THE ROLE OF AgfD-REGULATED THIN AGGREGATIVE FIMBRIAE AND CELLULOSE IN THE INTERACTIONS OF *Salmonella* Typhimurium ON THE TOMATO SURFACE

By

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To my brother Michael, a person who inspires and encourages those around him to continually strive for more
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<td>ANOVA</td>
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<td>BATH</td>
<td>Bacterial adherence to hydrocarbons</td>
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<tr>
<td>bdar</td>
<td>Brown, dry and rough</td>
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<tr>
<td>bp</td>
<td>Base pair</td>
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<td>BPW</td>
<td>Buffered peptone water</td>
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<td>c-di-GMP</td>
<td>Cyclic diguanylate</td>
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<td>CDC</td>
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<td>CI</td>
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<td>CIAP</td>
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<td>Calcofluor white</td>
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<td>GAPs</td>
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<td>IFAS</td>
<td>Institute of Food and Agricultural Sciences</td>
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<td>EBU</td>
<td>Evans Blue-Uranine</td>
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<tr>
<td>EDTA</td>
<td>Ethylene diamine tetraacetic acid</td>
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<td>EGTA</td>
<td>Ethylene glycol tetraacetic acid</td>
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<td>Gent</td>
<td>Gentamicin</td>
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<td>Kan</td>
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<td>LB</td>
<td>Luria-Bertani</td>
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OD$_{400}$  Optical density at 400 nm
PBS  Phosphate buffered saline
PCR  Polymerase chain reaction
pdar  Pink, dry, and rough
PFGE  Pulsed-field gel electrophoresis
rdar  Red, dry, and rough
RH  Relative humidity
RPM  Revolutions per minute
SAT  Salt agglutination test
saw  Smooth and white
TAE  Tris-Acetate-EDTA
T-BMPs  Tomato Best Management Practices
Tet  Tetracycline
T-GAPs  Tomato Good Agricultural Practices
TSB  Tryptic soy broth
US  United States
USDA  United States Department of Agriculture
UV  Ultraviolet
x-gal  5-bromo-4-chloro-3-indolyl-β-D-galactoside
XLD  Xylose lysine desoxycholate
THE ROLE OF AgfD-REGULATED THIN AGGREGATIVE FIMBRIAE AND CELLULOSE IN THE INTERACTIONS OF _Salmonella_ Typhimurium ON THE TOMATO SURFACE

By

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The rise of produce-linked salmonellosis outbreaks has directed attention towards the environmental persistence of _Salmonella_ spp. A complex phenotype of _Salmonella_, known as rdar (red, dry, and rough), has been linked to increased resistance to environmental desiccation and stress. Mutations in genes encoding thin aggregative fimbriae and/or cellulose biosynthesis result in a loss of the rdar phenotype. Thin aggregative fimbriae and cellulose are regulated by AgfD in _Salmonella_, either directly or indirectly. The transcription of _agfD_ is greatest in conditions that would be encountered outside of the animal host, including 28°C, low osmolarity, and nutrient starvation during stationary growth. Both thin aggregative fimbriae and cellulose have also been implicated as important factors in the attachment and persistence on abiotic, animal cell, and plant surfaces, as well as important structural components of biofilm formation. The roles of the AgfD regulator and AgfD-regulated thin aggregative fimbriae and/or cellulose in the interactions of _Salmonella_ Typhimurium on the tomato surface were assessed through deletion mutants. The fitness of the mutants was compared to the wild type _S_. Typhimurium in the initial attachment to the tomato surface, persistence
on tomatoes over 6 days, and biofilm formation on polystyrene, intact tomatoes, and tomato segments. The AgfD, thin aggregative fimbriae, and/or cellulose deficiencies in *Salmonella* produced no biological effect in the initial attachment to tomatoes within 5 min of contact, but significantly impaired the formation of biofilm on polystyrene, intact tomato surfaces, and tomato segments. Only the strains deficient in cellulose showed diminished persistence capabilities on the surface of both green and red tomatoes. In persistence, the cellulose deficient mutants were more sustainable on the surface of red, ripened tomatoes than green. These variations between tomato maturities indicate that both bacterial behavior and produce ripeness may be factors in *Salmonella* survival on the tomato surface. The importance of cellulose production in the persistence and biofilm formation on the tomato surface may also be useful in providing a possible target for preharvest treatments to reduce *Salmonella* contamination on produce.
CHAPTER 1
INTRODUCTION

The estimated accounts of foodborne disease in the US include 38.4 million illnesses, 71,878 hospitalizations, and 1,686 deaths annually. Non-typhoidal Salmonella is responsible for an estimated 1 million cases of illnesses, 20,000 hospitalizations, and over 350 deaths annually (68, 69). Previous to 1990, most cases of salmonellosis were attributed to contaminated poultry and poultry products. Recently there have been multiple Salmonella outbreaks associated with a variety of produce, including tomatoes, seed sprouts, cantaloupes, jalapeño and Serrano peppers, and unpasteurized fruit juices (12, 13, 15, 17, 33, 38, 53, 74). The most recent data estimates that 46% of US foodborne outbreaks were associated with contaminated produce (57).

The multi-state, tomato associated salmonellosis outbreak of S. Javiana in 1990 led to the recognition that raw tomatoes were possible vehicles for foodborne illness (15, 38). Numerous Salmonella based outbreaks linked to tomatoes have been reported since (12, 13, 15, 17, 33, 38). Tomatoes are susceptible to contamination with Salmonella spp. during various production stages, including cultivation, harvesting, packing, distribution, or preparation (19). There have even been cases of multiple outbreaks being traced back to the same contamination source. The S. Newport tomato outbreaks of 2002, 2005, and 2006 were grown in the eastern shores of Virginia, where an irrigation pond was suspected as the common contamination source (15, 33). A packing facility was implicated for the source of contamination in both the 1990 S. Javiana and 1993 S. Montevideo outbreaks (15, 33, 38).
The contamination of produce in the preharvest environment is challenging to eliminate since the commodity is often consumed as a raw, fresh food. The best method of minimizing the risk of foodborne illness is the prevention of contamination since there is no effective kill step for bacterial contaminants on raw, whole produce. Prevention methods are detailed within the Good Agricultural Practices (GAPs), which were established in 1998 with the US Food and Drug Administration (FDA) Guide to Minimize Microbial Food Safety Hazards for Fresh Fruits and Vegetables. In Florida, Tomato-GAPs (T-GAPs) and Tomato Best Management Practices (T-BMPs) have been mandatory since 2008 (27). GAPs are not yet implemented throughout the fresh produce industry, but the FDA Food Safety Modernization Act (FSMA) requires the implementation of prevention-based food safety programs, of which GAPs are usually a prerequisite (59). Establishing protective programs like (T-GAPS) is helpful in reducing the risk of pathogen introduction into produce fields (27), but there are too many gaps in knowledge to establish preharvest treatments targeted against enteric pathogens.

There are numerous routes for Salmonella contamination of produce in the field environment including contaminated irrigation water, soil, manure, or through animal reservoirs including birds, insects, reptiles, and amphibians (42, 67, 79). The field contamination of produce infers the ability of Salmonella spp. to survive on plants. In order for the pathogen to be transmitted through produce, the bacterium must first attach and persist on the plant surface. The genetic mechanisms employed by Salmonella in the survival on produce are not well known, but research has indicated the attachment of Salmonella on the tomato surface is rapid (44). Extracellular components of Salmonella, including thin aggregative fimbriae and cellulose, have been
implicated as important in the attachment and persistence on abiotic materials, animal cells, and plant surfaces including sprout seedlings and parsley leaves (3, 5, 45, 49). Thin aggregative fimbriae and cellulose are also important structural components of biofilm formation (80). It has been hypothesized that the environmental persistence of pathogens is achieved through biofilm formation and that 30-80% of bacterial populations on plant surface are present in biofilms (51, 80).

Thin aggregative fimbriae and cellulose are proposed to be regulated by AgfD in Salmonella, with direct regulation of thin aggregative fimbriae and indirect regulation of cellulose through adrA. Transcription of agfD is greatest in conditions that would be encountered outside of the animal host, including 28°C, low osmolarity, and nutrient starvation during stationary growth (63). Furthermore, the components regulated by AgfD contribute to the production of an extracellular matrix characterized by a rough, aggregative morphology in Salmonella grown in 25-30°C. The patterned morphology, referred to as rdar (red, dry, and rough), is proposed to be more resistant to desiccation and environmental stress, which are important in environmental survival of Salmonella (100). An understanding of the mechanisms Salmonella employ in the initial attachment, persistence, and biofilm formation on plant surfaces may allow for the development of intervention procedures and strategies to reduce the risk of preharvest tomato contamination.
Produce Associated Foodborne Illness

The Centers for Disease Control and Prevention (CDC) estimate that 95% of Salmonella-based infections originate from foodborne sources (29). Several Salmonella enterica serovars have been associated with both animal- and produce-based outbreaks, but two serovars, S. Enteritidis and S. Typhimurium, are associated with over half of the reported salmonellosis cases in the US (29). The primary routes of Salmonella infection in humans are through fecal-oral transmission or the ingestion of contaminated products. Salmonellosis typically manifests as vomiting, diarrhea, cramps, and fever; and while the illness is typically self-limiting, there are high-risk populations of young children, the elderly, and the immunocompromised where the illness may be fatal (60). The infectious dose is determined by physiological characteristics of the Salmonella strain and the health status of the host. The dose may also be affected by the composition of the contaminated food product (92). The typical infectious dose may range from $10^6$ to $10^8$ CFU, whereas the minimum infectious dose for high-risk members of the population has been estimated to be less than 100 CFU (21).

The infection of humans by non-typhoidal Salmonella spp. is based on the ability of the bacterium to survive the adverse conditions of the stomach and reach the site of colonization. Salmonella spp. survives a broad range of pH conditions from 4.05 to 9.5, with an optimum of pH 6.5-7.5. The optimum growth temperature is 37°C, but Salmonella spp. are able to proliferate between 7-48°C (21). The wide adaptability of Salmonella spp. allows the bacterium to persist outside of the animal host. Due to the
adaptability of *Salmonella* spp., salmonellosis is a leading foodborne illness in the US (18, 69).

It is estimated that foodborne diseases cause 38.4 million illnesses, 71,878 hospitalizations, and 1,686 deaths annually in the US (68, 69). The current trends indicate an increase in produce-based outbreaks. In the 1970’s, only 0.7% of foodborne outbreaks were determined to be produce related, while in the 1990’s, 6% of outbreaks were linked to produce (75). The most recent data estimated that 46% of US foodborne outbreaks were attributed to contaminated produce (57). The number of produce-based outbreaks associated with *Salmonella* spp. has also increased. Previous to 1990, most cases of salmonellosis were attributed to contaminated poultry and poultry products (83). In 2002-2003, 31 *Salmonella* spp. outbreaks of produce origin were reported in comparison to the 29 reported poultry-related outbreaks (16). *Salmonella* spp. have been associated with a variety of produce, with large salmonellosis outbreaks being linked to tomatoes, seed sprouts, cantaloupes, jalapeño and Serrano peppers, and unpasteurized fruit juices (12, 13, 15, 17, 33, 38, 53, 74).

In the US, there have been numerous *Salmonella* spp. based outbreaks linked to particular produce commodities, including tomatoes (12, 13, 15, 17, 33, 38). A significant, multi-state tomato associated salmonellosis outbreak occurred in 1990. The outbreak investigation led to the recognition that raw tomatoes were a possible vehicle for foodborne illness (15, 38). In the 1990 outbreak, consumption of contaminated whole round tomatoes resulted in 176 cases of *S. Javiana* infection. The outbreak was traced to a repacking facility (38). In 1993, another multi-state outbreak occurred with *S. Montevideo* and tomatoes. The source of contamination was traced back to the
same packing facility implicated in the 1990 outbreak (38). In 1999, another multi-state outbreak of salmonellosis was traced back to the consumption of raw, round tomatoes. *S. Baildon* was implicated as the etiological agent in the outbreak (22).

*S. Newport* was linked to tomato-associated outbreaks in 2002, 2005, and 2006. The implicated tomatoes of the outbreaks were grown and packed in the eastern shores of Virginia. The *S. Newport* strain was isolated from an irrigation pond near the tomato fields after the 2005 outbreak occurred. The *S. Newport* strain of the 2006 outbreak was not directly isolated from the irrigation pond, but the tomatoes were grown in the same area and the strain had an identical pulsed-field gel electrophoresis (PFGE) pattern as the strain in the previous outbreaks (15, 33). Contaminated irrigation water was also suspected to be the source of the 2008 *S. Saintpaul* outbreak associated with jalapeño and Serrano peppers (17). This outbreak is significant because there were not only over 1,400 illnesses in 42 states, but tomatoes were originally implicated as the suspected source of the outbreak. The early association of the outbreak with tomatoes resulted in a nationwide loss of 300 million dollars and a loss of 100 million dollars to Florida tomato growers alone (17, 36).

*Salmonella* in the Produce Environment

In the past, it was perceived that foodborne diseases were primarily linked to animal-based products rather than produce (75, 83). The current trends indicate an increase in produce-based outbreaks, with an estimated 46% of US foodborne illnesses from 1998-2008 associated with produce (57). Produce is often consumed as a raw, fresh commodity with little microbial reduction, processing, or packaging, thus increasing consumer risk from contaminants. The potential contamination of produce is possible during cultivation, handling, processing, and preparation of the products as
illustrated in previous produce-associated outbreaks (19, 75, 85). Once *Salmonella* has attached to the produce, the pathogen may persist throughout the shelf life of the product. *Salmonella* have been isolated from both green and red tomatoes as well as sprouts, mangoes, peppers, and cantaloupes, among other commodities (14, 17, 44, 89). The initial attachment of *S.* Montevideo on the surface of tomatoes has been documented to occur within only 30 sec of contact (44). Therefore, preharvest or postharvest contact between the produce and *Salmonella* spp. may result in contamination of the product.

The correlation between human pathogens and produce is not currently understood. *Salmonella* spp. are characterized as bacteria with wide pH and temperature tolerances, which aid in survival within various food matrixes as well as in the animal host (21, 92). The adaptability of the bacterium could be sufficient to allow persistence in the secondary niche of the produce environment (15, 38). The pathogen may utilize plants as a vehicle for transfer between animal hosts. The initial introduction of the bacterium into the animal host is often through a food or water source, where the animal further sheds the bacterium back into the environment. Once the bacterium has re-entered the environment, *Salmonella* spp. may persist on the plants until another animal ingests the plant. Thus, enteric pathogens may exhibit mechanisms to survive on plants to use them as a transfer medium between the environment and their animal hosts.

The phyllosphere is characterized as the aboveground surface of the plant, which is a potentially hostile environment for an enteric pathogen. The phyllosphere exposes bacteria to high doses of UV light, poor nutrients, an aerobic environment, and variable
temperature conditions (37, 96). These conditions are in contrast to the intestinal environment that is shielded from UV light, is nutrient rich, anaerobic, and has little temperature variability. A single study inoculated six enteric bacteria and viruses onto cantaloupe, lettuce, and bell pepper crops under controlled temperature and humidity conditions. The pathogens were still detectable in the plant environments after 14 days, demonstrating the survival of enterics within the phyllosphere (81). This study is important in demonstrating that enteric pathogens, including Salmonella spp., can survive in the phyllosphere despite the harsh and variable conditions.

While laboratory studies are important in illustrating the survival of enterics, it remains unclear how the bacteria adapt and survive in the plant environment. Furthermore, in vivo and laboratory studies cannot simulate the true produce environment. There are multiple factors involved in the interaction between Salmonella spp. and produce including the specific commodity, cultivar, physiological state of the plant, and the extrinsic environmental conditions (3, 43, 73, 95). There also appears to be Salmonella serovar depend factors facilitating plant colonization. Serovars of Salmonella typically associated with poultry, including S. Enteritidis and S. Dublin, appear less apt to grow and persist in tomatoes than S. Montevideo and S. Newport serovars (73). While current research methods cannot address all of the factors involved in enteric and plant interactions, the studies highlight the influences of each factor to better understand the overall interactions in the plant system.

The bacteria may persist in the plant environment through the formation of biofilms on the plants. S. Thompson was visualized on the surface and within cuts of lettuce leaves through episcopic differential interference contrast microscopy coupled
with epifluorescence to find that the aggregated cells appeared slimy, suggesting the formation of a biofilm on the lettuce (94). Protection and enhanced survival of human pathogens on the plant surface may also result from the association of the enterics within the biofilms of the plant microflora (51). While biofilm formation on the surface of produce has been observed, the formation of *Salmonella* biofilms within plant tissue remains to be shown (80).

The role of plant microflora in the survival of human pathogens on plants also remains unclear. A survey of supermarket produce revealed that *Salmonella* incidence was twice as likely in the presence of soft rot caused by plant pathogens, implicating a role of plant pathogens in the incidence of *Salmonella* (95). Another study showed that the microflora of carrots, green peppers, lettuce, green and purple cabbage, celery, and green and yellow onions were found to be inhibitory to human pathogens, including *S. Montevideo* (72). In the co-infection of damaged cilantro leaves, *Salmonella* populations were greater when co-inoculated with *Erwinia chrysanthemi* than with *Pseudomonas viridiflava* (9).

Another area of concern is that *Salmonella* spp. is reaching contamination levels on produce that are capable of causing illness. The typical infectious dose of *Salmonella* spp. ranges from $10^6$ to $10^8$ CFU (21). These are tremendous concentrations to be reached on the plant surface with limited water and nutrient availability. The infectious nature of the *Salmonella* spp. may be derived from the adaptations needed to survive on produce rather than reaching the infectious dose concentrations. One of the first barriers in human defenses against enteric pathogens is considered to be the acidity of the stomach. *Salmonella* spp. have an acid response
system which may enable survival in the acidic conditions of the stomach (28). The increased survival of *Salmonella* spp. in acidic conditions was observed after inoculation into fresh produce (8). *Salmonella* serovars adapted in apple, orange, and tomato juices also displayed enhanced survival in simulated gastric fluid in comparison to the non-adapted controls (103). The tolerance and adaptation to low pH environments within acidic produce or acidic wounds from plant pathogens may increase the fitness of *Salmonella* spp. in the low pH of the stomach and decrease the infectious dose.

**AgfD-Regulated Attachment Mechanisms of *Salmonella***

In understanding the survival of *Salmonella* spp. on the surface of plants and produce commodities, the first aspect to be studied is the mechanism of attachment. The aerial surface of plants are protected from desiccation and bacterial infiltration by the plant cuticle, waxes, and polysaccharides (77). Bacterial attachment to surfaces is characterized as a two-step process including a reversible phase and irreversible attachment. Reversible binding is hypothesized to be directed by physiochemical properties of the bacterium and surface, involving weak attractions including Van der Waals forces and hydrogen bonding. Flagella, pili, and fimbriae are suspected to influence this first step of attachment. The stronger, irreversible attachment is hypothesized to involve both chemical and physical properties with extracellular components, such as cellulose, contributing to attachment (44, 84). Current research implicates that *Salmonella* spp. may attach to the plant surface in a similar manner.

Thin aggregative fimbriae have been established to facilitate adhesion to solid surfaces in *Escherichia coli* (91). The thin aggregative fimbriae are filamentous proteinaceous non-flagella appendages expressed on the external surface of the bacterial cell (20), which are also referred to as Tafi in *Salmonella* (98). There are up to
15 putative fimbrial operons identified in *Salmonella* that can facilitate adhesion, such as *agf* (thin aggregative fimbriae), along with *fim* (type 1 fimbriae), *pef* (plasmid encoded fimbriae), and *lpf* (long polar fimbriae) (40, 86). The production of cellulose in *Salmonella* is generally used for protection (76). Thin aggregative fimbriae and cellulose also have structural roles in biofilm formation, with cellulose providing strength to biofilms (31, 80). *Salmonella* biofilm formation on *Aspergillus niger* has also been shown to include cellulose-chitin interactions, with insufficient attachment to the fungi by cellulose deficient mutants. Thin aggregative fimbriae and cellulose also contribute to the aggregative multicellular behavior of the bacteria (4, 5, 63, 100). Thin aggregative fimbriae, cellulose formation, and the O-antigen capsule were found to influence colonization of alfalfa seedlings (4, 5). In *Salmonella*, there is a regulator known as AgfD that directly regulates thin aggregative fimbriae and indirectly regulates cellulose biosynthesis through *adrA* gene activation (Figure 2-1) (100). The AgfD-regulated thin aggregative fimbriae and cellulose of *Salmonella* spp. may be significant in the attachment of the bacteria to plants (2, 4, 5, 63).

The AgfD transcriptional regulator is composed of a C-terminus DNA-binding domain homologous to those of the LuxR family of regulators and a N-terminus putative receiver domain (35, 64). AgfD is proposed to directly regulate thin aggregative fimbriae and indirectly regulate cellulose in *Salmonella* (100). The thin aggregative fimbriae and cellulose contribute to the production of an extracellular matrix, which produces the distinct rdar (red, dry, and rough) morphology of *Salmonella*. The rdar morphology is expressed as patterned, aggregative colonies of *Salmonella* when grown on Congo Red media at 25-30°C (31, 61). It has been hypothesized that *Salmonella*
exhibiting the rdar morphology are more resistant to desiccation and environmental stress, which is vital in environmental survival (100). It has also been proposed that non-rdar morphology promotes fitness of S. Typhimurium within an infected tomato (104).

The agfD promoter is regulated by different environmental factors through global regulators and the secondary messenger molecule, cyclic diguanylate (c-di-GMP). The transcriptional regulators of agfD include OmpR, RpoS, MlrA, CxpR, H-NS, and IHF (Figure 2-1). The regulators are able to activate agfD expression in response to environmental conditions including temperature, oxygen tension, nutrient stress, osmolarity, and ethanol presence (11, 47, 80, 100). AgfD is produced from the expression of agfD. The AgfD regulator stimulates the expression of agfBAC, which encodes for the production of the thin aggregative fimbriae (62, 100). RpoS and CxpR can also directly regulate the production of thin aggregative fimbriae without activation by AgfD (100). AgfD also indirectly regulates cellulose production through the expression of adrA. AdrA contains a GGDEF domain that results in the production of c-di-GMP. The transcription of the bscABZC-bcsEFG operon, which drives cellulose biosynthesis, is activated by c-di-GMP. AgfD activates expression of adrA; AdrA then activates the biosynthesis of cellulose through the production of c-di-GMP. The AgfD-dependent activation of bcsABZC through AdrA has been demonstrated in Salmonella at 28°C in Luria-Bertani media. There are AdrA-independent pathways of c-di-GMP production in Salmonella as well as AgfD-independent pathways of cellulose production in Salmonella at 37°C (23, 32, 100). The c-di-GMP molecule is also known to promote sessile behavior in Salmonella, increasing biofilm formation (100).
Thin aggregative fimbriae promote autoaggregation of cells and have a role in the attachment of *S. enterica* to plant seedlings and animal cells. The fimbriae are utilized by both *S. enterica* and *E. coli* during the attachment to alfalfa sprout seedlings and enhance the attachment of *S. enterica* to intestinal epithelial cells (4, 46, 82). Cellulose, along with thin aggregative fimbriae, contributes to both the aggregative multicellular behavior of *Salmonella* spp. and adherence to animal cells. The actions of cellulose and thin aggregative fimbriae also confer resistance against chlorination, acid, and possibly desiccation (32, 70, 100).

The AgfD-regulated thin aggregative fimbriae and cellulose may aid in the survival of *Salmonella* spp. in the harsh plant environment. Transcription of *agfD* is maximum during stationary growth at 28°C and low osmolarity, which are conditions that would be encountered outside of the host (62). While oxygen and nutrient availabilities interplay to dictate the activity of the transcription of *agfD*, there is maximum expression in both microaerophilic conditions in rich media and in aerobic conditions of minimal media. Nutrient starvation and ethanol presence also increase the expression of *agfD*, with the depletion of nitrogen and phosphate acting as a signal for activation during stationary growth (30). The thin aggregative fimbriae act to regulate aggregative behavior and short-range cell-to-cell interactions, while the *adrA* gene regulates long-range cell-to-cell interactions through activation of cellulose biosynthesis (63).

Previous studies have characterized the attachment of *S. enterica* to alfalfa seedlings and parsley leaves, but the AgfD-regulated gene system of *Salmonella* spp. has not been characterized on other produce (4, 5, 49). *In vitro* studies have shown...
that a $\Delta agfD$ mutant of S. Typhimurium ATCC 14028 completely lacked multicellular behavior and was unable to adhere to glass, form a pellicule in liquid media, or produce an extracellular matrix. In animal studies with the $\Delta agfD$ mutant, there was no significant influence on the virulence of the S. Typhimurium in a mouse model, indicating that the regulator is utilized outside of the animal host (63). On alfalfa seedlings, rpoS, agfD, and bcsA were identified as required genes for the attachment of S. Newport. The results also showed that no individual gene was responsible for attachment, indicating a cooperative mechanism (4, 5).

The studies also noted the downregulation of AgfD during the attachment and colonization of the alfalfa sprouts, which means that genetically based assays targeting upregulated genes during attachment may not correctly characterize the role of the AgfD regulator (4, 5). The genetic characterization of the attachment of Salmonella spp. to tomatoes and the identification of critical extracellular components will lead to a better understanding of the mechanisms used for colonization of plants. The understanding of the microbial behavior of Salmonella spp. and its association with tomatoes may provide specific targets for preharvest treatments for reducing the initial attachment of Salmonella spp. to tomatoes in the field environment.

**Research Aim and Objectives**

Understanding the initial interactions between Salmonella spp. and produce is crucial in establishing targeted preharvest measures to reduce produce contamination. Many preharvest techniques currently used with tomatoes aim to reduce the contact of pathogens and produce, such as the use of uncontaminated water sources and animal exclusion (27). Unfortunately, there are numerous sources of contamination and endless unforeseen circumstances that can introduce a pathogen into a produce field
including events that are out of human control such as storms and flooding.

Establishing protective programs such as the Florida Tomato Good Agricultural Practices (T-GAPS) are helpful in reducing the risk of pathogen introduction into produce fields (27), but there are too many gaps in the knowledge base to establish targeted preharvest treatments.

The main aim of this research is to determine the role of the extracellular components of *Salmonella* spp., specifically AgfD-regulated thin aggregative fimbriae and cellulose, in the initial interactions between the bacterium and the tomato surface of intact, unprocessed tomatoes. The significance of this aim is that produce contamination events cannot occur in the field without the attachment and persistence of the bacterial pathogen. There are numerous factors that contribute to the attachment and persistence of pathogens on produce, as previously stated, and not all of these components can be incorporated into this research. The aspect of *Salmonella* spp. and produce interactions being studied within the scope of this project are the roles of the AgfD-regulated thin aggregative fimbriae and cellulose production in the initial attachment, persistence, and biofilm formation of *S.* Typhimurium on the tomato surface.

The focus was directed towards the attachment and persistence mechanisms of *Salmonella* spp. on the intact tomato surface because T-GAPS currently mandates the culling, sorting, and removal of injured fruit to minimize microbial contamination (27). Therefore, the research focuses on the attachment and persistence of *S.* Typhimurium on the produce that reaches consumers. Furthermore, unprocessed tomatoes that received no sanitation or wax treatments were utilized in the studies to maintain the
condition of the tomatoes in the field at time of harvest. The current information about
the active environmental conditions of the AgfD system and the data demonstrating the
decreased attachment and biofilm formation of *agfD*, *agfB*, and *bcs* deletion mutants of
*Salmonella* supports the hypothesis that AgfD-regulated thin aggregative fimbriae and
 cellulose are critical to the initial attachment, persistence, and biofilm formation of
*Salmonella* spp. on the tomato surface. The overall goal is to determine the
extracellular components of *Salmonella* spp. that contribute to attachment and
persistence mechanisms. This knowledge may be used in the future to ascertain how
*Salmonella* are capable of evading the current washing the sanitation processes for
tomatoes and possibly establish preharvest prevention measures that target the actions
of these extracellular components to inhibit attachment and contamination of produce.

The main objectives of this research are as follows:

1. Determine the roles of AgfD-regulated thin aggregative fimbriae and cellulose in
   the initial attachment of *Salmonella enterica* serovar Typhimurium ATCC 14028
to the surface of intact, unprocessed green and red round tomatoes.

2. Characterize the roles of AgfD-regulated thin aggregative fimbriae and cellulose
   in the persistence of *Salmonella enterica* serovar Typhimurium ATCC 14028 on
   the surface of unprocessed green and red round tomatoes.

3. Assess the roles of AgfD-regulated thin aggregative fimbriae and cellulose in the
   biofilm formation of *Salmonella enterica* serovar Typhimurium ATCC 14028 on
   intact unprocessed green and red round tomatoes and tomato segments.
Figure 2-1. The AgfD regulation system. The components regulating agfD transcription are indicated in the boxes below agfD and agfBAC. HN-S and MrlA are also regulators not included in the diagram. As AgfD is expressed, the expression of agfBAC and adrA are activated. AdrA generates cyclic diguanylate (c-di-GMP), which further activates cellulose biosynthesis through bcsABZC.
CHAPTER 3
GENETIC MUTANT CONSTRUCTION AND CHARACTERIZATION

Background

The main aim of this research was to determine the role of the extracellular components of *Salmonella* spp., specifically AgfD-regulated thin aggregative fimbriae and cellulose, as well as the AgfD regulator, in the initial attachment, persistence, and biofilm formation of *Salmonella* on the tomato surface. *Salmonella* mutants of *S. Typhimurium* ATCC 14028 deficient in AgfD production, thin aggregative fimbriae, and/or cellulose production were designed to assess the role of these extracellular components in the interactions between *Salmonella* spp. and tomatoes. The advantages of using *Salmonella* Typhimurium ATCC 14028 as the parent *Salmonella* strain rather than an outbreak strain include the access to the full genome sequence, susceptibility to phage P22, and lack of antibiotic resistances (1, 84). *S. Typhimurium* 14028 has also been used to research *Salmonella* with lettuce (71), alfalfa (25), and tomatoes (56, 73). *S. Typhimurium* 14028 was compared with tomato associated outbreak strains on the surface and within tomatoes to find the strains displayed similar behaviors (56). This section describes the methodologies used in the construction and characterization of the deletion mutants as well as the genetic complements and vector control strains.

The AgfD-regulated thin aggregative fimbriae and cellulose of *Salmonella* are components that contribute to the production of the extracellular matrix, characterized as rdar (red, dry, and rough) colony morphology. The rdar morphology is expressed as the patterned, aggregative colonies of *Salmonella* when grown on Congo red (CR) media at 25-30°C (31, 61). It has been hypothesized that the rdar morphology of
Salmonella may be part of an environmental persistence strategy, since the morphology has not been shown to increase virulence capabilities (99). The disruption of thin aggregative fimbriae and/or cellulose production in Salmonella spp. can be observed through alternative aggregative patterns of the bacterial colonies on CR media. The β-strand structure of the thin aggregative fimbriae subunits and the glucose linkages of cellulose bind to the hydrophobic dye of the CR media, contributing to the rdar appearance. The lack of intact thin aggregative fimbriae results in pdar (pink, dry, and rough) colony morphology (62). The lack of cellulose biosynthesis results in bdar (brown, dry, and rough) colony morphology, and the lack of both thin aggregative fimbriae and cellulose results in saw (smooth and white) morphology (61, 62). The Salmonella mutants utilized in these studies were phenotypically characterized by their morphology on CR media, as well as on Calcofluor white (CW) agar for cellulose visualization.

Construction of Salmonella Deletion Mutants

DNA Amplification and Visualization

In the construction of the Salmonella enterica serovar Typhimurium ATCC 14028 deletion mutants, polymerase chain reaction (PCR) was performed for confirmation and amplification of specific genes using DNA of individual colonies or plasmid DNA. The recipe routinely used for a single PCR reaction of 25 µl is as follows, 20.5 µl sterile DNA grade water (Fisher Scientific, Pittsburgh, PA), 2.5 µl of 10X Standard Taq buffer with MgCl₂ (New England Biolabs, Ipswich, MA), 1 µl of 2.5 mM deoxynucleotide triphosphates (dNTPs), 0.5 µl of 50 µM forward primer, 0.5 µl of 50 µM reverse primer, and 0.625 units/µl Taq polymerase enzyme (New England Biolabs, Ipswich, MA). To perform colony PCR, an individual colony was lightly touched on an agar plate with
appropriate antibiotics with a sterile pipette tip and was added to the PCR reaction.

PCR of plasmid DNA was performed by adding 1 µl of DNA to the reaction mix. The DNA was amplified in either the TC412 (Techne, Minneapolis, MN) or MJ mini (Bio-Rad, Hercules CA) thermocycler on a cycle running for 10 min at 95°C, 35 cycles of 1 min at 95°C, 1 min at 53°C, 2 min at 72°C, and a final extension at 72°C for 10 min, unless otherwise specified. The primers utilized in these studies are listed in Table 3-1.

The DNA amplified from the PCR reaction was visualized through agarose gel electrophoresis. Tris-Acetate-Ethylene diamine tetraacetic acid (EDTA) buffer (1X), referred to as TAE buffer, was used to make 0.9% (w/v) agarose gels containing 1-3% ethidium bromide. The gels were poured into 7 cm x 10 cm trays in a mini-sub cell GT electrophoresis system (Bio-Rad, Hercules CA). The gel was loaded with 4 µl of exACTGene 1kb Plus DNA Ladder (Fisher Scientific, Pittsburgh, PA) and 8-15 µl of sample. The FB300 power supply (Fisher Scientific, Pittsburgh, PA) was used to complete the electrophoresis. The gels were visualized with the Molecular Imager Gel Doc XR+ System running version 3.0 of the Image Lab software (Bio-Rad, Hercules CA).

The desired Salmonella deletion mutants included the following targeted genes, agfD, the gene producing the AgfD regulator, agfB, the gene encoding the fimbrin protein, AgfB, which is the proposed anchor site of the fimbrial assembly, and bcs, the promoter region and cellulose synthase gene, bcsA (63, 97, 106). The three genes were targeted and amplified in S. Typhimurium 14028 through the PCR methods described in the previous section to ensure major mutations were not present. Due to the large size of the bcs segment (3.7kb), the following thermocycler conditions were
used, 10 min at 95°C, 35 cycles of 2 min at 95°C, 2 min at 53°C, 5 min at 72°C, and a final extension at 72°C for 10 min. The primers used to target each gene are listed in Table 3-1. The PCR products resulted in the predicted gene sizes, indicating no significant mutations or defects in the genes of the parent strain. PCR was also repeated in the Salmonella strains containing the desired mutations.

**Phage-Mediated Transduction**

Transduction was used to transfer mutations between Salmonella strains utilizing bacteriophage, P22. After confirmation of the desired mutations, cell lysates were made from the mutant donor strains. The lysates were made by growing the individual donor strains overnight at 37°C, shaking at 200 revolutions per minute (rpm) in 5 ml of Luria-Bertani (LB) broth (Fisher Scientific, Atlanta, GA) with the appropriate antibiotics (media composition listed in Appendix A). The overnight cultures were washed three times in phosphate buffered saline (PBS) (Fisher Scientific, Pittsburgh, PA). For each donor strain, a series of eight cultures tubes were set up, each containing 5 ml of LB without antibiotics. The overnight culture was used to inoculate each of the 8 tubes, with 100 µl of culture per tube. One hundred µl of the P22 phage was added to the first tube and was serially diluted (1/10) to the following six tubes by transferring 500 ml to each. The remaining tube was the bacterial control. The culture tubes were incubated for 2 hr at 37°C, shaking at 200 rpm. After incubation, the eight culture tubes were observed and the culture tube exhibiting the greatest amount of clearing was centrifuged with the Eppendorf Centrifuge 5415 D at 12,000 x g for 15 min. The supernatant was removed, 100 µl of chloroform was added to the supernatant, and the solution was centrifuged at 12,000 x g for 15 min. The final supernatant was placed in a sterile glass tube with an additional 100 µl of chloroform. Each donor lysate was stored at 4°C. The Salmonella
cultures utilized as donor strains for lysate construction include S. Typhimurium LT2 \(\Delta agfD11::FRT\)-Kan-FRT mutant from Santiviago et al. (66) and S. Typhimurium JSG1748 (58) to result in P22/\(\Delta agfD11::FRT\)-Kan-FRT and P22/bcs::FRT-Kan-FRT, respectively. The full descriptions of the strains are listed in Table 3-2.

The transductions were performed by growing the wild-type recipient culture (S. Typhimurium 14028) overnight in 5 ml of LB at 37°C, shaking at 200 rpm. The overnight culture was washed three times in PBS. A set of eight culture tubes were set up, each containing 100 µl of LB without antibiotics. One hundred µl of culture was added to seven of the eight tubes, with the uninoculated tube being set aside. The first tube was then mixed with 10 µl of P22 Phage and was serially diluted (1/10) in the next five tubes by subsequently transferring 20 µl to each tube. The inoculated tube without the P22 phage was the bacterial control. The tube that was previously set aside was supplemented with 10 µl of P22 phage and was the phage control.

After incubation of the culture tubes at 37°C for 25 min, the tubes were supplemented with 1 ml of LB with 10 mM ethylene glycol tetraacetic acid (EGTA). The cultures were incubated at 37°C for 1 hr and were then centrifuged at 12,000 x g for 1 min. The culture was resuspended in 100 µl of the supernatant and each solution was plated onto LB agar supplemented with kanamycin (50 µg/ml) and EGTA (10 mM). The EGTA was added to the transduction reactions to prevent further phage absorption. The EGTA strongly chelates calcium (Ca\(^{++}\)) that is necessary for the P22 phage attachment (39). The plates were incubated at 37°C overnight and resulting colonies were purified on LB agar supplemented with kanamycin and EGTA three times before being tested on Evans Blue-Uranine (EBU) agar (Appendix A) by being cross-streaked
against P22 phage. The EBU agar test confirms that the culture has been cured of all phage while maintaining phage sensitivity. The antibiotic utilized in the phage-mediated transduction studies was kanamycin because the deletion mutations of all of the donor strains contained kanamycin resistance cassettes.

The *Salmonella* deletion mutants were constructed to result in strains deficient in AgfD regulation, thin aggregative fimbriae, and/or cellulose synthesis. The two *Salmonella* strains used to make the P22 lysates resulted in P22/Δ*agfd*11::FRT-Kan-FRT and P22/bcs::FRT-Kan-FRT. The first lysate, P22/Δ*agfd*11::FRT-Kan-FRT was transduced with the wild-type, S. Typhimurium 14028 to result in the strain, MKF1 (Table 3-2). The second lysate, P22/bcs::FRT-Kan-FRT was constructed from a cellulose deficient strain, JSG1748, provided from the previous work of Prouty and Gunn (58). The lysate was transduced with the wild-type, S. Typhimurium 14028 to result in MKF2 (Table 3-2). The transduction was done to ensure the deletion mutant was in a 14028 strain. The P22/bcs::FRT-Kan-FRT was also transduced with S. Typhimurium TIM2265, a strain deficient in thin aggregative fimbriae production. The final construct, WJZ2, lacks both bcs and agfB genes, making it deficient in both cellulose and thin aggregative fimbriae synthesis (Table 3-2).

**Construction of Genetic Complements and Empty Vector Controls**

**Cloning**

To support the results obtained from the use of MKF1 (14028 Δ*agfd*::FRT-Kan-FRT), MKF2 (14028 bcs::FRT-Kan-FRT), and WJZ2 (14028 bcs::FRT-Kan-FRT Δ*agfb*::FRT) on the tomato surface, control strains of the deletion mutants including complementation vectors expressing the deleted genes were necessary. The genetic complements were used to restore the original phenotypes of the strains when the
deleted genes were expressed on extrachromosomal plasmids. Restoration of the phenotype confirms that the results observed from the deletion mutants were due to the disruption of the specific gene. The primers listed in Table 3-1 were utilized to amplify the three target areas of \textit{agfD}, the \textit{bcsA} gene and upstream promoter, and \textit{agfB}, in \textit{S. Typhimurium 14028}. The respective PCR products were isolated through gel electrophoresis, as previously described, and were purified utilizing the Illustra™ DNA and gel band purification kit (GE Healthcare, Buckinghamshire, UK). The PCR products were each cloned into pCR2.1-TOPO (Invitrogen, Carlsbad, CA) to make pMKF1, pMKF2, and pMKF3, respectively. The pCR2.1 plasmids containing the gene inserts were individually transformed into chemically competent \textit{E. coli} DH5α (Tables 3-2 and 3-3). The \textit{E. coli} DH5α was prepared as described in Inoue et al. (41). The transformation was performed by adding the ligation reaction to a frozen aliquot of \textit{E. coli} DH5α. The mixture was incubated on ice for 30 min, when the cells were heat shocked for 30 sec at 42°C and then recovered on ice for 2 min. The cells underwent incubation in 1 ml of NZY+ for 1 hr at 37°C (NZY broth from Fisher Scientific, Pittsburgh, PA) (media composition in Appendix A). The transformed cells were centrifuged and plated onto LB agar supplemented with kanamycin (50 μg/ml) and 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) (40 μg/ml) for blue-white screening analysis. Primers specific to each insert and M13 primers were used for PCR confirmation (Table 3-1).

Construction of the complementation vectors proceeded with the subcloning of the pMKF1 (pCR2.1 \textit{agfD}), pMKF2 (pCR2.1 \textit{bcs}), and pMKF3 (pCR2.1 \textit{agfB}) into their respective recipient plasmids. The vector and plasmid containing the gene insert of interest were purified using the QIAprep spin mini-prep kit (Qiagen, Santa Clarita, CA)
and were prepared for subcloning by restriction digest with the appropriate restriction enzymes (New England BioLabs, Ipswich, MA). The vectors were digested in parallel overnight at 37°C in NEB 10x Buffer (New England BioLabs, Ipswich, MA). In the case where only one restriction enzyme was used, the vector sample was treated after incubation with 1 µl of calf intestinal alkaline phosphatase (CIAP) in NEB Buffer 3 for 1 hr at 37°C to prevent self-ligation through removal of the 5' phosphate groups. After separation through gel electrophoresis, the desired fragments of the linear plasmid and the DNA insert were excised from the gel and purified using the Illustra™ DNA and gel band purification kit. The restriction fragments were quantified using a Nanodrop 1000 spectrophotometer with software version 3.6.0 (Thermo Scientific, Wilmington, DE).

Ligation reactions contained 4 µl of the recipient vector, 4 µl of the DNA insert, 1 µl of the 10x T4 ligase buffer, and 0.5 µl of the T4 ligase (New England BioLabs, Ipswich, MA). Two control reactions were also performed, one reaction lacking the insert to assess for self-ligation and another reaction lacking the T4 ligase to assess for undigested vector. After overnight incubation, the ligations were transformed into chemically competent E. coli DH5α. Subcloning was confirmed through blue-white screening on LB agar supplemented with X-gal (40 µg/ml) and the appropriate antibiotic and PCR analysis.

The agfD complementation vector was constructed using pMKF1 and the plasmid, pWSK29. Since AgfD is a regulator of Salmonella, it was desirable to use a low copy number plasmid for complementation. EcoRI and SalI were used for the restriction digest of pMKF1 and pWSK29 in NEB 10x Buffer 3 to result in pMKF4. Construction of pMKF4 was confirmed through blue-white screening on LB agar
supplemented with X-gal (40 µg/ml) and ampicillin and PCR analysis with MKF1, MKF2, and M13 primers (Table 3-1). Several attempts were made to subclone the bcs insert from pMKF2 into pWSK29 using EcoRI, but subcloning remained unsuccessful. Therefore, the original pCR2.1 vector containing the bcs insert, pMKF2, was used as the complementation vector. The agfB complementation vector was constructed using MKF3 and the plasmid, pBBR1-MSC5-Gent. The pMKF3 and pBBR1-MSC5 vectors were digested with Clal in NEB 10x Buffer 4 and were treated with CIAP to prevent self-ligation to result in pMKF5. Confirmation of pMKF5 was achieved through blue-white screening on LB agar supplemented with X-gal (40 µg/ml) and gentamicin (50 µg/ml) and PCR analysis with MKF11, MKF13, and M13 primers (Table 3-1).

The first step of confirming pMKF4 (pWSK29 carrying agfD), pMKF2 (pCR2.1 carrying bcs), and pMKF5 (pBBR1-MSc5 carrying agfB) was PCR screening white colonies from the LB agar with X-gal with M13 primers (Table 3-1). The primers bind to Plac up- and downstream from the insertion sites of pWSK29, pCR2.1, and pBBR1-MSC5. A successful gene insertion was indicated by PCR products of approximately 1560 bp for pMKF4, 3900 bp for pMKF2, and 1100 bp for pMKF5. Without a gene insert, the M13 primers result in a product of 200 bp, so the size of the desired PCR product was determined by adding the 200 bp to the size of each gene insert. Once a positive PCR sample was obtained, PCR was repeated with primers M13F and MKF2 for pMKF4, M13F and MKF13 for pMKF2, and M13 and MKF6 for pMKF5. M13F is a forward primer of Plac and MKF2, MKF13, and MKF6 are the reverse primers specific to each gene. The resulting PCR products from these primer pairs indicated that the gene was inserted in the same orientation as Plac. An internal reverse primer was utilized for
pMKF5 to allow use of the standard thermocycler conditions and result in a smaller PCR product of 1400 bp. After PCR confirmation, a diagnostic digest was performed with each of the plasmids with EcoRI and Sall for pMKF4, EcoRI for pMKF2, and Clai for pMKF5 as previously described. Gel electrophoresis was used to visualize the size of the gene insert. The original gene inserts were recovered with products of ~1360 bp for *agfD* from pMKF4, 3700 bp for *bcs* from pMKF2, and 910 bp for *agfB* from pMKF5.

**Electroporations**

Salmonella cannot be made chemically competent. Therefore, transformations by electroporation were used to shuttle plasmids from *E. coli* into Salmonella and between Salmonella strains. Electrocompetent transformations were utilized to move plasmids between the bacterial strains in the construction of the Salmonella complement and empty vector control strains. Electrocompetent recipient strains were prepared from overnight cultures grown in LB with the appropriate antibiotics. The overnight cultures were incubated on ice for 15 min, followed by a washing step to remove all nutrient and salts. The cultures were each washed 4 times with ice-cold DNA grade water, with a final resuspension in 200 µl of cold DNA water. The washed cells were then incubated on ice for an additional 10 minutes. With the cells remaining on ice, the electroporations were performed with 50 µl of the culture and 3 µl (15 ng/µl) of the appropriate plasmid in chilled 2 mm gap electroporation cuvettes (Eppendorf, Hamburg, Germany). The cuvettes were dried and the cells were shocked (25 μF, 200 Ω, 2.5 kV) using a MicroPulser electroporator (Bio-Rad, Hercules, CA). The cells were recovered in 1 ml of NZY+ and incubated at 37°C for 1 hr. After incubation, the cells were centrifuged at 12,000 x g for 1 min and resuspended in 100 µl of supernatant. The cells were then plated onto LB with appropriate antibiotics.
The *Salmonella* mutant complementation and empty vector controls were constructed by first purifying the necessary plasmids, pMKF4 (pWSK29 *agfD*), pMKF2 (pCR2.1 *bcs*), and pMKF5 (pBBR1-MSC5 *agfB*), from *E. coli* DH5α using the QIAprep spin mini-prep kit. To transfer the plasmids from an *E. coli* strain to *Salmonella*, the plasmids were individually passed through an intermediate host, the restriction-minus modification-plus *Salmonella* strain, JS198, which was derived from *S. Typhimurium* 14028 prior to transformation into the final *S. Typhimurium* host strains (26). From JS198, the plasmids were again purified utilizing the QIAprep spin mini-prep kit and were transformed into their final host strains. The pMKF4 plasmid was transformed with MKF1 to construct the *agfD* complement strain, MKF1 pMKF4. The pMKF2 plasmid was transformed with both MKF2 and WJZ2. The final *bcs* complement strain was MKF2 pMKF2 (Tables 3-2 and 3-3). The double mutant complement was completed by transforming pMKF5 into WJZ2 pMKF2 to complement both of the deleted genes of WJZ2 pBBR1-MSC5 was used for the vector of pMKF5 because of the encoded gentamicin resistance so both complementation plasmids of WJZ2 could be selected for with the use of different antibiotics (Tables 3-2 and 3-3). As a negative control, the vectors without gene inserts were also transformed into the appropriate strains to result in MKF1 pWSK29, MKF2 pCR2.1, and WJZ2 pCR.21 pBBR1 (Table 3-2).

**Phenotypic Characterization of Salmonella Strains**

Phenotypic assays were performed on *S. Typhimurium* 14028 and the mutants to assess the morphology on Congo red (CR) media and the cellulose production of the strains. The extracellular components, including thin aggregative fimbriae and cellulose, bind the hydrophobic dyes of the CR media, contributing to the resulting color
of the colonies. The colonies may also form complex surface patterns that can be completely removed from the agar plates. The presence of the extracellular components of *Salmonella* and the rough, aggregative pattern results in the rdar (red, dry, and rough) morphology on CR agar. The lack of intact thin aggregative fimbriae and/or cellulose result in alternative morphologies including pdar (pink, dry, and rough) from a lack of intact thin aggregative fimbriae, bdar (brown, dry, and rough) from deficient cellulose production, and saw (smooth and white) from a lack of both extracellular components (61, 62).

CR media was prepared with 40 μg/ml of Congo red dye (MCB, Norwood, OH) and 20 μg/ml of Brilliant Blue R-250 (Acros Organics, Geel, Belgium) in salt-less LB agar (as described in Appendix A) (23, 62). Cultures were started from frozen glycerol stocks and used to inoculate 5 ml of LB broth with appropriate antibiotics. Inoculated cultures tubes were incubated overnight at 30°C, shaking at 200 rpm and then 6 µl of each culture were spotted onto CR agar. The plates were incubated at 30°C for 48 hr. Photographs of cultures on CR media are displayed in Figure 3-1. The S. Typhimurium 14028 has the rdar morphology, with the modified 14028 strains, MKF5 and JS246, having similar rdar phenotypes. The MKF1 strain expressed as pdar morphology, MKF2 appeared as bdar, and the double mutant, WJZ2, appeared as a light pink to saw phenotype. The phenotypes of the mutant colonies on CR media confirm the successful deletion of the targeted genes. The phenotype of MKF1 pMKF4 showed exaggerated rdar morphology, likely due to multiple expression of the AgfD regulator on the pMKF1 plasmids. The MKF2 pMKF2 and WJZ2 pMKF2 pMKF5 complement strains indicate only partial restoration of the rdar phenotype with an increase in red color, but
little patterned, aggregative appearance. The three strains carrying the empty vectors controls do not exhibit phenotype restoration, indicating that the phenotype restoration of the complements was a result of the expression of the vector gene insert.

Cellulose production of the *Salmonella* strains was assessed through both qualitative and quantitative assays. Salt-less LB agar supplemented with Calcofluor white with Evans Blue dye (Sigma Aldrich, St. Louis, MO) to a final concentration of 40 μg/ml resulted in Calcofluor white (CW) media (Appendix A). The calcofluor detects the β-1,4-glucose linkages of the cellulose and fluorescence under a 366-nm ultraviolet (UV) light source (106). Glycerol stocks of *Salmonella* strains were used to inoculate 5 ml of LB broth with appropriate antibiotics. Cultures were incubated overnight at 30°C, shaking at 200 rpm. The cultures were streaked onto the CW agar plates and incubated without light exposure at room temperature for two days. After 48 hr, the plates were placed on the UV trans-illuminator and were photographed (Figure 3-2). The plates were then placed back into the dark for 30 min. After the dark treatment, the cultures were suspended in sterile deionized (DI) water and samples were loaded into a 96-well polystyrene plate along with a control of DI water from an uninoculated CW agar plate. The optical density and fluorescence readings of the cultures were measured and recorded as performed in Da Re and Ghigo (23). Final cellulose production was quantified using the following equation, ((fluorescence of sample) - (fluorescence of control) / (OD_{600} of sample – OD_{600} of control)). Photographs of the CW plates are displayed in Figure 3-2, with cellulose quantification in Figure 3-3.

The greatest fluorescence was observed from the 14028, MKF5, JS246, and MKF1 pMKF4 strains (Figures 3-2 and 3-3). It was expected to see high cellulose
production from the 14028 strain and the modified 14028 strains, MKF5 and JS246. A reduction in fluorescence was recorded for the MKF1, MKF2, and WJZ2 strains. A greater amount of fluorescence was observed in the S. Typhimurium MKF1 than in MKF2 and WJZ2 because cellulose production through adrA can be expressed in the absence of AgfD. Cellulose production was directly affected in the MKF2 and WJZ2 strains with the deletion of a portion of the bcs operon. Full to partial restoration of cellulose production restoration was observed in the S. Typhimurium MKF1 pMKF4, MKF2 pMKF2, and WJZ2 pMKF2 pMKF5 strains by an increase in recorded fluorescence. The three strains carrying the empty vectors (controls) did not exhibit phenotype restoration, indicating that the phenotype restoration of the complements was not the result of the transformation of the plasmid(s) alone.

**Growth Curves of Salmonella Strains**

The growth rates and patterns of S. Typhimurium 14028 and the strains derived from it were assessed before the use of the strains in studying the roles of AgfD, thin aggregative fimbriae, and/or cellulose production in the attachment, persistence, and biofilm formation of Salmonella on the tomato surface. Growth curves were used to establish if the genetic mutations affected the growth rate of the cultures. The use of cultures with slowed or altered growth patterns could result in inaccurate data on the fitness of the cultures on the tomato surface.

Glycerol stocks of the Salmonella strains were used to inoculate 5 ml of LB broth with appropriate antibiotics and incubated overnight at 30°C, at 200 rpm. Overnight cultures were diluted in sterile PBS to a concentration of $10^6$ CFU/ml. A volume of 1 ml of the diluted culture was added to 99 ml of sterile LB, resulting in a starting culture of approximately $10^4$ CFU/ml. Cultures were incubated at 30°C for 12 hr, while shaking at
200 rpm. Cultures were serially diluted each hour, including time 0 (10 minutes after inoculation), to the dilutions of the countable range (25-250 CFU/ml) and plated onto LB agar. The original cultures were also plated. Plates were incubated overnight at 37°C and CFU were counted. The average log_{10} CFU/ml population was calculated for each time point. Analysis of variance (ANOVA) was performed using JMP 9.0 Pro statistical software (SAS Institute Inc., Cary, NC) to determine significant differences among resulting concentrations at each hour. With a significance of p<0.05, no significant differences were found between the S. Typhimurium 14028 and isogenic mutant strains depicted in Figure 3-4.
<table>
<thead>
<tr>
<th>Name</th>
<th>Primer Sequence</th>
<th>Primer Target</th>
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</thead>
<tbody>
<tr>
<td>MKF1</td>
<td>GTCGACAGTGAACTCTGCTGCTACAATCCAG</td>
<td>Forward primer of <em>agfD</em> in <em>S. Typhimurium</em>, SalI site at 5’ end</td>
</tr>
<tr>
<td>MKF2</td>
<td>GTACGATGAGAGTATGTCCCTG</td>
<td>Reverse primer of <em>agfD</em> in <em>S. Typhimurium</em></td>
</tr>
<tr>
<td>MKF5</td>
<td>CATTAACCCTGGACGCAAAAGAC</td>
<td>Forward primer of <em>agfB</em> in <em>S. Typhimurium</em></td>
</tr>
<tr>
<td>MKF6</td>
<td>AGACTGCCTGAAAATACGATT</td>
<td>Reverse primer of <em>agfB</em> in <em>S. Typhimurium</em></td>
</tr>
<tr>
<td>MKF11</td>
<td>ATCGATATACCTCACAGCAGCTTGTACACTGCC</td>
<td>Forward primer of <em>bcs</em> in <em>S. Typhimurium</em>, Clal site at 5’ end</td>
</tr>
<tr>
<td>MKF12</td>
<td>ATCGATCACGTATATGTGTCAGATCCAG</td>
<td>Reverse primer of <em>bcs</em> in <em>S. Typhimurium</em>, Clal site at 5’ end</td>
</tr>
<tr>
<td>MKF13</td>
<td>AGGTCTTGTATAGGTCGTTACA</td>
<td>Internal primer of <em>bcs</em> in <em>S. Typhimurium</em></td>
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<tr>
<td>M13F</td>
<td>GTAAAACGACGGCCAG</td>
<td>Forward primer of <em>lacZY</em> (Invitrogen)</td>
</tr>
<tr>
<td>M13R</td>
<td>CAGGAAACAGCTATGAC</td>
<td>Reverse primer of <em>lacZY</em> (Invitrogen)</td>
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### Table 3-2. Bacterial strains

<table>
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<tr>
<th>Isolate or strain</th>
<th>Relevant characteristics*</th>
<th>Source, construction, or reference</th>
</tr>
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<tbody>
<tr>
<td><em>Salmonella</em> Typhimurium Isolates</td>
<td></td>
<td></td>
</tr>
<tr>
<td>14028</td>
<td>Wild type</td>
<td>American Type Culture Collection</td>
</tr>
<tr>
<td>MKF5</td>
<td>14028 Kan&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Spontaneous Kan&lt;sup&gt;R&lt;/sup&gt; of <em>S.</em> Typhimurium 14028</td>
</tr>
<tr>
<td>JS246</td>
<td>14028 <em>yjeF</em>:res1-tetR-res1, Tet&lt;sup&gt;R&lt;/sup&gt; LT2 metE551 metA22 ilv452 trpB2 hisC527(am) galE496 xyl-404 rpsL120</td>
<td>(55)</td>
</tr>
<tr>
<td>JS198</td>
<td>flaA66 hsdL6 hsdSA29 zjg8103::pir+recA1</td>
<td></td>
</tr>
<tr>
<td>MKF1</td>
<td>14028 Δ<em>agfD11</em>:FRT-Kan-FRT, Kan&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This study, <em>agfD11</em>:frt-kan-frt mutation from Santiviago et al. (66) transduced into <em>S.</em> Typhimurium 14028</td>
</tr>
<tr>
<td>MKF1 pMKF4</td>
<td>14028 Δ<em>agfD11</em>:FRT-Kan-FRT pWSK29 carrying *agfD, Kan&lt;sup&gt;R&lt;/sup&gt;, Amp&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>MKF1 pWSK29</td>
<td>Kan&lt;sup&gt;R&lt;/sup&gt;, Amp&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>JSG1748</td>
<td><em>bcs::FRT</em>-Kan-FRT, Kan&lt;sup&gt;R&lt;/sup&gt;</td>
<td>(58)</td>
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<tr>
<td>MKF2</td>
<td>14028 <em>bcs::FRT</em>-Kan-FRT, Kan&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This study, <em>bcs::FRT</em>-Kan-FRT from JS1748 transduced into <em>S.</em> Typhimurium 14028</td>
</tr>
<tr>
<td>MKF2 pMKF2</td>
<td>14028 <em>bcs::FRT</em>-Kan-FRT pCR2.1 carrying *bcs, Kan&lt;sup&gt;R&lt;/sup&gt;, Amp&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>MKF2 pCR2.1</td>
<td>Kan&lt;sup&gt;R&lt;/sup&gt;, Amp&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>WJZ2</td>
<td>14028 <em>bcs::FRT</em>-Kan-FRT Δ<em>agfB15::FRT</em>, Kan&lt;sup&gt;R&lt;/sup&gt;, Gent&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>WJZ2 pMKF2 pMKF5</td>
<td>14028 <em>bcs::FRT</em>-Kan-FRT Δ<em>agfB15::FRT</em> pCR2.1 *agfB, Kan&lt;sup&gt;R&lt;/sup&gt;, Gent&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>WJZ2 pCR2.1 pBBR1</td>
<td>14028 <em>bcs::FRT</em>-Kan-FRT Δ<em>agfB15::ftr</em> pCR2.1 pBBR1-MSC5, Kan&lt;sup&gt;R&lt;/sup&gt;, Gent&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>Isolate or strain</td>
<td>Relevant characteristics*</td>
<td>Source, construction, or reference</td>
</tr>
<tr>
<td>------------------</td>
<td>---------------------------</td>
<td>-------------------------------------</td>
</tr>
<tr>
<td>JTN176</td>
<td>Spontaneous non-rdar mutant of S. Typhimurium 14028</td>
<td>(104)</td>
</tr>
<tr>
<td>JTN212</td>
<td>Spontaneous non-rdar mutant of S. Typhimurium 14028</td>
<td>(104)</td>
</tr>
<tr>
<td><em>TetR</em> designates tetracycline resistance, KanR, kanamycin resistance; AmpR, ampicillin resistance; GentR, gentamicin resistance</td>
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</table>

Escherichia coli isolates

| DH5α             | F-φ80lacZ∆M15 ∆(lacZYA-argF) U169 recA1 endA1 hsdR17 (rk-, mk+) phoA supE44 λ-thi-1 gyrA96 relA1 | Invitrogen (55) |

Table 3-2. Continued
<table>
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<th>Plasmid name</th>
<th>Relevant characteristics*</th>
<th>Source or reference</th>
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<tr>
<td>pCR2.1 TOPO</td>
<td>Cloning vector, Kan$^R$, Amp$^R$</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>pWSK29</td>
<td>Low copy number cloning vector, Amp$^R$</td>
<td>(93)</td>
</tr>
<tr>
<td>pBBR1-MSC5</td>
<td>Cloning vector, Gent$^R$</td>
<td>(48)</td>
</tr>
<tr>
<td>pMKF1</td>
<td>pCR2.1 containing <em>agfD</em> amplified by MKF1 and MKF2 from 14028 with expression from the native promoter, Kan$^R$, Amp$^R$</td>
<td>This study</td>
</tr>
<tr>
<td>pMKF2</td>
<td>pCR2.1 containing <em>bcs</em> amplified by MKF11 and MKF12 from 14028 with expression from the native promoter region and cellulose synthase gene, <em>bcsA</em>, that are in the same orientation as Plac on pCR2.1, Kan$^R$, Amp$^R$</td>
<td>This study</td>
</tr>
<tr>
<td>pMKF3</td>
<td>pCR2.1 containing <em>agfB</em> amplified by MKF5 and MKF6 from 14028 with expression from the native promoter, Kan$^R$, Amp$^R$</td>
<td>This study</td>
</tr>
<tr>
<td>pMKF4</td>
<td>pWSK29 containing <em>agfD</em> subcloned from pMKF1 from 14028 with expression from the native promoter and in the same orientation as Plac on pWSK29, Amp$^R$</td>
<td>This study</td>
</tr>
<tr>
<td>pMKF5</td>
<td>pBBR1-MSC5 containing <em>agfB</em> subcloned from pMKF3 from 14028 with expression from the native promoter and in the same orientation as Plac on pBBR1-MSC5, Gent$^R$</td>
<td>This study</td>
</tr>
</tbody>
</table>

*Kan$^R$ designates kanamycin resistance; Amp$^R$, ampicillin resistance; Gent$^R$, gentamicin resistance
Figure 3-1. Depictions of *Salmonella* Typhimurium strains on Congo red media. A) *S. Typhimurium* 14028, B) MKF5, C) JS246, D) MKF1, E) MKF2, F) WJZ2, G) MKF1 pMKF4, H) MKF1 pWSK29, I) MKF2 pMKF2, J) MKF2 pCR2.1, K) WJZ2 pMKF2 pMKF5, and L) WJZ2 pCR2.1 pBBR1. *Salmonella* cultures spotted onto Congo red media and incubated at 30°C for 48 hr. An Olympus MVX10 confocal microscope and Olympus MVX-TV1XC camera was used to photograph the strains. The *S. Typhimurium* 14028 has the rdar morphology, with MKF5, the spontaneous 14028 kanamycin resistant strain, and JS246 have similar rdar phenotypes. The MKF1 strain appears as the pdar morphology, MKF2 displays bdar, and the double mutant, WJZ2, has a light pink to saw phenotype. The phenotype of MKF1 pMKF4 is an exaggerated rdar morphology. The *bcs* and double mutant complement strains indicate only partial restoration of the rdar phenotype with an increase in red color, but little patterned, aggregative appearance. The three empty vector controls do not indicate phenotype restoration from the plasmid presence alone.
Figure 3-2. Qualitative assessment of cellulose production of *Salmonella* Typhimurium strains. A) S. Typhimurium 14028, B) MKF5, C) JS246, D) MKF1, E) MKF2, F) WJZ2, G) MKF1 pMKF4, H) MKF1 pWSK29, I) MKF2 pMKF2, J) MKF2 pCR2.1, K) WJZ2 pMKF2 pMKF5, and L) WJZ2 pCR2.1 pBBR1. Above are the *S. Typhimurium* strains under dark conditions on Calcofluor White (CW) agar. Photographs were taken with a digital camera on the UV trans-illuminator after the plates were incubated for 48 hr at room temperature.
Figure 3-3. Quantitative assessment of cellulose production of *Salmonella* Typhimurium strains. The graph represents the quantified relative fluorescence units of cellulose produced by each strain. Cells were resuspended on the CW agar in DI water and the fluorescence was recorded in 96 well plates at an excitation of 360 ± 40 nm and emission of 460 ± 40 nm. Optical density of the cultures was measured at 600 nm. Relative fluorescence was then calculated by fluorescence of sample minus the fluorescence of control divided by the OD$_{600}$ of sample minus the OD$_{600}$ of control and is expressed as fluorescence per unit of OD$_{600}$ ± standard deviation from three replicate studies.
Figure 3-4. Growth curves of *Salmonella* Typhimurium 14028 and mutant strains for 12 hr at 30°C. A) Illustrates the growth curves of the *S*. Typhimurium 14028 strain along with the three deletion mutants deficient in AgfD, cellulose, and thin aggregative fimbriae production. B) Illustrates the growth curves of *S*. Typhimurium MKF5 and JS246, two modified strains of 14028, as well as the genetic complements and empty vector control strains of the three deletion mutants. Overnight cultures of each strain were diluted to the approximate concentration of $10^4$ CFU/ml and incubated at 30°C, shaking at 200 rpm for up to 12 hr. Samples were diluted and plated once per hour, including hour 0. The final growth curves were the average of three replicates per strain with error bars indicating standard deviation.
CHAPTER 4
THE ROLES OF AgfD-REGULATED THIN AGGREGATIVE FIMBRIAE AND CELLULOSE IN THE INITIAL ATTACHMENT AND PERSISTENCE OF *Salmonella* Typhimurium ON THE TOMATO SURFACE

**Background**

There are numerous routes of contact between produce and *Salmonella* spp. in the field environment including contaminated irrigation water, soil, manure, fecal matter, or through animal reservoirs (42, 67, 79). The microbiological quality of irrigation water is crucial for maintaining the safety of produce. *Salmonella* spp. also survive in water sediments, making flooding of fields, either seasonally or from storm events, a potential source of both produce and soil contamination (6, 79). The introduction of *Salmonella* spp. in produce fields can also occur through the use of improperly composed manure. The use of compost is beneficial in the maintenance of soil fertility, but improperly composed manure may contain gastrointestinal pathogens from the animal source(s), including *Salmonella* spp. *S. Typhimurium* was found to survive for more than 200 days in soil amended with contaminated compost (42). Field contamination from feces may result from domestic or wild animals roaming in the fields as well.

*Salmonella* spp. are able to colonize both cold-blooded and warm-blooded animals. The colonization of the animals does not necessarily infer illness for the host, resulting in animal reservoirs or carriers. *S. Typhimurium* and *S. Enteritidis* are considered to have a broad host range since they cause disease in a wide range of animals. *S. Typhi* and *S. Gallinarum* are host specific serovars, with the capability of causing disease in only humans and fowl, respectively. The initial introduction of the bacterium into the animal host is often through a food or water source, where the animals further shed the bacterium back into the environment (101). The shedding
events may occur whether the *Salmonella* is virulent or non-virulent in the host. Birds and flies are among many animal vectors capable of widespread dissemination of *Salmonella* spp. in the environment (24). Once the bacterium has re-entered the environment, plants may be utilized as a vehicle for pathogen transfer. Thus, enteric bacteria may exhibit mechanisms to survive on plants to use them as a transfer medium between animal hosts (7).

The genetic mechanisms employed in the interactions between *Salmonella* spp. and produce are not yet well understood. Through any of the previously mentioned routes of contamination, the *Salmonella* spp. must attach and persist in the plant environment in order to cause an outbreak event. In previous research on the attachment and survival of *Salmonella* spp. on produce, Ukuku and Sapers reported *S. Stanley* to be firmly attached to the surface of cantaloupes within 4 hr (87) and Iturriaga et al. indicated that 0.3-0.7% of *Salmonella* inoculum ($10^5$-$10^8$ CFU/ml) attached to the tomato surface within 30 sec of contact (44). It has also been demonstrated that *S. Montevideo* persisted on tomato surfaces at 20-30°C and survived at 10-30°C at 45 to 60% relative humidity (RH) for up to 18 days (105). *S. Montevideo* persistence on tomatoes was also associated with humidity conditions, with increased persistence at higher relative humidity (60-97% RH) (43).

The aboveground surfaces of plants are protected from desiccation and bacterial infiltration by the plant cuticle, waxes, and polysaccharides (77). Bacterial attachment to surfaces is characterized as a two-step process including reversible phase and an irreversible attachment. Reversible binding is hypothesized to be directed by physiochemical properties of the bacterium and surface with flagella, pili, and fimbriae...
suspected to influence this first step of attachment. The stronger, irreversible attachment is hypothesized to involve both chemical and physical properties with extracellular components, such as cellulose, contributing to attachment. There is also evidence that there are locations of favored attachment on plant surfaces, including the cuticle, stomata, and/or trichomes (44, 84).

In *Salmonella*, AgfD directly regulates thin aggregative fimbriae and indirectly regulates cellulose biosynthesis through *adrA* (100). The study of a Δ*agfD* mutant of *S. Typhimurium* 14028 demonstrated reduced adherence to glass, pellicule formation in liquid media, and extracellular matrix production at 30°C (63). *Salmonella* strains lacking thin aggregative fimbriae production have demonstrated significantly reduced attachment to polystyrene and glass surfaces (45). The production of cellulose in *Salmonella* is generally used for protection and plays a structural role in the strength of biofilms (31, 76). *Salmonella* mutants deficient in cellulose failed to attach or form biofilm to chitin beads or fungi (10). The attachment of *Salmonella* mutants deficient in both thin aggregative fimbriae and cellulose to parsley were significantly reduced (49). Previous research also indicated the importance of thin aggregative fimbriae and cellulose in the attachment of *S. enterica* to alfalfa sprout seedlings (4, 5).

In this study, the aim was to characterize the roles of the AgfD regulator and AgfD-regulated thin aggregative fimbriae and cellulose in the initial attachment and persistence of *Salmonella* spp. on the surface of green and red tomatoes. Previous research indicates that *Salmonella* spp. is capable of rapidly attaching to the surface of tomatoes. Furthermore, the AgfD-regulated thin aggregative fimbriae and cellulose production of *Salmonella* spp. are proposed to have important roles in the attachment to
both abiotic and plant surfaces. This information was used to develop the hypothesis that S. Typhimurium deficient in AgfD-regulated thin aggregative fimbriae and cellulose production would demonstrate reduced initial attachment and persistence on the tomato surface. The role of the AgfD regulator was also assessed to maintain if AgfD-independent thin aggregative fimbriae and cellulose production could be achieved in the strains.

**Materials and Methods**

**Culture Maintenance**

All *Salmonella* strains were maintained as frozen glycerol stocks in LB broth with 35% glycerol. The strains were grown through sub-culturing from stocks into 5 ml of LB broth with appropriate antibiotics. Antibiotics were utilized at the following concentrations: ampicillin (Amp), 100 µg/ml; gentamicin (Gent), 50 µg/ml; kanamycin (Kan), 50 µg/ml, and tetracycline (Tet), 10 µg/ml. Cultures were prepared through overnight incubation at 30°C, shaking at 200 rpm prior to all experiments, unless specified. The determination that incubation while shaking did not alter the attachment and biofilm formation abilities of the strains is detailed in Appendix B.

**Acquisition of Tomato Samples**

All produce utilized in the study was unwaxed. Red, vine-ripe, round tomatoes cultivar Campari were obtained from the local supermarket (Publix Grocery). According to the packaging label, the tomatoes originated from Mexico, Canada, or New Jersey, USA. The green and red unwaxed, unprocessed tomatoes cultivars Florida 47 and Quincy were obtained from various locations throughout Florida, including the University of Florida Institute of Food and Agricultural Sciences (IFAS) North Florida Research and Education Center in Marianna, FL; DiMare Fresh in Tampa, FL; Pacific Tomato Growers
in Palmetto, FL; West Coast Tomato in Palmetto, FL; and Gadsden Tomato Company in Quincy, FL. Green tomatoes refers to ripeness stage 1 and red tomatoes refers to ripeness stage 6 of the US Department of Agriculture (USDA) color classification in the US Standards for Grades of Fresh Tomatoes (88).

**Evaluation of Hydrophobicity of *Salmonella* Strains**

Evaluation of hydrophobicity was performed using the bacterial adherence to hydrocarbons (BATH) method first described by Rosenberg et al. (65) and a modified salt agglutination test (SAT) (52, 102). In preparation of the BATH assay, overnight cultures of *S. Typhimurium* 10428, MKF1, MKF2, WJZ2, and JS246 were prepared and washed twice in PUM buffer (Appendix A) and diluted to an optical density (OD) of 0.8 when measured at 400 nm (OD$_{400}$). Round bottom test tubes were filled with 1.2 ml of the washed cells suspended in PUM buffer. Volumes ranging from 50 μl to 200 μl of *n*-hexadecane (Acros Organics, Geel, Belgium) were added to the washed cells. Following a preincubation at room temperature (22°C) for 10 min, the solutions were vortexed for 120 sec and incubated at room temperature for 15 min to allow for hydrocarbon separation. The aqueous phase was carefully removed with a Pasteur Pipette and transferred to a 1 ml cuvette where the optical density was read at 400 nm by the BioSpec-mini spectrophotometer (Shimadzu, Kyoto, Japan). The results were recorded as the percentage absorbance of the aqueous phase after treatment relative to the initial absorbance of the bacterial suspension. The equation is diagramed as follows, $[(\text{OD}_{400} \text{ original bacterial suspension} – \text{OD}_{400} \text{ aqueous phase sample})/(\text{OD}_{400} \text{ original bacterial suspension})] \times 100$. The assay was performed with three biological replicates.
For the SAT assay, *S. Typhimurium* 14028, JS246, MKF1, MKF2, and WJZ2 were grown on LB agar at 37°C overnight. The cells were suspended in 0.002 M sodium phosphate buffer (Appendix A), washed twice and diluted to $10^8$ CFU/ml based on OD$_{600}$ and plate counts. Once diluted, 25 μl of the bacterial suspensions were mixed with an equal volume of varying concentrations of ammonium sulfate ranging from 0.2 to 4.2 M in 0.5 M increments in 0.002 M sodium phosphate buffer. The bacterial and ammonium sulfate solutions were mixed for two minutes and incubated at room temperature for 30 min. The lowest molar concentration of ammonium sulfate producing visible aggregation was scored as the numerical value for the bacterial surface hydrophobicity, the SAT value.

**Desiccation of *Salmonella* on the Tomato Surface**

Overnight cultures of *Salmonella Typhimurium* 14028 and MKF1 were washed three times in sterile DI water and diluted to a starting concentration of $10^7$ CFU/ml. The initial inocula were diluted and plated onto XLD. Vine-ripened, red Campari tomatoes were then spot inoculated with 10 spots in volumes of 10 µl on each tomato. The tomatoes were stored at approximately 22°C, 60-80% RH. After 5 min, two tomatoes of each inoculum were placed into individual stomacher bags filled with 100 ml of PBS. Each bag was shaken for 30 sec and the rinsates were diluted and plated onto xylose lysine deoxycholate (XLD) agar (Becton Dickson, Franklin Lakes, NJ). This procedure was repeated at 15, 30, 60, and 120 min. The plates were incubated overnight at 37°C to obtain plate counts of the surface concentrations of recovered *Salmonella*. Statistical analysis was completed through JMP 9.0 Pro with paired t-tests of the recovered concentrations at each time point with a significance set to $p<0.05$. 
Assessment of Tomato Inoculation Procedures

The study was performed to compare the concentrations of recoverable *Salmonella* from the surface of vine-ripened, red Campari tomatoes after either spot or dip inoculation. Overnight cultures of *S. Typhimurium* 14028, MKF2, and WJZ2 were washed three times in sterile DI water. The strains were diluted with 1 ml of each strain added to 9 ml of sterile DI water to result in three separate inocula. The initial inocula were diluted and plated onto XLD. The blossom end of the Campari tomatoes were dip inoculated for 2 min in *S. Typhimurium* 14028, MKF2, or WJZ2 inocula. For the spot inoculation studies, the washed cultures were directly used to inoculate 10 spots of 10 µl each onto the blossom end of the Campari tomatoes. The initial inocula were diluted and plated onto XLD agar. The studies were performed in triplicate, with three biological replicates.

Tomatoes rested at room temperature for 5 min and were then rinsed in 10 ml of sterile DI. The rinsates were diluted and plated onto XLD agar. The tomatoes were stored in a two level environmental chamber maintained at 22°C, 60-80% relative humidity (RH). After 2 hr, the blossom ends of the tomatoes were rinsed in 10 ml of sterile DI and the rinsates were plated onto XLD. Tomatoes were then sterilily moved into individual stomacher bags (Whirl-Pak, Fort Atkinson, WI) containing a membrane filter and 100 ml of PBS. The tomatoes were rubbed by hand for 60 sec with the blossom scar facing the membrane filter. Rinsate from the filtered side of the bag was collected and plated onto XLD. Plates were incubated overnight at 37°C to obtain plate counts of recovered bacterial concentrations from the tomato surface. Preliminary tests were also performed to show the membrane filter was not resulting in decreased plate
counts due to bacterial binding to the filter. The final results were averaged from three biological replicates performed in triplicate for a sample size of 9 tomatoes per strain.

**Initial Attachment of *Salmonella* Typhimurium 14028, MKF1, MKF2, and WJZ2 to the Tomato Surface**

Overnight cultures of *S. Typhimurium* 14028, MKF1, MKF2, and WJZ2 were washed three times in sterile DI water and diluted to a concentration of 1.0 x 10^8 CFU/ml. The initial inocula were diluted and plated onto XLD agar. The washed cultures were used to inoculate the blossom end of unwaxed, unprocessed green Florida 47 tomatoes 10 times in 10 µl volumes (for a total of 100 µl per tomato). The inoculated tomatoes remained at room temperature for 5 min. The blossom end of the tomatoes underwent an initial rinse in individual containers of 10 ml of sterile DI water. The rinsates were diluted and spread on XLD. Tomatoes were stored in a two level environmental chamber at 22°C, 60-80% RH for 2 hr. After 2 hr, the blossom end of the tomatoes were rinsed in 10 ml of PBS and were placed into Whirl-Pak stomacher bags containing 100 ml of PBS. Each tomato was rubbed by hand in the stomacher bags for 60 sec. The rinsates from the secondary rinse and from the stomacher bag were diluted and plated onto XLD agar. The plates were incubated overnight at 37°C to obtain plate counts of the recovered *Salmonella* concentrations. Average results were obtained from three biological replicates, performed in triplicate. Analysis of variance (ANOVA) comparisons were performed for each the initial rinse, secondary rinse, and tomato rinse results with JMP 9.0 Pro statistical software.

**Competitive Co-infections of *Salmonella* Strains on the Tomato Surface**

Overnight cultures of *S. Typhimurium* 14028, JS246, MKF1, MKF2, and WJZ2 were washed three times in sterile DI water. The washed cells were measured at 600
nm in a spectrophotometer and a 1:1 concentration ratio inoculum was generated for 14028:JS246, 14028:MKF1, 14028:MKF2, and 14028:WJZ2. The initial inocula were diluted and plated onto XLD. The blossom end of unwaxed, unprocessed, green and red Florida 47 tomatoes were each dip inoculated into a co-inoculum for 2 min. The tomatoes rested, blossom end up, at room temperature for 5 min. The tomatoes then underwent an initial rinse in a container of 10 ml of sterile DI. The rinsate was diluted and plated onto XLD. The tomatoes were stored in an environmental chamber at 22°C, 60-80% RH for 2 hr.

After 2 hr, the blossom ends of the tomatoes were again rinsed in 10 ml of sterile DI. A sample of the rinsate was directly plated onto XLD. The blossom end of the tomato was then swabbed and spread on a section of an XLD plate and the sample was streaked for isolation with a sterile, metal loop. The plates were incubated at 37°C overnight. One hundred colonies from each of the initial co-inoculum, initial rinse, and tomato swab plates were patched onto either LB agar with tetracycline or kanamycin. Samples of the 14028:JS246 co-inoculum were patched onto LB agar with tetracycline to determine the number of tetracycline resistant JS246 colonies. The remaining samples were patched onto LB agar with kanamycin to determine the number of kanamycin resistant colonies. The numbers of antibiotic resistant colonies from the plates were used to determine the wild type to mutant ratios. Changes in the wild type to mutant ratio between the initial co-inoculum and the samples recovered from the tomato surface were used to calculate the competitive index with the following equation, competitive index (CI) = [Mutant_{out}/14028_{out}]/ [Mutant_{in}/14028_{in}]. The sample size was 15 green or red tomatoes for each strain. The resulting CI values were log transformed.
and statistical analysis was performed in JMP 9.0 Pro by assessing pairwise t-tests of the CI results for the 14028:mutant studies versus the 14028:JS246 control at a significance of $p<0.05$.

**Persistence of *Salmonella* Strains in Buffered Solution**

Overnight cultures of *S. Typhimurium* 14028, MKF1, MKF2, and WJZ2 were washed three times in sterile PBS with a final wash and suspension in 0.1 M potassium phosphate buffer (Appendix A). A set of five test tubes were filled with 5 ml of sterile 0.1 M potassium phosphate buffer and 5 µl of each washed culture was added to result in a starting culture of $10^6$ CFU/ml. The remaining uninoculated tube served as a negative control. After vortexing, a 100 µl sample was removed from each culture tube. The sample was diluted and plated onto LB agar. The plates were incubated at 37°C overnight to obtain plate counts. The culture tubes were left at 22°C for 20 days and were sampled on days 0, 1, 2, 4, 6, 8, 10, 12, 14, 16, 18, and 20. The experiment was performed with three biological replicates.

**Persistence of *Salmonella* Strains on the Tomato Surface**

Overnight cultures of *S. Typhimurium* 14028, MKF1, MKF2, and WJZ2 were washed three times and serially diluted twice in 0.1 M potassium phosphate buffer to result in an approximate concentration of $10^7$ CFU/ml. The initial inocula were diluted and plated onto XLD. The blossom end of five green and five red Florida 47 tomatoes were spot inoculated 10 times in 10 µl volumes with each inoculum. After 10 min of rest at room temperature, one tomato of each inoculum was rinsed in a container 10 ml of sterile PBS. The rinsate was diluted and was plated onto XLD. The plates were incubated overnight at 37°C. The tomatoes were also sampled on days 1, 2, 4, and 6. The tomatoes were stored in an environmental chamber at 22°C, 60-80% RH until
culture recovery. The average recovery data was obtained from six biological replicates. Statistical analysis of the persistence data was performed using JMP 9.0 Pro. ANOVA and post hoc pairwise t-tests were completed with a significance of p<0.05.

A control assay was also performed alongside the previously described recovery process. After the tomatoes were sampled and the rinsates were plated, the tomatoes were weighed. A volume of PBS solution equal to that of the tomato weight was added to the stomacher bag to make a 1:1 volume to weight ratio. The tomatoes were stomached in a Stomacher 4000 Circulator (Seward, West Sussex, UK) at 250 rpm for 30 sec and dilutions were plated onto XLD. The XLD plates were incubated at 37°C overnight to obtain plate counts. Control tomatoes of each maturity were also inoculated on day 0 with each inoculum with 10 spots of 10 µl each and were sampled without rinsing after 10 min of rest at room temperature. The control tomatoes were used to determine if the original inoculum could be recovered from the stomaching recovery process. The control assay was performed to determine if a decrease in persistence over time was due to the loss of viable culture on the tomato surface or due to attached cells that could not be removed from the tomato surface though the rinsing process alone.

Persistence studies of the *Salmonella* complementation and vector controls were also performed on unwaxed, unprocessed green and red Florida 47 tomatoes. The assay was completed with S. Typhimurium MKF1 pMKF4, MKF2 pMKF2, WJZ2 pMKF2 pMKF5, and the empty vector controls, MKF1 pWSK29, MKF2 pCR2.1, and WJZ2 pCR2.1 pBBR1-MSC5. There were a total of four biological replicates for each strain at
each maturity level, and to ensure plasmid retention while on the tomato surface, the recovered colonies were patched onto LB agar supplemented with the appropriate antibiotics. The plates were incubated at 37°C overnight.

**Results**

**Initial Attachment of *Salmonella* to the Tomato Surface**

The investigation of the role of the AgfD regulator and AgfD-regulated thin aggregative fimbriae and cellulose in the surface attachment of the pathogen to the surface of tomatoes was performed with three deletion mutants derived from *S. Typhimurium* 14028. Since it was proposed that the initial, reversible attachment of bacteria might be mediated by physiochemical properties, the overall surface properties of the mutant strains were assessed using the BATH and SAT assays. There were no significant differences between the percent absorbance for the strains under all conditions, indicating no overall changed in adherence properties to hydrocarbons was displayed by the mutant strains (data not shown). *S. Typhimurium* 14028, JS246, MKF1, MKF2, and WJZ2 all had minimal absorption with a maximum of only 7.6%, indicating very hydrophilic properties. The SAT assay resulted with values of 4.2 for the surface hydrophobicity of all the cultures, also indicating hydrophilic properties (50, 54).

After the characterization of the adherence properties of the *Salmonella* strains, initial studies were performed to determine the optimal conditions to investigate *Salmonella* attachment. The contact time between the *S. Typhimurium* and tomatoes in the studies was minimalized to maintain conditions that may occur in the field. A study was performed to determine if MKF1 (Δ*agfD::FRT-Kan-FRT*) was more susceptible to desiccation on the tomato surface over time in comparison to *S. Typhimurium* 14028. The results indicated that desiccation does occur over time, but there was no significant
difference in recovery of 14028 or MKF1 at any time point (Figure 4-1). Furthermore, at 5 min, there was a modest reduction of $\sim 0.5 \log_{10}$ CFU/ml in recovery from the initial inoculum concentration for both S. Typhimurium 14028 and MKF1. The modest reduction of recovery could be attributed to the attachment of the Salmonella to the tomato surface since it has been documented that attachment of S. Montevideo occurs within 30 sec on the tomato surface (44). The results of Iturriaga et al. (44) along with the desiccation study indicated that a contact time of 5 min of Salmonella with the tomato surface was sufficient to study initial attachment. The ideal inoculation procedures for the attachment assays were also assessed through comparison of spot versus dip inoculation. The results show a 10-fold loss of recovery with the dip inoculation compared to spot inoculation procedures (Figure 4-2). There is also uncertainty of the bacterial concentrations that actually contact the tomato surface and the surface area of the tomato that is inoculated with the dip inoculation procedures. The results indicate that spot inoculation should be used in any assay resulting in plate counts as the measure of attachment to the tomato surface.

The investigation of the initial attachment of S. Typhimurium 14028, MKF1, MKF2, and WJZ2 on the tomato surface was completed on unwaxed, unprocessed, green field grown Florida 47 tomatoes. The assay was designed to compare overall attachment of the strains side-by-side. The strains were recovered at three time points. The first recovery was the initial rinse, which was performed to remove all cells that did not initially attach to the tomato within 5 min of contact. The initial rinse step utilized DI water rather than PBS to not alter the condition or add any buffering capacity to the cells remaining attached to the tomato surface. After 2 hr at 22°C, 60-80% RH, the strains
were recovered through a secondary rinse which was performed to remove cells that had initially, reversibly attached but did not remain attached. The final tomato rinse, which involved the physical removal of the cells by rubbing the tomato, was completed to obtain the final counts of attached cells. Therefore, in terms of this investigation, attachment is defined as the *Salmonella* remaining attached after two surface rinses but can be subsequently removed through physical abrasion including hand rubbing and swabbing. The results of the surface attachment studies indicated there were no significant differences in the initial attachment of *S. Typhimurium* 14028, MKF1, MKF2, and WJZ2 to the tomato surface (Figure 4-3).

To more directly determine the effect of the mutations in the initial attachment of *Salmonella*, a competitive index (CI) assay was employed. A competitive index assay uses ratios that indicate the fitness of a genetic mutant in comparison to the wild type within a specific environment. In this case, the fitness of the deletion mutants on the tomato surface was compared to that of *S. Typhimurium* 14028 on the tomato surface. The tomatoes were inoculated with a 1:1 ratio of mutant and wild type culture. The fitness of the mutant is determined by comparing the initial 1:1 ratio to the ratio of the cells recovered from the tomato surface in the initial rinse, secondary rinse, and tomato swab. The data from the secondary rinse are not shown since the information from the initial rinse and tomato swab were most informative in determining initial attachment. Dip inoculation was utilized in the CI studies since bacterial concentrations were not determined from plate counts. The results of Figure 4-4 and Tables 4-1, 4-2, and 4-3 show that the gene deficiencies do alter the fitness of the *Salmonella*, despite not changing the overall attachment in the side-by-side studies (Figure 4-3).
In Figure 4-4, graphs A and B show the CI values of the initial rinses of the green and red tomatoes, respectively. A CI greater than zero indicates an increase of fitness for the mutant strain in comparison to the wild type, and a CI value less than zero indicates a decreased fitness for the mutant strain. For the initial rinses, all strains except for S. Typhimurium MKF1 on red tomatoes resulted in a CI greater than zero (Figure 4-4). This dictates that the ratios obtained from the initial rinses contained a higher ratio of mutant cells than wild type. The cells recovered from the initial rinse of the tomatoes represent the cells that did not initially attach, indicating that strains deficient in thin aggregative fimbriae and/or cellulose production did not attach to the tomato surface as efficiently as the wild type strain. Statistical significance was determined for the initial rinse data for WJZ2 within the green tomato samples and MKF1 within the red tomato data (Tables 4-1 and 4-2). Statistical analysis was completed through comparative t-tests at a significance of p<0.05 with the modified S. Typhimurium 14028 strain, JS246, acting as a control. The CI results for the 14028:JS246 co-inoculum are minimal since the res-tet-res insertion into JS246 should not alter attachment; the 14028:JS246 results were used to represent the variability present within the assay.

Graphs C and D of Figure 4-4 show the CI values of the *Salmonella* recovered from the tomato swabs of the green and red tomatoes, respectively. The three deletion mutants all show a similar change in fitness, with all CI values being less than zero in the tomato swab results (Figure 4-4). The cells recovered from the tomato swabs represent the attached cells on the tomato surface, so the negative CI values indicate that more wild type cells had been attached to the tomato surface than the mutant
strains. The results of both the initial rinse and tomato swab recovery steps show a decrease in fitness of the MKF1, MKF2, and WJZ2 mutant strains in the attachment to the tomato surface. For the *Salmonella* attachment to green tomatoes, statistical significance was determined for WJZ2 recovered from the tomato swab (Table 4-1). Furthermore, the deficiency of the AgfD regulator in MKF1 had a greater effect on red tomatoes than green, while cellulose deficiencies of MKF2 and WJZ2 reduced the fitness of the strains on green tomatoes more than on red tomatoes (Figure 4-4). In comparing the CI values of each strain from green and red tomatoes, the CI values of WJZ2 recovered from the tomato swab were significantly different in green versus red tomatoes (Table 4-3).

**Persistence of *Salmonella* on the Tomato Surface**

In the persistence of *Salmonella* on the tomato surface, several suspension medias were compared. The suspension media utilized in the initial attachment studies was sterile DI water, which was suitable due to the short-term nature of the study (2 hr). In testing the persistence of *S. Typhimurium* on the tomato surface 5 days, the concentrations of *S. Typhimurium* 14028, MKF1, MKF2, and WJZ2 in the DI suspension fell below the level of detection after day 1 (data not shown). Two other solutions were used to assess the impact of the carrier medium, 0.1 M potassium phosphate buffer and 0.1% buffered peptone water (BPW) (Becton Dickson, Franklin Lakes, NJ). In both media, the *Salmonella* was recoverable from the tomato surface over 5 days. The concentration of the *Salmonella* control, strain 14028, in 0.1% BPW modestly increased by 1 log_{10} CFU/ml from day 2 to day 5. This indicated that the *Salmonella* was utilizing the nutrients provided in the BPW to sustain on the tomato surface. This suggests that *Salmonella* may be nutrient limited on the tomato surface and the reduction of viable
cell numbers (when applied in DI or PBS) may be due to a combined effect of nutrient limitation and desiccation. The S. Typhimurium 14028 suspended in the 0.1 M potassium phosphate buffer is buffered from osmotic stress, but is not provided with sufficient growth nutrients. In the buffer, the concentration of recovered Salmonella on the tomato surface was also within the recoverable range throughout the 5 days and did not increase over time. These results concluded that the persistence assays would be performed with culture suspended in 0.1 M phosphate buffer to minimize osmotic stress while determining persistence of Salmonella on the tomato without an artificial nutrient source.

The results of the persistence assay indicate an advantage for cultures producing cellulose on the surface of tomatoes (Figure 4-6). The resulting colony forming units of the cellulose deficient mutants diminished 5-fold on green tomatoes and 2.5- to 3.0-fold on red tomatoes over 6 days. A comparison of the recovered concentrations on green and red tomatoes resulted in a significant difference on day 6 for both MKF2 and WJZ2 strains. The resulting average concentrations for MKF2 and WJZ2 on day 6 of the green tomato samples fell below the level of detection because three of the replicate studies resulted in recovered concentrations just above the level of detection, the remaining three replicates had concentrations below detection limits of the assay. Only a modest reduction was observed in the wild-type culture, indicating the cellulose deficiency was responsible for the reduced persistence as opposed to desiccation stress. The S. Typhimurium MKF1 strain sustained on the tomato surface similar to the wild type (Figure 4-6). ANOVA assays of the green tomato data indicated significant differences in the recovered cultures on days 4 and 6, with only day 6 recovered
concentrations being significant within the red tomato data. The post hoc t-test results are documented in Tables 4-4 and 4-5. In comparing the concentrations recovered from the green and red tomatoes, significance was determined for MKF2 and WJZ2 on day 6, as previously stated.

The control studies of the stomached tomatoes indicated that the S. Typhimurium was being effectively removed from the tomato surface through the rinsing steps (data not shown). In the control studies, there was a slight loss in the recovery of the initial inoculum, but in testing the tomatoes that had undergone the rinsing and sampling for the persistence assay, the resulting concentrations were below the level of detection for all samples.

The persistence of the S. Typhimurium complement and empty vector strains were also investigated (Figure 4-7). Graphs A and C illustrate the persistence of the complemented mutants, S. Typhimurium MKF1 pMKF4, MKF2 pMKF2, and WJZ2 pMKF2 pMKF5, on green and red tomatoes, respectively. The strains maintain in initial population levels until day 4 on both green and red tomatoes. On day 4, S. Typhimurium MKF1 pMKF4 sustained, while the recovered concentrations of S. Typhimurium MKF2 pMKF2 and WJZ2 pMKF2 pMKF5 decreased by 1 log_{10} CFU/ml on both green and red tomatoes. Recovery of all three strains decreased on day 6 on both green and red tomatoes, but the recovered colony forming units for the MKF2 pMKF2 and WJZ2 pMKF2 pMKF5 strains were 1.5- to 2.0-fold greater than those of the respective deletion mutants for days 2-6, indicating a partial restoration of the phenotype. The empty vector control strains tested on green and red tomatoes did not restore the phenotype to the level of the complemented strains, as shown in graphs B
and D of Figure 4-7. The patch plating of the complement and vector control strains on LB agar supplemented with antibiotics selecting for the plasmids resulted in 100% growth for all samples. This indicated that the plasmids were maintained under the test conditions and that plasmid loss was not a confounding factor in the experiments despite the lack of antibiotic pressure.

**Discussion**

The first stage of produce contamination is the initial attachment of the bacteria to the produce surface. The objectives of the studies were to characterize the roles of AgfD-regulated thin aggregative fimbriae and cellulose production, as well as the AgfD regulator in the initial attachment and persistence of *Salmonella* on the surface of tomatoes. The scope of the research focused on the field environment and surface contamination of the produce surface in the field. Therefore, the tomatoes used in the experiments were unwaxed tomatoes, either vine-ripened Campari tomatoes or field tomatoes from various areas in Florida. It was important to use unwaxed tomatoes, since the waxed surface may alter the attachment and persistence of the bacterium. The use of waxed, processed tomatoes would also not effectively simulate field contamination. The vine-ripened red, Campari tomatoes were utilized for initial preliminary studies due to their availability. The attachment and persistence studies were completed with the green field tomatoes since this is the stage of maturity round tomatoes are harvested. Red field tomatoes were further tested to discover if there was a significant difference in the attachment and persistence of *Salmonella* to green (unripened) versus red (ripened) tomatoes. Lastly, the attachment studies used sterile DI water as the suspension and initial rinse medium to remove external factors such as nutrient accessibility or pH buffering that would be provided by nutrient rich or buffered
solutions. The buffering was necessary to maintain *Salmonella* on the tomato surface for more than 2 days, so 0.1 M potassium phosphate buffer was utilized for the persistence assays. The media supplied the necessary buffering capacity but did not support growth by providing nutrients. The suspension solutions simulated extreme conditions with a lack of nutrients and organic debris, which is not likely to occur in the field, but implies that the results seen in the lab may be attainable in the field environment where the contamination conditions may be more favorable to the bacteria.

It was hypothesized that the *S. Typhimurium* strains deficient in AgfD-regulated thin aggregative fimbriae and cellulose production would demonstrate reduced initial attachment and persistence on the tomato surface. Complete loss of attachment was not expected since the strains were only single or double deletion mutants. Attachment is a cooperative mechanism where no individual gene is responsible for initial attachment ([4], [5]). The BATH and SAT assays indicated that any change in initial attachment was not due to modifications of the overall surface properties of the strains; but the side-by-side attachment studies showed that there was no overall change in the initial attachment of *Salmonella* strains (Figure 4-3). The recovery process was not sensitive enough to determine the effect of the genetic deficiencies of the *S. Typhimurium* strains. The question being addressed was the role of thin aggregative fimbriae and cellulose in the initial attachment to the tomato surface. The variability between tomatoes, tomato microflora, and dilution and plating procedures of the assay might have been greater than the effect caused by the deletion mutations. The original question of the role of thin aggregative fimbriae and cellulose deficiencies in initial attachment was reassessed through competitive index assays. The CI assays indicated
that impairing \textit{agfD}, \textit{agfB}, and/or \textit{bcs} in \textit{S. Typhimurium} 14028 reduced the fitness and attachment capabilities of the \textit{Salmonella} on the surface of both green and red tomatoes. While there were some statistically significant results from the CI assay, the results do not indicate the differences would be biologically significant. The attachment studies indicate that while these extracellular components are involved in the initial attachment of \textit{Salmonella} to the tomato surface, they do not predominantly dictate attachment to tomatoes.

The initial attachment assays were performed over a time frame of 2 hr, with initial rinses occurring after only 5 min of contact. Under these time limitations, cellulose may not have been adequately produced. To assess the role of \textit{AgfD}-regulated thin aggregative fimbriae and cellulose over a long time period on the surface of tomatoes, persistence assays were completed over 6 days. The extrinsic, environmental conditions are very important in the persistence of \textit{Salmonella} on produce \((43, 105)\), so conditions were maintained at 22\(^\circ\)C, 60-80\% RH throughout the persistence studies. The \textit{Salmonella} strains were assessed in the suspension media, 0.1 M phosphate buffer, over 20 days to ensure any loss of persistence on tomatoes was not due to a deficiency of the strain. All four strains persisted in the media over the 20 days, with no growth in the negative control, assuring the long-term viability of the strains (Figure 4-5). The studies on the tomato surface indicated that cellulose production has a role in the persistence of \textit{Salmonella}. The cellulose deficient strains resulted in a 5-fold decrease in concentration on green tomatoes and 2.5- to 3.0-fold loss on red tomatoes (Figure 4-6). The partial restoration of persistence in the complement strains, and lack of phenotype restoration in the empty vector controls, also indicates that the decrease in
persistence of MKF2 and WJZ2 was due to the gene deletions rather than artifacts of the mutation process (Figure 4-7).

As covered in the introduction, the thin aggregative fimbriae and cellulose of the AgfD-regulated system have alternative regulators beyond AgfD. It is hypothesized that the alternative regulators activated thin aggregative fimbriae and cellulose production under the conditions of the tomato surface since the MKF1 strain sustained at the same concentrations as the wild type, 14028. The influence of tomato ripeness did not affect persistence of S. Typhimurium 14028 or MKF1, so the differential persistence of MKF2 and WJZ2 on suggests that cellulose deficient Salmonella are more sustainable on red, ripe tomatoes versus green tomatoes. On both green and red tomatoes, the 14028 and MKF1 strains experience a decrease of 1 log\(_{10}\) CFU/ml over the 6-day trial.

In all of the analyses, there was no statistical difference in the recovered concentrations of MKF2 and WJZ2, indicating that thin aggregative fimbriae had no significant role in the persistence of S. Typhimurium under the tested conditions. A study of rdar and non-rdar Salmonella strains obtained from human or poultry samples found that thin aggregative fimbriae deficient (bdar) strains exhibited an attachment deficiency, but had greater attachment and persistence to polystyrene and glass surfaces than the saw strain deficient in thin aggregative fimbriae and cellulose production (45). The difference in results between the two studies could be explained by the undetermined mutations of the bdar strain in their study. The S. Typhimurium strain was obtained from human samples and was characterized as bdar on CR and CW agar, but the mutation causing this morphology may not have been in the agf operon, resulting in different attachment results.
It is hypothesized that either the recovery conditions were not precise enough to distinguish slight differences in persistence between the MKF1 and WJZ2 strains or the alternative fimbriae of the WJZ2 strain were utilized in initial attachment, making the gene deletion inconsequential in the attachment and persistence process. In the initial genetic analysis of fimbrial genes of Salmonella, up to 15 putative fimbrial operons were identified (40, 86). Selective pressures from animal hosts may contribute to the multiplicity of fimbriae in Salmonella. Fimbriae are externally expressed features that can trigger antigenic effects in a host. The partially redundant functionality of fimbrial operons allows for environmental regulators to change fimbriae expression and evade immune detection. The inactivation of single fimbrial operons in S. Typhimurium did not affect overall virulence of the strain, but simultaneous inactivation of lpf, pef, and agf operons resulted in attenuated virulence (90). Understanding the redundant functionality of fimbriae in the animal host leads to the conclusion that the same may be true on the plant surface.

Overall, the production of AgfD-regulated cellulose in S. Typhimurium 14028 has a significant role in persistence on the tomato environment, but little effect in the initial attachment of Salmonella under the tested conditions. There was no significant reduction of initial attachment or persistence attributed to AgfD or thin aggregative fimbriae deficiencies. In both the competitive index and persistence assays, the cellulose deficient mutants, S. Typhimurium MKF2 and WJZ2, were more sustainable on the surface of red, ripened tomatoes than green. This reduced persistence on green tomatoes indicates that both bacterial behavior, including cellulose production, and produce ripeness are factors in persistence of S. Typhimurium on the tomato surface.
Table 4-1. Statistical analysis of competitive index values of *Salmonella* recovered from the green tomato surface

<table>
<thead>
<tr>
<th>Sample comparison</th>
<th>Sample recovery step</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>14028:JS246 and 14028:MKF1</td>
<td>Initial rinse</td>
<td>0.769</td>
</tr>
<tr>
<td></td>
<td>Tomato swab</td>
<td>0.389</td>
</tr>
<tr>
<td>14028:JS246 and 14028:MKF2</td>
<td>Initial rinse</td>
<td>0.299</td>
</tr>
<tr>
<td></td>
<td>Tomato swab</td>
<td>0.331</td>
</tr>
<tr>
<td>14028:JS246 and 14028:WJZ2</td>
<td>Initial rinse</td>
<td>0.015</td>
</tr>
<tr>
<td></td>
<td>Tomato swab</td>
<td>0.050</td>
</tr>
</tbody>
</table>

Table 4-2. Statistical analysis of competitive index values of *Salmonella* recovered from the red tomato surface

<table>
<thead>
<tr>
<th>Sample comparison</th>
<th>Sample recovery step</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>14028:JS246 and 14028:MKF1</td>
<td>Initial rinse</td>
<td>0.011</td>
</tr>
<tr>
<td></td>
<td>Tomato swab</td>
<td>0.103</td>
</tr>
<tr>
<td>14028:JS246 and 14028:MKF2</td>
<td>Initial rinse</td>
<td>0.151</td>
</tr>
<tr>
<td></td>
<td>Tomato swab</td>
<td>0.621</td>
</tr>
<tr>
<td>14028:JS246 and 14028:WJZ2</td>
<td>Initial rinse</td>
<td>0.062</td>
</tr>
<tr>
<td></td>
<td>Tomato swab</td>
<td>0.411</td>
</tr>
</tbody>
</table>

Table 4-3. Statistical analysis comparing competitive index values of *Salmonella* recovered from green versus red tomato surface

<table>
<thead>
<tr>
<th>Co-inoculum</th>
<th>Sample recovery step</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>14028:JS246</td>
<td>Initial rinse</td>
<td>0.054</td>
</tr>
<tr>
<td></td>
<td>Tomato swab</td>
<td>0.176</td>
</tr>
<tr>
<td>14028:MKF1</td>
<td>Initial rinse</td>
<td>0.120</td>
</tr>
<tr>
<td></td>
<td>Tomato swab</td>
<td>0.442</td>
</tr>
<tr>
<td>14028:MKF2</td>
<td>Initial rinse</td>
<td>0.606</td>
</tr>
<tr>
<td></td>
<td>Tomato swab</td>
<td>0.379</td>
</tr>
<tr>
<td>14028:WJZ2</td>
<td>Initial rinse</td>
<td>0.191</td>
</tr>
<tr>
<td></td>
<td>Tomato swab</td>
<td>0.042</td>
</tr>
</tbody>
</table>
Table 4-4. Statistical analysis of recovered *Salmonella* concentrations from the green tomato surface over 6 days

<table>
<thead>
<tr>
<th>Sample Day</th>
<th>Significantly different strains</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>No significant difference</td>
<td>N/A</td>
</tr>
<tr>
<td>1</td>
<td>No significant difference</td>
<td>N/A</td>
</tr>
<tr>
<td>2</td>
<td>No significant difference</td>
<td>N/A</td>
</tr>
<tr>
<td>4</td>
<td>14028 and WJZ2</td>
<td>0.0483</td>
</tr>
<tr>
<td></td>
<td>MKF1 and WJZ2</td>
<td>0.0456</td>
</tr>
<tr>
<td>6</td>
<td>14028 and MKF2</td>
<td>0.0035</td>
</tr>
<tr>
<td></td>
<td>14028 and WJZ2</td>
<td>0.0040</td>
</tr>
<tr>
<td></td>
<td>MKF1 and MKF2</td>
<td>0.0038</td>
</tr>
<tr>
<td></td>
<td>MKF1 and WJZ2</td>
<td>0.0043</td>
</tr>
</tbody>
</table>

Table 4-5. Statistical analysis of recovered concentrations of *Salmonella* from the red tomato surface over 6 days

<table>
<thead>
<tr>
<th>Sample Day</th>
<th>Significantly different strains</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>No Significant Difference</td>
<td>N/A</td>
</tr>
<tr>
<td>1</td>
<td>No Significant Difference</td>
<td>N/A</td>
</tr>
<tr>
<td>2</td>
<td>No Significant Difference</td>
<td>N/A</td>
</tr>
<tr>
<td>4</td>
<td>No Significant Difference</td>
<td>N/A</td>
</tr>
<tr>
<td>6</td>
<td>14028 and MKF2</td>
<td>0.0015</td>
</tr>
<tr>
<td></td>
<td>14028 and WJZ2</td>
<td>0.0043</td>
</tr>
<tr>
<td></td>
<td>MKF1 and MKF2</td>
<td>0.0044</td>
</tr>
<tr>
<td></td>
<td>MKF1 and WJZ2</td>
<td>0.0134</td>
</tr>
</tbody>
</table>
Figure 4-1. Desiccation of *Salmonella* Typhimurium 14028 and MKF1 on the tomato surface over time. This study was performed to determine if significant desiccation of *Salmonella* was observed over 120 min on the tomato surface. The study assessed the recovered concentrations of *S*. Typhimurium at 5, 15, 30, 60, and 120 min. The recovered concentrations were obtained through plate counts. The study compiled data from three biological replicates, each performed in triplicate. The strain designations are labeled in the legend and the error bars denote the standard error.
Figure 4-2. Recoverable *Salmonella* Typhimurium from the tomato surface through spot or dip inoculation. A) Illustrates the results from spot inoculation. B) Illustrates the results from dip inoculation. The study was designed to determine if there was a difference in recoverable *Salmonella* from the two inoculation procedures. The studies were performed with three biological replications in triplicate. The strain designations are labeled in the legends and the error bars represent the standard error.
Figure 4-3. The initial attachment of *Salmonella* Typhimurium 14028, MKF1, MKF2, and WJZ2 on the tomato surface. The recovery of *Salmonella* from the surface of green, unprocessed Florida 47 tomatoes was performed after 5 min (initial rinse) and 2 hr (secondary rinse), followed by abrasive removal of *Salmonella* through rubbing the tomato (tomato rinse). Recovered *Salmonella* concentrations were obtained through plate counts on XLD. The represented data is the average of three biological replicates performed in triplicate. The error bars indicate standard error.
Figure 4-4. Competitive co-infections of *Salmonella* strains on the tomato surface. A) The CI values recovered from the initial rinse of green Florida 47 tomatoes. B) The CI values recovered the initial rinse of red Florida 47 tomatoes. C) The CI values recovered from the tomato swab of green Florida 47 tomatoes. D) The CI values recovered from the tomato swab of red Florida 47 tomatoes. The competitive index (CI) studies were performed to specifically measure the effect of the deletion mutants on the fitness of the *Salmonella* on the tomato surface. CI values greater than zero indicate an increase in fitness for the deletion mutants in comparison to the wild-type, while CI values less than zero indicate a decrease in fitness for the deletion mutant on the tomato surface. The data encompasses the results from five biological replicates, each performed in triplicate. The *res-tet-res* insertion of JS246 should not alter the competitive fitness and was used as a control for statistical analysis. The box plots around the data points denote the 10%, 25%, 75% and 90% quantiles as well as the median. The diamonds represent the group mean as well as 95% confidence intervals. Student’s t-test were used to test for significance at p<0.05. Statistical results represented in Tables 4-1, 4-2, and 4-3.
Figure 4-5. Persistence of *Salmonella* Typhimurium 14028, MKF1, MKF2, and WJZ2 in 0.1 M potassium phosphate buffer. The assay was performed to determine if the genetic mutants decreased in viability over a period of time. The mutants deficient in AgfD, thin aggregative fimbriae, and/or cellulose production were sampled from the suspension media utilized for the persistence studies on the tomato surface for 20 days. The data was averaged from three biological replicates. The strain designations are assigned in the legend; the error bars indicate standard deviation.
Figure 4-6. Persistence of *Salmonella* Typhimurium 14028, MKF1, MKF2, and WJZ2 on the green and red tomato surface. A) Illustrates the persistence of *Salmonella* on green tomatoes. B) Illustrates the persistence of *Salmonella* on red tomatoes. The assay was performed to determine if deficiency of AgfD, thin aggregative fimbriae, and/or cellulose production would alter the persistence of *Salmonella* on either green or red tomatoes. The data was averaged from six biological replicates. The strains are labeled in the legend. The error bars indicate standard error. The results were statistically analyzed through ANOVA and pairwise t-tests at a significance of p<0.05.
Figure 4-7. Persistence of *Salmonella* complement and vector control strains on the surface of green and red tomatoes. A) Illustrates the persistence of *Salmonella* complements on green tomatoes. B) Illustrates the persistence of *Salmonella* empty vector controls on green tomatoes. C) Illustrates the persistence of *Salmonella* complements on red tomatoes. D) Illustrates the persistence of *Salmonella* empty vector controls on red tomatoes. The assay was performed to determine if the persistence deficiencies observed by the deletion mutants were the result of the gene deletion. The strain designations are detailed in the legends between the graphs, with A and C sharing a legend and B and D sharing the other legend. The data was obtained through six biological replicates and error bars indicate standard error.
CHAPTER 5

Background

The formation of biofilms facilitate bacterial survival in both host and non-host environments. Biofilms are structured communities of bacterial cells enclosed in a self-produced polymeric matrix adherent to either abiotic or biotic surfaces (80). The bacteria present in biofilms are generally protected from environmental stress, antibiotics, and disinfectants, making them difficult to eradicate. Salmonella spp. are known to form biofilm on numerous abiotic surfaces including plastic, rubber, glass, and stainless steel (58, 76, 80). Salmonella spp. may persist in the plant environment through the formation of biofilms on the plants. S. Thompson was visualized on the surface and within cuts of lettuce leaves through episcopic differential interference contrast microscopy coupled with epifluorescence to find what appeared to be biofilm formation on the lettuce (94). Salmonella have also demonstrated biofilm formation on the surface of parsley in studies that resulted in decreased biofilm from strains deficient in thin aggregative fimbriae and cellulose (49). Human pathogens may integrate into the biofilms of the plant microflora for enhanced protection and survival (51). Salmonella also form biofilm on the hyphae of fungi, including Aspergillus niger, which may serve in the environmental survival of the pathogen (10). Biofilm formation may have an integral role in the persistence of human pathogens in the plant environment.

Thin aggregative fimbriae and cellulose are important structural components of biofilm formation and Salmonella deficient in these components demonstrate reduced biofilm on glass, polystyrene, leafy greens, and Aspergillus niger (10, 45). Transcription
of agtD is also greatest in conditions that would be encountered outside of the animal host, including 28°C, low osmolarity, and nutrient starvation during stationary growth (63). It has been implicated the environmental persistence of pathogens is achieved through biofilm formation and that 30-80% of bacterial populations on plant surface are present in biofilms (51, 80). It has been hypothesized that Salmonella exhibiting the rdar morphology are more resistant to desiccation and environmental stress, which is vital in environmental survival (100). It has also been proposed that non-rdar morphology promotes fitness of S. Typhimurium within an infected tomato (104).

If Salmonella spp. are forming biofilms on the plant surface to persist in the environment, there may be a correlation between the rdar morphology and increased fitness in the plant environment. Of 204 Salmonella isolates acquired from food, environmental, and clinical samples, as well as some included laboratory strains, 66% resulted in the rdar morphology. In assessing the strains for biofilm formation, only 3% were identified as biofilm deficient (76). Another research group determined the percentage of rdar positive Salmonella strains based on the source of the strain. The results included rdar strains in 3% of the clinical strains, 84% of strains obtained from meat samples, and 56% of produce related strains. In assessing the biofilm formation of the produce related strains, all but one rdar strain exhibited strong biofilm formation in 96-well polystyrene microtiter plates; the non-rdar strains lacked biofilm formation (78).

Both the rdar morphology and biofilm formation of Salmonella may contribute to the environmental survival of the pathogen, which leads to the question of whether the rdar morphology corresponds to biofilm formation capabilities. While evidence suggests that Salmonella displaying the rdar morphology may have increased biofilm formation
and protection from environmental stress, there were a high percentage of non-r达尔 strains (44%) isolated from produce samples, suggesting that non-r达尔 strains are persisting on plants as well. *S. Typhimurium* 14028, MKF1, MKF2, and WJZ2, as well as genetic complements, negative controls, and produce outbreak non-r达尔 *Salmonella* strains will be assessed for biofilm formation on both abiotic and green and red tomatoes to determine the roles of the AgfD regulator, AgfD-regulated thin aggregative fimbriae, and/or cellulose in biofilm formation and evaluate the roles of the rd达尔 morphology and biofilm formation in the environmental survival of *Salmonella*.

**Materials and Methods**

**Biofilm Formation of *Salmonella* Typhimurium on Polystyrene**

Maintenance and growth of the *Salmonella* strains, as well as tomato acquisition are as detailed in the previous chapter, unless stated otherwise. Overnight cultures of *S. Typhimurium* 14028, MKF5, MKF1, MKF2, WJZ2, MKF1 pMKF4, MKF1 pWSK29, MKF2 pMKF2, MKF2 pCR2.1, WJZ2 pMKF2 pMKF5, and WJZ2 pCR2.1 pBBR1-MSC5 were each washed in sterile PBS three times. Cultures were diluted 1:100 in 1/10 tryptic soy broth (TSB) (Becton Dickson, Franklin Lakes, NJ) and were pipetted (100 μl per well) into appropriate wells of a 96-well polystyrene plate (Costar, Washington, DC). The 96-well plate also contained wells containing 1/10 TSB media that acted as negative controls. The plates were enclosed in zip lock bags and were incubated at 30°C.

After 24 hr, 25 μl of 1% crystal violet (Acros Organics, Geel, Belgium) solubilized in 100% ethanol was added to each well of the polystyrene plates. Plates were incubated at ambient temperature for 15 min. The crystal violet solution was decanted from plates and plates were gently rinsed in DI water. The crystal violet was solubilized
in 120 μl of 33% acetic acid (Fisher Scientific, Pittsburgh, PA). From each well, 100 μl of solubilized crystal violet was transferred to a new 96-well polystyrene plate. Optical density of the samples was read at 595 nm in the microtiter plate reader, Victor3 (Perkin Elmer, Fremont, CA) and the Wallac 1420 Manager work-station software. The results were baselined to the negative control samples. The study was performed with four technical replications for each of the three biological replications. The data was analyzed for significance in JMP 9.0 Pro through ANOVA and post hoc Tukey-Kramer HSD analysis with a significance of p<0.05.

**Biofilm Formation of *Salmonella Typhimurium* MKF5, MKF1, MKF2, and WJZ2 on the Tomato Surface**

Overnight cultures of *S. Typhimurium* MKF5, MKF1, MKF2, WJZ2, MKF1 pMKF4, MKF1 pWSK29, MKF2 pMKF2, MKF2 pCR2.1, WJZ2 pMKF2 pMKF5, and WJZ2 pCR2.1 pBBR1-MSC5 were washed in sterile DI water three times. All cultures were diluted to ~10^6 CFU/ml in sterile DI water and were supplemented with the appropriate antibiotic(s) to reduce proliferation and biofilm formation of tomato microflora. Field grown, unwaxed, unprocessed green and red tomatoes cultivar Florida 47 or Quincy were wiped with ethanol and dried in the biological hood for 10 min. Small, plastic containers that were washed with bleach and UV sterilized were each filled with 70 ml of inoculum. The blossom ends of field tomatoes were submerged in the inocula, with 70-90 mm of the blossom end of each tomato being submerged. The tomatoes were incubated at 30°C for 24 hr. After 24 hr, the tomatoes were moved to a container of 2 ml of 1% crystal violet. The staining and measurement of biofilm was performed as previously described by staining the blossom ends of the tomato, rinsing in DI, and
solubilizing in 30 ml of 33% acetic acid. Negative controls were performed with each study. The study was performed in triplicate, with three biological replications.

For the biofilm formation of S. Typhimurium MKF5, MKF1, MKF2, and WJZ2, significance was determined in JMP 9.0 Pro through pairwise t-tests comparing each strain on green versus red tomatoes and two Dunnett’s tests were completed with MKF5 as the control, one for the green tomato data and the other for the red tomato data. An effect test was also performed to determine the variable (Salmonella strain and/or tomato maturity) most significantly effecting the Salmonella biofilm formation.

For the biofilm formation of S. Typhimurium MKF1 pMKF4, MKF1 pWSK29, MKF2 pMKF2, MKF2 pCR2.1, WJZ2 pMKF2 pMKF5, and WJZ2 pCR2.1 pBBR1-MSC5, significance was determined in JMP 9.0 Pro. Pairwise t-tests comparing the biofilm formation of genetic complements, S. Typhimurium MKF1 pMKF4, MKF2 pMKF2, and WJZ2 pMKF2 pMKF5, to the biofilm formation of MKF5 and the vector controls, S. Typhimurium MKF1 pWSK29, MKF2 pCR2.1, and WJZ2 pCR2.1 pBBR1-MSC5, were compared to MKF1, MKF2, and WJZ2, respectively. The green tomato and red tomato data were compared separately for the statistical analysis of the genetic complements and controls.

Biofilm Formation of Salmonella Typhimurium MKF5, MKF1, MKF2, and WJZ2 on Tomato Segments

Overnight cultures of S. Typhimurium MKF5, MKF1, MKF2, WJZ2, MKF1 pMKF4, MKF1 pWSK29, MKF2 pMKF2, MKF2 pCR2.1, WJZ2 pMKF2 pMKF5, and WJZ2 pCR2.1 pBBR1-MSC5 were washed three times in sterile DI water. All cultures were diluted to ~10^6 CFU/ml in either sterile DI water or sterile DI water supplemented with MnCl₂ (5mg/L) and MgSO₄ (50mg/L) and supplemented with the appropriate
antibiotic(s). Two field grown, unwaxed, unprocessed tomatoes cultivar Florida 47 or Quincy, one green and one red, were wiped with ethanol and dried in the biological hood for 5 min. Six 15 mm x 15 mm squares were cut from the surface of each tomato with a scalpel and tweezers that were flame sterilized. The tissue was removed from the 15 mm x 15 mm squares to result in a height of ~2 mm. The tomato segments were added to each well of two 6 well polystyrene plates (Costar, Washington, DC) to result in a plate of green tomato segments and a plate of red tomato segments. Diluted cultures were pipetted into the wells of the polystyrene plate, 4 ml per well. Two wells were only inoculated with media to serve as negative controls. Plates were placed in zip lock bags and incubated for 24 hr at 30°C.

After 24 hr, the tomato segments were removed from the wells and placed into the individual wells of new, sterile 6 six well polystyrene plates containing 1 ml of 1% crystal violet in each well. The tomato segments were stained and optical density readings were recorded as previously described. Statistical analysis was performed as described in the previous section with pairwise t-tests, Dunnett’s tests, and an effect test.

**Characterization of *Salmonella* Outbreak Strains**

Several produce-associated *Salmonella* outbreak strains were electroporated with plasmid pWSK29, as previously described, to add antibiotic resistance to the outbreak strains. The strains are detailed in Table 5-1. The strains were assessed for rdar morphology and cellulose production by streaking the *Salmonella* outbreak strains with and without the pWSK29 plasmid onto CR and CW agar plates. CR agar was incubated at 30°C for two days, while CW agar plates were incubated without light exposure at room temperature for two days. After 48 hr, pictures were taken of the CR
plates and the cultures on the CW agar were suspended in sterile deionized (DI) water and samples were loaded into a 96-well polystyrene plate along with a control of DI water from an uninoculated CW agar plate. The optical density and fluorescence readings of the cultures were measured and recorded as performed in Da Re and Ghigo (23). Growth curves were also performed over 12 hr at 30°C, as previously described. Pairwise t-tests with 5% significance were performed to determine if the plasmid insert altered cellulose production. Of the strains that were not significantly different in their cellulose production, an ANOVA and pairwise t-tests were performed with a significance of p<0.05. After analysis of cellulose production and growth curves, the strains were utilized for biofilm assays. The biofilm formation of each strain containing the pWSK29 plasmid was assessed on tomato segments of green, Florida 47 tomatoes, as previously described. The biofilm data was analyzed in JMP 9.0 Pro through an ANOVA with significance at p<0.05.

Results

The biofilm studies were conducted at 30°C rather than 37°C due to the enhanced transcription of agfD at 25-30°C and to more effectively simulate the environmental conditions as opposed the temperatures within an animal host. As displayed in Figure 5-1, the biofilm formation of S. Typhimurium 14028, MKF5, JS246, as well as MKF1 pMKF4 were indicated as significantly greater in the Tukey-Kramer HSD analysis. This indicates that the biofilm formation of both MKF5 and JS246 were comparable to the wild-type 14028 culture, making either suitable for use in place of 14028 in future biofilm studies. An antibiotic resistant wild-type surrogate culture was necessary for biofilm studies on tomatoes to allow for control of the natural tomato microflora during the studies. S. Typhimurium MKF5 was chosen for use in the
remaining biofilm studies in place of 14028 because the strain was resistant to kanamycin, which was the same resistance carried by MKF1, MKF2, and WJZ2. S. Typhimurium MKF1, MKF2, and WJZ2 demonstrated significantly reduced biofilm, expressing up to a 3-fold loss of optical density in comparison to MKF5. The biofilm formation of MKF1 pMKF4 indicated a full restoration of biofilm formation capabilities with the genetic complementation of agfD on the extrachromosomal pWSK29 plasmid. The biofilm formation of MKF2 pMKF2 and WJZ2 pMKF2 pMKF5 indicated partial restoration of biofilm formation capabilities with a 2-fold increase in OD in comparison to MKF2 and WJZ2, respectively. The empty vector controls, S. Typhimurium MKF1 pWSK29, MKF2 pCR2.1, and WJZ2 pCR2.1 pBBR1-MSC5 demonstrated similar biofilm formation as the MKF1, MKF2, and WJZ2 strains, respectively.

The assessment of biofilm formation of S. Typhimurium MKF5, MKF1, MKF2, WJZ2, MKF1 pMKF4, MKF1 pWSK29, MKF2 pMKF2, MKF2 pCR2.1, WJZ2 pMKF2 pMKF5, and WJZ2 pCR2.1 pBBR1-MSC5 on intact tomatoes and tomato segments were completed on green and red field tomatoes in DI water. The resulting formation of biofilm on intact tomatoes is illustrated in Figures 5-2 and 5-3. There were no significant differences in the biofilm formation of each Salmonella strain on green versus red intact tomatoes or tomato segments, as determined through pairwise t-tests with a significance of p<0.05. In a comparison of the data through a Dunnett’s test with a significance of p<0.05, the biofilm formation of S. Typhimurium MKF1, MKF2, and WJZ2 on both green and red intact tomatoes were significantly less than the biofilm formed by MKF5, with a 2-fold reduction in OD. An effect test with significance of 5% indicated that the significant effector of the biofilm formation was the Salmonella strain, as
opposed to the tomato maturity in the intact tomato studies. The comparison of S. Typhimurium MKF1 pMKF4, MKF2 pMKF2, and WJZ2 pMKF2 pMKF5 to the biofilm formation of MKF5 and the comparison of S. Typhimurium MKF1 pWSK29, MKF2 pCR2.1, and WJZ2 pCR2.1 pBBR1-MSC5 to the respective deletion mutants on either green or red intact tomatoes resulted in no significant differences.

Figures 5-4 and 5-5 illustrate the biofilm formation of Salmonella on green and red tomato segments suspended in DI water and the appropriate antibiotic. The biofilm formation of S. Typhimurium MKF5 was larger than the MKF1, MKF2, and WJZ2 deletion mutants, but the difference in biofilm formation was not significant on green tomatoes according to the Dunnett’s test. In the biofilm formation on red tomato segments, S. Typhimurium MKF2 and WJZ2 displayed significantly decreased biofilm in comparison to MKF5, with optical density levels measured below the negative controls. The pairwise t-tests for the strains on green or red tomato segments resulted in a significant difference for biofilm formation of S. Typhimurium MKF1 pMKF4 on green versus red tomatoes, with greater biofilm formation on green tomato segments. There were no significant differences in the biofilm formation of S. Typhimurium MKF1 pMKF4, MKF2 pMKF2, and WJZ2 pMKF2 pMKF5 compared to MKF5 or S. Typhimurium MKF1 pWSK29, MKF2 pCR2.1, and WJZ2 pCR2.1 pBBR1-MSC5 compared to MKF1, MKF2, and WJZ2, respectively, on either green or red tomato segments. An effect test indicated that the Salmonella strain was a significant factor in biofilm formation on tomato segments, while the tomato maturity was found to be insignificant.

Analysis of Salmonella biofilm on green and red tomato segments was also performed in DI water supplemented with MnCl₂ (5 mg/L) and MgSO₄ (50 mg/L) and the
appropriate antibiotic(s) to determine if the presence of the compounds altered biofilm formation (Figure 5-6). Pairwise t-tests were performed to determine that there was no significant difference in the biofilm formation of the *Salmonella* strains in the presence or absence of MnCl₂ and MgSO₄ on either green or red tomato segments, with the exception of *S. Typhimurium* MKF1 pMKF4 on green tomatoes. An effect test also indicated that in considering the factors of *Salmonella* strain, tomato maturity, and presence or absence of MnCl₂ and MgSO₄, the only significant factor effecting the formation of biofilm on tomatoes was the *Salmonella* strain.

The biofilm studies showed that the non-rdar *S. Typhimurium* MKF1, MKF2, and WJZ2 were deficient in biofilm formation on the tomato surface. The deficiency of targeted deletion mutants in the formation of biofilm does not establish there is a correlation between the rdar morphology and biofilm formation. Several produce outbreak *Salmonella* strains of clinical and environmental origin were also used to study the correlation between the rdar morphology, biofilm formation, and environmental persistence (Table 5-1). The outbreak strains were assessed for the rdar or non-rdar morphology on CR agar along with three lab-derived controls, *S. Typhimurium* 14028, the wild-type strain, *S. Typhimurium* JTN176, an rdar-like strain, and *S. Typhimurium* JTN212, a spontaneous *rpoS* mutant with saw (smooth and white) morphology. The *S. Typhimurium* JTN176 and JTN212 strains were strains of 14028 passed through tomatoes three times (Table 3-2). The three controls, along with the outbreak strains underwent electroporations with pWSK29 to add extrachromosomal antibiotic resistance to the strains for use in the biofilm studies.
The strains were characterized for morphology on CR media, growth curves were performed, and cellulose was quantified through fluorescence on CW agar (Figures 5-7, 5-8, and 5-9). The *S. Typhimurium* 14028 pWSK29 and JTN176 pWSK29 strains demonstrated rdar or rdar-like morphologies, with the JTN212 pWSK29 demonstrating the saw morphology. The outbreak strains all expressed non-rdar morphologies that were a slight pdar (pink, dry, and rough) with irregular or curled edging (Figure 5-7). The growth curves in Figure 5-8 showed no irregular or deficient growth patterns for the *Salmonella* strains. The cellulose quantification resulted in significant differences between the wild-type and pWSK29 containing strains for *S. Typhimurium* JTN176, *S. Braenderup* 04E01347, and *S. Michigan* LJH521. The three strains were removed from the study. The cellulose quantifications for the remaining *Salmonella* strains were analyzed to determine the only strains that did not significantly differ in cellulose production were *S. Newport* C4.2 and C6.3 (Figure 5-9). The cellulose was quantified for each strain to determine if biofilm formation could be linked to cellulose production. The biofilm formations of the strains were only assessed on green tomato segments. The ANOVA analysis of the biofilm data indicated there is no significant difference between the outbreak strains or the *S. Typhimurium* 14028 and JTN212 controls.

**Discussion**

The analysis of the biofilm formation of *S. Typhimurium* MKF5, MKF1, MKF2, and WJZ2 established that the deletion of the AgfD regulator, AgfD-regulated thin aggregative fimbriae, and/or the cellulose genes did significantly reduce biofilm formation on polystyrene and intact tomato surfaces of both green and red tomatoes (Figures 5-1 and 5-2). The TSB media utilized in the biofilm assays was diluted 1:10 because nutrient limitation has been shown to promote biofilm formation and
transcription of *agfD* (78). On the polystyrene surface, the deletion mutants had an approximate 3-fold reduction in biofilm formation (as measured by optical density) (Figure 5-1). While on the intact tomato surface, the loss of biofilm formation was approximately 2-fold for each of the three deletion mutants in comparison to *S.* Typhimurium MKF5 (Figure 5-2). The formation of biofilm by *S.* Typhimurium MKF5 and reduced production from MKF1, MKF2, and WJZ2 on both polystyrene and tomato surfaces indicated that the AgfD regulator and cellulose have important roles in surface-independent biofilm formation of *Salmonella*. The lack of significant difference between *S.* Typhimurium MKF2 and WJZ2 indicate that the thin aggregative fimbriae do not have a major role under these experimental conditions, as was observed in the previous initial attachment and persistence studies.

The previously described persistence studies determined that the lack of the AgfD regulator did not decrease the persistence of *Salmonella* on the tomato surface, but the deletion of *agfD* alters the biofilm forming capacity of the strain on polystyrene and the intact tomato surface. There are AgfD-independent routes of cellulose production and numerous fimbriae operons of *Salmonella*, which were proposed to contribute to the null effect of the deletion of *agfD* in the persistence studies. The deletion of *agfD* is hypothesized to result in the significant loss of biofilm formation because *agfD* is regulated by environmental cues of nutrient scarcity, oxygen tension, temperature, pH, osmolarity, and ethanol presence through numerous global regulators including RpoS, OmpR, MlrA, CxpR, H-NS, and IHF (11, 47, 100). It has been proposed that the environmentally based regulation of *agfD* allows the regulator to trigger the transition between planktonic behavior and the sessile community structure.
of biofilm formation in response to changing environmental conditions (31). The Salmonella could not effectively form biofilm due to the loss of regulation by AgfD.

The biofilm formation of Salmonella on tomato segments differed from the biofilm formed on the tomato surface. On the tomato segments, the Salmonella had contact with the tomato tissue and any exudates remaining on the exposed tissue and surface. As previously discussed, the plant surface has minimal nutrients available and is a location where Salmonella may persist but do not typically thrive, while studies have demonstrated a 5-fold increase in Salmonella populations within red tomatoes in less than 1-2 days (56). The presence of the exposed tomato tissue may have provided nutrients to the bacterium, therefore, reducing nutrient limitation and biofilm formation.

S. Typhimurium MKF5 exhibited decreased biofilm formation on green tomato segments in DI water in comparison to the biofilm formation on the green intact tomato or the red tomato segment, resulting in a lack of significant differences between the biofilm formation of S. Typhimurium MKF5 and MKF1, MKF2, and WJZ2 (Figure 5-4). The biofilm formation of S. Typhimurium MKF5 on red tomato segments exceeded that on the intact tomato surface, but the biofilm formation of MKF1, MKF2, and WJZ2 were minimal on the red tomato compared to the green tomato segments. Biofilm studies in 96-well polystyrene microtiter plates were performed using rinsates from the tomato segments to establish if there were antagonistic or synergistic exudates causing the difference in biofilm formation of S. Typhimurium MKF5, but the biofilm in the rinsates did not differ (data not shown). The data indicates that while biofilm formation on tomato surfaces and segments may be variable, there is strong biofilm formation on the intact tomato surface of both green and red tomatoes.
The biofilm assays on the tomato segment were also performed with a suspension solution of DI water supplemented with \( \text{MnCl}_2 \) and \( \text{MgSO}_4 \) to determine if the compounds would alter the development of biofilms. The only significant difference was between the biofilm formation of \( S. \) Typhimurium MKF1 pMKF4 on green tomato segments in DI versus the supplemented DI (Figure 5-6). The biofilm formation of \( S. \) Typhimurium MKF1 pMKF4 was also the only strain to significantly differ on green versus red segments, so the significance is likely attributed to the high optical density measured on the green tomato segments in DI water rather than an effect from the \( \text{MnCl}_2 \) and \( \text{MgSO}_4 \). The effect tests of the tomato surface, tomato segment, and variable suspension solution studies all only indicated that the \( \text{Salmonella} \) strain was significantly directing biofilm formation, indicating that the characteristics of the bacterial strain may be more important than the ripeness or presence of plant microflora in the environmental persistence of \( \text{Salmonella} \) in the produce environment.

In all three biofilm studies on tomatoes, the genetic complements including \( S. \) Typhimurium MKF1 pMKF4, MKF2 pMKF2, WJZ2 pMKF2 pMKF5 demonstrated a restored phenotype to the biofilm capabilities of MKF5 with no significant differences between the complements and MKF5 for any of the assays. The biofilm formation of the vector controls, \( S. \) Typhimurium MKF1 pWSK29, MKF2 pCR2.1, and WJZ2 pCR2.1 pBBR1-MSC5, also did not significantly differ from their respective mutants, MKF1, MKF2, and WJZ2 (Figures 5-3, 5-5). The results for the genetic complements and controls indicated that the decrease in biofilm formation of MKF1, MKF2, and WJZ2 was due to the deficiency of the AgfD regulator and AgfD-regulated thin aggregative fimbriae, and/or cellulose in the strains.
The characterization and assessment of biofilm formation of *Salmonella* outbreak strains was done to assess the correlation between the rdar morphology, biofilm formation, and fitness in the plant environment. The cellulose for the wild-type and pWSK29 containing strains were compared and the strains significantly differing in cellulose production were removed from the study since the insertion of pWSK29 altered a phenotype that contributes to biofilm formation. The outbreak strains assessed for biofilm formation were linked to tomato outbreaks of either clinical or environmental origin (Table 5-1). The *S. Typhimurium* 14028 pWSK29 was the control for the rdar morphology, with JTN212 pWSK29 as a saw control. The *Salmonella* outbreak strains exhibit environmental survival, so the characterization of the strains as non-rdar and having decreased cellulose production indicates that the rdar morphology does not directly correlate with environmental survival (Figures 5-5 and 5-9).

The maximum biofilm formation on the tomato segments was produced by *S. Typhimurium* 14028 pWSK29, the rdar control, but the biofilm formation was not significantly different from the saw control, *S. Typhimurium* JTN212 pWSK29, or the remaining outbreak strains (Figure 5-10). The resulting studies with the outbreak strains indicated that rdar morphology and biofilm formation may correlate, but not significantly so. It is difficult to correlate the rdar morphology with a specific phenotype, such as biofilm or cellulose production. This lack of correlation may be due to the unknown mutation causing the loss of the rdar morphology. The loss of rdar can be an evolutionary loss, which is often seen in laboratories where strains are continuously passed through nutrient rich media. The loss of the rdar morphology may be due to a mutation in the cellulose biosynthesis pathway, but it is more likely to be derived from a
mutation of one of the upstream regulators of \textit{agfD}, which may alter cellulose production along with other crucial factors of biofilm formation. The complexity of the rdar morphology and the survival of \textit{Salmonella} outside of the animal host results in the conclusion that there is no indication that rdar morphologies, biofilm formation, or cellulose production can singularly dictate the environment fitness of \textit{Salmonella} in the produce environment.
Table 5-1. *Salmonella enterica* cultures obtained from produce associated outbreaks

<table>
<thead>
<tr>
<th>Serovar and strain</th>
<th>Outbreak event and characteristics</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Braenderup 04E00783</td>
<td>Isolated from 19 yr old male, stool sample, early in Roma Tomato outbreak</td>
<td>(34)</td>
</tr>
<tr>
<td>Braenderup 04E01347</td>
<td>Isolated from 24 yr old female, stool sample, sample obtained in middle of Roma Tomato outbreak</td>
<td>(34)</td>
</tr>
<tr>
<td>Braenderup 04E01556</td>
<td>Isolated from 5 yr old male, stool sample, sample obtained late in Roma Tomato outbreak</td>
<td>(34)</td>
</tr>
<tr>
<td>Michigan LJH521</td>
<td>Spontaneous rifamycin resistant derivative of the strain isolated from cantaloupe associated <em>Salmonella</em> outbreak</td>
<td>L. J. Harris, University of California Davis</td>
</tr>
<tr>
<td>Montevideo LJH519</td>
<td>Spontaneous rifamycin resistant derivative of the strain isolated from patient with salmonellosis in tomato outbreak</td>
<td>M. D. Dany luk, University of Florida</td>
</tr>
<tr>
<td>Newport C4.2</td>
<td>Isolated from tomato field in Delmarva area during round tomato associated outbreak, identical PFGE BlnI and XbaI pattern as C6.3 strain</td>
<td>(33)</td>
</tr>
<tr>
<td>Newport C6.3</td>
<td>Isolated from tomato field in Delmarva area during round tomato associated outbreak, identical PFGE BlnI and XbaI pattern as C4.2 strain</td>
<td>(33)</td>
</tr>
</tbody>
</table>
Figure 5-1. Biofilm formation of *Salmonella* on polystyrene. The *Salmonella* strains were diluted 1:100 in 1/10 tryptic soy broth (TSB) and were incubated in polystyrene plates for 24 hr at 30°C. The plates were stained with crystal violet, which was solubilized in 33% acetic acid. The optical density of the samples was measured at 595 nm. The error bars denote the standard deviation. The statistical grouping of the data as determined by Tukey-Kramer HSD analysis with significance set to p<0.05 is denoted by the letters, with a significance difference in samples of different letters.
Figure 5-2. Biofilm formation of *Salmonella* Typhimurium MKF5, MKF1, MKF2, and WJZ2 on the surface of intact green and red tomatoes. The blossom end of field grown green and red, round tomatoes were submerged in *Salmonella* inocula suspended in DI water for 24 hr at 30°C. The biofilm was quantified through crystal violet staining and optical density readings at 595 nm. The error bars denote standard error. The stars denote significant difference in biofilm formation of the strains in comparison to MKF5 on both green or red tomatoes as indicated by a Dunnett’s test with a significance of p<0.05.
Figure 5-3. Biofilm formation of *Salmonella* Typhimurium complement and vector control strains on the surface of intact green and red tomatoes. The blossom end of field grown green and red, round tomatoes were submerged in *Salmonella* inocula in DI water for 24 hr at 30°C. The biofilm was quantified through crystal violet staining and optical density readings at 595 nm. The error bars denote standard error.
Figure 5-4. Biofilm formation of *Salmonella* Typhimurium MKF5, MKF1, MKF2, and WJZ2 on green and red tomato segments. Tomato segments measuring 15 mm x 15 mm x 2 mm were submerged in *Salmonella* inocula of DI water for 24 hr at 30°C. The biofilm was quantified through crystal violet staining and optical density readings at 595 nm. The error bars denote standard error. The stars denote significant difference in biofilm formation of the strains in comparison to MKF5 on both green or red tomatoes as indicated by a Dunnett’s test with a significance of p<0.05.
Figure 5-5. Biofilm formation of *Salmonella* complement and vector control strains on green and red tomato segments. Tomato segments measuring 15 mm x 15 mm x 2 mm were submerged in *Salmonella* inocula of DI water for 24 hr at 30°C. The biofilm was quantified through crystal violet staining and optical density readings at 595 nm. The error bars denote standard error.
Figure 5-6. Biofilm formation of *Salmonella* strains on green and red tomato segments in DI water supplemented with MnCl$_2$ and MgSO$_4$. A) Biofilm formation of *Salmonella* on green tomato segments. B) Biofilm formation of *Salmonella* on red tomato segments. Tomato segments measuring 15 mm x 15 mm x 2 mm were submerged in *Salmonella* inocula of DI water for 24 hr at 30°C. The biofilm was quantified through crystal violet staining and optical density readings at 595 nm. The error bars denote standard error.
Figure 5-7. Depictions of *Salmonella* outbreak strains on Congo red media. A) S. Typhimurium 14028 pWSK29, B) JTN176 pWSK29, C) JTN212 pWSK29, D) Braenderup 0783 pWSK29, E) Braenderup 1347 pWSK29, F) Braenderup 1556 pWSK29, G) Michigan pWSK29, H) Montevideo pWSK29, I) Newport C4.2 pWSK29, J) Newport C6.3 pWSK29. *Salmonella* cultures spotted onto Congo red media and incubated at 30°C for 48 hr. An Olympus MVX10 confocal microscope and Olympus MVX-TV1XC camera was used to photograph the strains. The *Salmonella* 14028 pWSK29 and JTN176 pWSK29 are rdar controls, while JTN212 pWSK29 is a saw control. The outbreak strains all appear to be varying non-rdar morphology.
Figure 5-8. Growth curves of produce related *Salmonella* outbreak strains for 12 hr at 30°C. Overnight cultures of each strain were diluted to the approximate concentration of $10^4$ CFU/ml and incubated at 30°C, shaking at 200 rpm for up to 12 hr. Samples were diluted and plated once per hour, including hour 0. The final growth curves were the average of three replicates per strain with error bars indicating standard deviation.
Figure 5-9. Cellulose quantification of *Salmonella* outbreak strains. The graph represents quantified relative fluorescence units of cellulose produced by each strain on the left axis. The streaked colonies for each of the strains were resuspended on the CW agar in DI water and the fluorescence was recorded in 96 well plates at an excitation of 360 ± 40 nm and emission of 460 ± 40 nm. Optical density of the cultures was measured at 600 nm. The relative fluorescence is expressed as fluorescence per unit of OD$_{600}$ ± standard deviation from three replicate studies. The statistical grouping of the data as determined by t-test analysis with significance set to p<0.05 is denoted by the letters, with a significance difference in samples of different letters.
Figure 5-10. Biofilm formation of *Salmonella* outbreak strains on green tomato segments. Tomato segments measuring 15 mm x 15 mm x 2 mm were submerged in *Salmonella* inocula of DI water for 24 hr at 30°C. The biofilm was quantified through crystal violet staining and optical density readings at 595 nm. The error bars denote standard error.
CHAPTER 6
CONCLUSIONS AND FUTURE STUDIES

In this work, the AgfD regulator, AgfD-regulated thin aggregative fimbriae, and/or AgfD-regulated cellulose were investigated for their roles in the interactions of *Salmonella* and the tomato surface. The research concluded that AgfD-regulated thin aggregative fimbriae were not found to have a significant role in initial attachment, persistence, or biofilm formation on the tomato surface under these experimental conditions. Future research could be directed towards the role of thin aggregative fimbriae in the interactions between *Salmonella* and produce surfaces as there have been reports of thin aggregative fimbriae mutants (both targeted deletion mutants and spontaneous mutants) deficient in attachment and biofilm. A continuation of this research could assess the role and expression of the fimbrial operons of *Salmonella* during the attachment to abiotic and biotic surfaces to determine which specific fimbriae are active in attachment to produce.

Along with thin aggregative fimbriae, the AgfD regulator and AgfD-regulated cellulose were also found to be insignificant in the initial attachment to the tomato surface, indicating that initial attachment may be directed more by physiochemical properties than the biological factors studied in this research. In the persistence of *Salmonella* on the tomato surface, AgfD-regulated cellulose was determined to be a significant factor in persistence over 6 days. Understanding the components necessary for the environmental survival and persistence of *Salmonella* may provide targets for preharvest treatments to reduce contamination of produce and while the application of cellulase may reduce persistence of *Salmonella* on plant surface, the cellulose may also damage the plants since cellulose is a component of cell walls in green plants. Despite
the lack of an effective target to reduce *Salmonella* persistence on plants, the research assessing the necessary components for human pathogen survival and persistence in the environment should be continued for future development of targeted preharvest prevention strategies.

The establishment of *Salmonella* biofilms on the surface of both green and red tomatoes is also important in the food industry. A future direction of this work could be the investigation of current sanitizer concentrations against *Salmonella* biofilms on the tomato surface and in simulated flume systems to establish if current standards are effective in removing the pathogen. The biofilm studies were performed by submerging the materials in solutions of *Salmonella*, so these conditions are unlikely to occur in the field environment but it is possible for *Salmonella* to form biofilm on the surface of intact tomatoes without additional nutrient sources in the field during a flooding event. The formation of biofilms should also be considered in the tomato packing facilities where flume systems are used. The potential for biofilm formation may support the use of brush-roller system rather than flumes. The AgfD regulator and AgfD-regulated cellulose were found to be significant factors in the formation of biofilm on the surface of polystyrene and intact tomatoes. The AgfD regulator is active under a variety of stimuli including temperatures of 25-30°C, low nutrient conditions, and in the presence of ethanol. Packing facilities should deter the use conditions in the flume systems that are favorable for transcription of *agfD* to reduce AgfD-directed biofilm formation. Cellulase could also be used along with chemical sanitizers in the sanitation process of the flumes and other food contact surfaces to reduce the persistence of any viable *Salmonella* before biofilms are formed.
The persistence of *Salmonella* on the tomato surface was also found to be more favorable on red versus green tomatoes for the cellulose deficient strains. The 2-fold reduction in the persistence on green tomatoes indicates that both bacterial behavior, including cellulose production, and produce ripeness could be factors in the persistence of *S. Typhimurium* on the tomato surface. This should be considered in determining the best time to harvest the produce. The taste of a product is important to consumers, and higher demand is being seen for fresher produce that are harvested closer to the peak maturity and ripeness. Additional research would be needed to determine if the persistence of *Salmonella* is favorable as the tomatoes ripen, because if so, harvesting pink tomatoes to meet consumer demand may decrease the safety of the product.

*Salmonella* has the ability to persist and form biofilm on the tomato surface under the conditions of this research. The work should be applied to greenhouse and field studies to determine the persistence and potential biofilm formation in these environments. The determination of environmental fitness through screening methods such as rdar or non-rdar classification could also be used to characterize *Salmonella* isolated from the environment. While this was attempted, the rdar morphology and environmental persistence did not correlate. The current direction of food safety is one of prevention. Understanding the biological factors necessary for *Salmonella* survival in the plant environment may provide targets for specific preharvest treatments in addition to the general safety strategies set forth by GAPs and FSMA.
APPENDIX A
MEDIA COMPOSITION

Buffered Peptone Water (0.1%)
1.0 g peptone
0.5 g NaCl
0.35 g Na₂HPO₄
0.15 g NaH₂PO₄
1.0 L DI H₂O
Autoclave sterilize

Calcofluor White (CW) Agar
9.6 g tryptone
4.8 g yeast extract
960 ml DI H₂O
Autoclave sterilize
Add: 40 ml of Calcofluor white with Evans Blue dye

Colonization Factor Antigen (CFA) Medium
10 g casamino acids
1.5 g yeast extract
50 mg MgSO₄
5.0 mg MnCl₂
1.0 L DI H₂O
Adjust to pH 7.4
Autoclave sterilize

Congo Red (CR) Agar
10 g tryptone
5.0 g yeast extract
1.0 L DI H₂O
Autoclave sterilize
Add: 4.0 ml of 100 mg/ml Congo red in methanol
2.0 ml of 100 mg/ml Brilliant Blue in ethanol

Evans Blue-Uranine (EBU) Agar
10 g tryptone
5.0 g yeast extract
5.0 g NaCl
2.5 g glucose
15 g agar
960 mL DI H₂O
Autoclave sterilize
Add: 28.71 mL of 1M K₂HPO₄
1.25 mL of 1% Evans Blue in DI H₂O
2.5 mL of 1% Uranine in DI H₂O
Light sensitive, store properly
**Luria-Bertani (LB) Lennox Agar**
- 10 g tryptone
- 5.0 g yeast extract
- 5.0 g NaCl
- 15 g agar
- 1.0 L DI H$_2$O
- Autoclave sterilize

**Luria-Bertani (LB) Lennox Broth**
- 10 g tryptone
- 5.0 g yeast extract
- 5.0 g NaCl
- 1.0 L DI H$_2$O
- Autoclave sterilize

**NZY+**
- Per 100 ml DI H$_2$O
- 1.0 g NZ amine
- 0.5 g yeast extract
- 0.5 g NaCl
- Autoclave sterilize
- Add: 1.25 mL of 1 M MgCl$_2$
- 2.0 mL 20% glucose in DI H$_2$O

**Potassium Phosphate Buffer (0.1M)**
- 61.5 ml of 1 M K$_2$HPO$_4$ in DI H$_2$O
- 38.5 ml of 1 M KH$_2$PO$_4$ in DI H$_2$O
- Adjust pH to 7.0
- Dilute 1:10 in DI H$_2$O
- Autoclave sterilize

**PUM Buffer (65)**
- 22.2 g K$_2$HPO$_4$H$_2$O
- 7.26 g KH$_2$PO$_4$
- 1.8 g urea
- 0.2 g MgSO$_4$H$_2$O
- 1.0 L DI H$_2$O
- Adjust to pH 7.1
- Autoclave sterilize

**Sodium Phosphate Buffer (0.002 M)**
- 46.3 ml of 1 M Na$_2$HPO$_4$ in DI H$_2$O
- 53.7 ml of 1 M NaH$_2$PO$_4$ in DI H$_2$O
- Adjust pH to 6.8
- Dilute 0.4 ml in 199.6 ml DI H$_2$O
- Autoclave sterilize
Tryptic Soy Broth (TSB)
1.7 g tryptone
0.3 g soy peptone
0.25 g dextrose
0.5 g NaCl
0.25 g Na$_2$HPO$_4$
1.0 L DI H$_2$O
Autoclave sterilize

Xylose Lysine Desoxycholate (XLD) Agar
3.0 g xylose
5.0 g L-lysine
7.5 g lactose
7.5 g sucrose
5.0 g NaCl
3.0 g yeast extract
0.08 g phenol red
2.5 g sodium desoxycholate
6.8 g sodium thiosulfate
0.8 g ferric ammonium citrate
13.5 g agar
1.0 L DI H$_2$O
Heat media until reaching boiling, do not autoclave
APPENDIX B
ATTACHMENT AND BIOFILM FORMATION OF *Salmonella* STRAINS AFTER INCUBATION UNDER STATIC OR SHAKING CONDITIONS

Before studying the attachment, persistence, and biofilm formation of *Salmonella*, the incubation conditions for growing the *Salmonella* strains were assessed. The cultures were grown at 30°C to encourage activation of *agfD* and the AgfD-regulated system (64). Preliminary attachment and biofilm studies were performed with *S. Typhimurium* 14028, MKF2, and WJZ2 to determine if incubating the strains while shaking at 200 rpm would disrupt the extracellular components of the bacteria and alter the results in the tomato studies. The MKF1 strain was not included in the study because the scope of the assay was on the effect of incubation conditions on the condition of the extracellular components, so the culture with the highest and lowest expression of thin aggregative fimbriae and cellulose were compared. The 14028, MKF2, WJZ2 strains were grown in parallel at 30°C either static or shaking at 200 rpm. After overnight incubation, the strains grown under shaking conditions were diluted to the measured OD$_{600}$ (optical density at 600 nm) of the static cultures. The strains were washed three times in PBS. The strains were then used to assess attachment to tomatoes and biofilm formation.

For the attachment assay, the blossom end of Campari, vine ripe tomatoes (obtained from Publix Grocery, Gainesville, FL) were spot inoculated with 10 spots of 10 μl of inoculum for a total of 100 μl per tomato. Tomato experiments were run in triplicate, with two biological replications. After inoculation, the tomatoes rested on the bench for five minutes before being rinsed in 10 ml of sterile DI water. The rinsate was diluted and spread plated onto XLD and the tomatoes were stored at 22°C, 60-80%
relative humidity for 4 hr. The blossom ends of the tomatoes were then rinsed in 10 ml of sterile DI water. The tomatoes were rubbed by hand for 60 sec in a stomacher bag containing 100 ml of PBS. The secondary rinsates and tomato rinse from the stomacher bags were diluted and plated onto XLD. Plates were incubated at 37°C overnight to obtain plate counts of the recovered *Salmonella* concentrations (Figure B-1). The recovered concentrations of 14028, MKF2, and WJZ2 in the initial rinse, secondary rinse, and tomato attachment do not differ.

For the biofilm assay, the washed strains were diluted 1:100 in colonization factor antigen (CFA) medium and loaded into 96-well polystyrene plates (Appendix A). The plates were incubated at 30°C for 24 hr. After incubation, the plates were treated with crystal violet, rinsed with DI water, and the crystal violet dye was solubilized in 33% acetic acid for optical density measurement at 595 nm. The results of the biofilm assay are illustrated in Figure B-2. T-test analysis for each culture pair was performed with JMP 9.0 Pro statistical software to show there was no significant difference (with p<0.05) in the attachment or biofilm formation of *S. Typhimurium* 14028, MKF2, or WJZ2 when grown at 30°C in static or shaking conditions.
Figure B-1. Attachment of *Salmonella* on the surface of Campari tomatoes after static or shaking overnight growth. The assay was performed by incubating the *S. Typhimurium* 14028, MKF2, and WJZ2 strains overnight in duplicate at 30°C, either static or shaking at 200 rpm. The cultures were recovered in an initial rinse after 5 min a secondary rinse after 4 hr, and a final recovery through physical abrasion of the tomato surface by rubbing. The three graphs depict the attachment results based on the resulting plate counts, with the growth conditions and strains labeled within the legends of each graph.
Figure B-2. *Salmonella* biofilm formation in polystyrene plates after static or shaking overnight growth. The assay was performed by incubating the *S. Typhimurium* 14028, MKF2, and WJZ2 strains overnight in duplicate at 30°C, either static or shaking at 200 rpm. The cultures grown under shaking conditions were diluted to maintain similar starting concentrations, and the cultures were inoculated into polystyrene plates in parallel. After overnight incubation at 30°C, the plates were stained and the optical density of the solubilized solution was recorded at an OD of 595 nm. The biofilm readings for each *Salmonella* strain were baselined to uninoculated samples within each biofilm plate to account for any crystal violet binding to the polystyrene.
LIST OF REFERENCES


BIOGRAPHICAL SKETCH

Marianne Kathryn Fatica was born in 1986 in Albany, New York and grew up in Cape Coral, Florida. After graduating from Mariner High School in 2004 as valedictorian, Marianne attended the University of South Florida in Tampa, Florida. During her undergraduate studies, she became involved in laboratory research through the Research Experience for Undergraduates (REU) program. Through the Department of Chemistry, Marianne analyzed protein composition of European and Africanized Honeybees as part of a project to develop an enzyme-linked immunosorbent assay (ELISA) test for the rapid identification of Africanized Honeybees. She was also involved in the Anthropology department, where she completed her undergraduate honors thesis, titled, “The Application of the Anthropological Perspective in Medicine to Address Problems within Physician-Patient Communication.” Marianne graduated from the University of South Florida in 2008 with a Bachelor of Science in microbiology and a minor in biological anthropology.

Marianne began her graduate degree in the Department of Food Science and Human Nutrition at the University of Florida in 2008. As someone new to the field of Food Science, Marianne felt it was important to become certified in Good Agricultural Practices and HACCP for juice processors to better understand the application of these safety programs. Along with her doctoral program, Marianne also diversified her studies by earning a certification in public health, with an emphasis in environmental health, from the University of Florida Public Health Department in 2011. Along with her graduate work, Marianne was highly involved in teaching microbiology courses within the department. Marianne has also been honored with several awards throughout her graduate studies including the Agriculture Women’s Club, Robert H. Olsen, and Florida
Association of Food Protection Scholarships. After completing her doctoral degree in May 2013, Marianne will continue her professional interests in food microbiology.