI	-4.26
STM14_3211	-4.22
ycdX	-4.21
citG2	-4.18
ssaK	-4.16
Intergen_0349	-4.14
gcvR	-4.11
proQ	-4.07
Intergen_0504	-4.05
ytfG	-4.02
STM14_0650	-4.01
Intergen_2619	-4.01
yjcC	10.48

Table 3. Results of the 40K random insertion transposon mutant library screen shows *S. enterica* genes that influence both growth and colonization in germinating alfalfa by 72 hpi. Numbers represent fold change compared to input pool at 0 hpi. Color of cells correspond to continuum of fold decrease (red) to fold increase (green) compared to input pool. Statistical cutoff for data shown is $p<0.001$ for seed colonization results, with change cutoff of greater than +4 or less than -4 compared to the input pool.

	Fold Change	
Gene	**Colonization**	**Growth**
apbA	-3337.00	-42.37
bioA	-3022.00	-27.41
bioA	-2363.00	-210.04
yhhK	-2288.00	-10.04
prc	-2229.00	-743.00
ddl_2	-2166.00	-40.11
tatC	-2013.00	-6.16
pnp	-2008.00	-178.49
tolC	-1645.00	-2193.33
trxA	-1549.00	-11.16
thrC	-1426.00	-2.68
cysP	-1413.00	-16.53
ompA	-1348.00	-599.11
prc	-1313.75	-33.85
minE	-1292.00	-287.11
Intergen_3017	-1291.00	-1.04
yhbE	-1198.00	-31.02
cysU	-1138.25	-8.89
cysI	-1100.00	-162.96
mraW	-1070.00	-43.23
Intergen_0887	-1059.00	-15.02
pitA	-1027.00	-6.17
metQ	-1022.50	-4.14
panB	-1019.75	-36.50
manA	-988.00	-105.39
cysJ	-959.33	-47.97
bioF	-946.00	-111.29
cysJ	-919.00	-245.07
rpe	-914.00	-2.59
panD	-875.00	-16.91
STM14_5533.J	-852.00	1.02
Intergen_3963	-852.00	1.02
cysW	-800.00	-17.78
Intergen_3840	-800.00	1.07
folC	-769.00	-4.48

	Fold Change	
Gene	**Colonization**	**Growth**
yjgA	-738.00	-140.57
oadG	-721.00	-1.18
STM1034	-715.00	-1.12
STM14_2433	-714.00	-6.66
glnG	-713.00	-158.44
IsrM [P]	-700.00	-1.03
dcrB [D]	-697.00	-58.08
ybfE	-686.00	-1.64
fumX [J]	-635.00	1.00
yjiS	-627.00	-2.98
cysQ	-626.50	-278.44
araA	-623.00	-1.25
galE	-616.00	1.34
mrcB	-615.75	-9.72
panC	-613.00	-51.08
pagP	-613.00	-1.13
Intergen_3900	-611.00	-1.82
moeB_2	-609.00	-1.19
bioH	-599.50	-77.98
bioD_1	-592.00	-41.54
ompR	-581.00	-7.07
dsbA_2	-574.00	-12.15
dsbB	-568.00	-2.07
ugtL	-554.00	1.22
serC	-537.83	-226.46
Intergen_3858	-535.00	-1.43
STM14_0088.J	-531.00	-1.22
solA	-529.00	-1.61
nlpI	-522.00	-11.23
STM_1650	-505.00	1.05
Intergen_1388	-500.00	-1.45
bioC	-483.60	-161.20
fliD	-472.00	1.03
STM14_5496	-471.00	-1.57
bioH	-462.59	-79.43

	Fold Change	
Gene	**Colonization**	**Growth**
rfbI_2	-460.50	-409.33
Intergen_0482	-460.00	-1.32
STM14_1448	-455.00	-1.53
citG2	-453.00	-1.45
Intergen_2404	-445.00	-1.05
parE [J]	-439.00	-1.38
STM14_0292	-429.00	-2.48
rfaP	-407.00	-6.96
typA	-400.50	-67.81
citC2	-397.00	-1.01
ssaL	-396.00	-1.71
Intergen_2786	-395.00	1.06
STM14_0178.RJ	-394.00	-1.33
Intergen_0070	-382.50	-1.18
hflC	-376.00	-1.94
yhbJ	-375.00	-2.30
Intergen_1988	-367.50	-2.01
thrV	-364.00	-3.96
Intergen_0305	-351.00	1.24
STM14_0112.J	-347.00	-1.37
STM14_0113	-347.00	-1.37
Intergen_3551	-340.00	-1.90
panB	-332.29	-55.38
STM3131	-332.00	-1.14
yeiA	-322.00	1.62
Intergen2367	-317.00	-1.12
mexF [J]	-317.00	-1.06
rlmI [R]	-312.00	-1.15
Intergen_0897	-312.00	-1.15
STM14_2248.J	-309.50	-1.17
Intergen_2297	-285.00	-1.18
celD	-275.00	1.06
copS	-273.00	1.02
Intergen_1208	-271.00	-4.88
STM_4390	-270.00	-1.42
oppC_1	-268.33	1.10
modB	-268.00	-2.04
Intergen_1971	-266.00	-1.04
STM14_3197	-262.00	1.25

	Fold Change	
Gene	**Colonization**	**Growth**
STM_2668	-261.00	-1.49
STM14_1055.J	-261.00	-1.45
argR [R]	-257.50	-1.20
ccmC_1	-254.00	-1.48
Intergen_0811	-242.00	1.18
STM1869	-240.50	1.38
idi	-235.00	-1.08
pmrM	-233.50	-12.71
Intergen_3104	-225.00	-1.30
Intergen_3208	-224.50	-1.33
Intergen_3032	-224.25	-2.69
apaH	-214.00	-10.19
fhuC [R]	-212.00	-1.12
nusA	-200.00	-1.29
Intergen_2217	-199.67	-159.73
yhdA	-193.33	-1.91
Intergen_3655	-189.00	-1.59
STM2593	-137.67	-1.00
Intergen_1182	-107.00	1.48
nhoA	-99.00	-1.01
ybhO	-94.00	-1.51
yaiY	-76.57	-1.33
rpmJ_1	-74.25	-1.41
yfcL	-60.17	-1.48
yncC	-55.82	-1.02
hemF	-47.54	-2.63
fis	-37.04	-4.85
aroE_2	-24.53	-3.22
wecB	-22.80	-12.99
damX	-20.56	-8.92
proC	-19.03	-3.85
STM14_0457	-19.03	-3.85
slyD	-18.69	-4.09
pfkA	-18.00	-7.80
pfkA	-17.11	-4.04
dam	-15.86	-4.55
rluD	-15.04	-8.93
Intergen_3018	-13.87	-5.43
tktA	-13.52	-2.42

	Fold Change	
Gene	**Colonization**	**Growth**
secG	-12.89	-3.01
rrfe	-12.81	-5.47
IsrM [P]	-12.26	1.00
STM14_3329.P2	-12.26	1.00
Intergen_1053	-11.39	-1.22
ndh	-11.20	-1.66
smtA	-11.14	-2.30
recG	-10.31	-8.61
Intergen_3020	-10.26	-2.16
tatC	-9.79	-4.91
cysD	-9.75	-2.52
yggX	-9.71	-8.83
dam	-9.70	-5.38
STM14_3756.J	-9.64	-8.77
mdoH	-9.59	-1.95
dedD	-9.30	-4.61
srmB	-9.13	-4.01
Intergen_2571	-9.03	1.11
Intergen_2147	-8.96	-5.46
Intergen_0510	-8.53	-1.75
cysD	-8.32	-5.28
Intergen_3202	-8.11	-19.56
STM0028	-7.94	-2.47
pcnB	-7.25	-3.90
STM14_4770.J	-6.84	-2.10
STM14_4771	-6.84	-2.10
ftsN	-6.72	-2.70
proA	-6.70	-1.91
STM1001	-6.68	-3.67
mrcB	-6.25	-5.20
gshB	-6.25	-4.28
tktA	-6.23	-2.27
apbE	-6.17	-1.33
deaD	-6.13	-7.26
mdoH	-6.03	-1.84
proQ	-5.67	-5.23
Intergen_1085	-5.62	-1.14
mdoG	-5.15	-2.02
asmA	-5.10	-3.74

	Fold Change	
gene	**Colonization**	**Growth**
cysN	-4.98	-19.47
erfK [J]	-4.95	-4.35
minC	-4.92	-1.75
fepA	-4.90	-2.87
cysC	-4.86	-5.59
ssrB	-4.83	1.00
mrp	-4.78	-2.42
wecB	-4.74	-6.73
cfa	-4.72	-1.18
ispB	-4.67	-1.55
cheA	-4.46	-1.31
Icc	-4.45	-1.43
rfbC	-4.40	1.04
tatB	-4.37	-3.11
yaeE	-4.25	-3.87
gcvR	-4.22	-1.09
entE	-4.22	-1.13
ptsG	-4.15	-1.32
STM2689	-4.12	-1.13
rfe	-4.09	-3.92
dedD	-4.08	-2.72
fliR	4.88	5.65
nlpD_1	6.80	7.05
yhjS	8.03	5.24
rpoS	10.00	14.97
yhjL	10.49	6.94
bcsA	10.73	6.94
yhjL	11.21	7.34
yhjQ	12.77	7.83
yhjN	13.03	7.57
yhjN	14.40	7.35
yhjR	17.84	5.87

Table 4. Summary of genes of interest for this study based on 40K mutant library screen. Numbers represent abundance of both the input (0 hpi) and output pool (24 hpi) based on transposon sequencing. Input pool and exudate growth values represent an average (n=3) of samples. *Denotes mutant with a two-fold or greater decrease in seed colonization in at least 4 out of 6 or †less than 4 out of 6 samples. ∞Role in seed colonization undetermined due to absence from input pool.

		Input Pool	Seed Colonization Samples						Exudate Growth
	Mutation		**1**	**2**	**3**	**4**	**5**	**6**	
	Δ*serA**	266	1	7	58	0	204	12	40
	Δ*manA**	659	1	0	108	0	0	0	3
	Δ*yncC**	115	36	73	26	17	0	38	96
SPI-2	Δ*ssaU**	212	191	31	63	1	144	6	69
	Δ*ssaT*∞	0	x	x	x	x	x	x	x
	Δ*ssaS**	239	86	23	68	19	265	20	64
	Δ*ssaR**	75	48	14	139	0	5	0	33
	Δ*ssaQ*†	319	147	110	241	31	279	76	78
	Δ*ssaP**	106	0	0	27	34	29	72	29
	Δ*ssaO*∞	0	x	x	x	x	x	x	x
	Δ*ssaN*†	265	238	49	2763	53	245	112	98
	Δ*ssaV**	802	360	173	138	398	1252	123	191
	Δ*ssaM**	21	18	0	44	0	0	0	3
	Δ*ssaL**	132	284	13	6	24	0	11	24
	Δ*ssaK**	83	23	0	35	4	4	19	6
	Δ*ssaJ**	32	0	0	13	2	0	5	2
	Δ*ssaI*∞	0	x	x	x	x	x	x	x
	Δ*ssaH**	120	6	43	94	15	155	42	33
	Δ*ssaG**	345	141	52	603	65	228	24	76
	Δ*ssaE**	37	2	4	19	2	29	41	7
	Δ*ssaD**	543	402	44	266	155	51	148	107
	Δ*ssaC**	435	199	47	229	50	378	65	129
	Δ*ssaB*∞	0	x	x	x	x	x	x	x
	Δ*sseG*∞	0	x	x	x	x	x	x	x
	Δ*sseF**	143	0	13	79	43	283	62	42
	Δ*sscB**	374	495	101	240	77	579	50	102
	Δ*sseE**	185	65	23	200	4	80	36	54
	Δ*sseD*†	310	456	163	392	70	223	103	90
	Δ*sseC*∞	0	x	x	x	x	x	x	x
	Δ*sscA**	343	76	32	299	14	472	52	101
	Δ*sseB*∞	0	x	x	x	x	x	x	x
	Δ*sseA**	16	0	0	0	0	0	0	2
	Δ*ssrA**	772	3095	165	121	80	335	78	165
	Δ*ssrB**	727	297	94	297	23	1349	123	172