Survival and Growth of Attenuated *Salmonella enterica* Serovars Newport and Typhimurium in Growth Media and Tomatoes

Lily L Yang

Thesis submitted to the faculty of the Virginia Polytechnic Institute and State University
in partial fulfillment of the requirements for the degree of

Master of Science
In
Food Science and Technology

Renee R. Boyer
Robert C. Williams
Ganyu Gu
Steven Rideout

June 9, 2014
Blacksburg, VA

Keywords: *Salmonella enterica*, Typhimurium, Newport, tomato(es), attenuated, media

Survival and Growth of Attenuated *Salmonella enterica* Serovars Newport and Typhimurium in Media Culture and Tomatoes

Lily L Yang

ABSTRACT

Fresh market tomatoes have been associated with 15 multistate *Salmonella* outbreaks between 1973 and 2010. While, *S. enterica* survival has been studied in tomato plants, field studies have been limited. To understand pathogen growth and survival, in crop fields, surrogate or attenuated organisms must be developed and validated. The purpose of this study was to compare the growth and survival of seven attenuated *S. enterica* Typhimurium and Newport strains against virulent strains *S.* Typhimurium ATCC14028 and *S.* Newport J1892 in optimum (TSB and TSB+kan) and minimal M9 growth media, and in commercial, red ripe tomatoes. Bacterial growth in media was assessed via BioScreen. Tomatoes were separately inoculated with 7 Log CFU/g of each isolate via vacuum infiltration, surface spot inoculation, or diced inoculation. Populations of each strain were determined on Days 0, 1, 3, and 5. In media, there were few differences in overall growth and growth rates between mutant isolates and wild-type ($P<0.05$). Growth in M9 was less ($P<0.01$), while growth rates were higher ($P<0.01$) than in TSB. In tomatoes (per treatment), there were no significant differences between growth rates of each isolate compared to WT ($P>0.05$); however, S*almonella* strains in diced tomatoes had a higher growth rate than that in spot treated tomatoes ($P>0.05$). The

growths of all the isolates in tomatoes indicated that under the tested conditions, isolates acted similarly to their WT counterparts. Thus, these strains may be able to be used as surrogate organisms in field studies.

Acknowledgements

First and foremost, with the utmost sincerity and with the deepest humility, I wish to thank Dr. Renee Boyer, my graduate advisor, for taking a chance with me, and accepting me into graduate school and into her research group. Of all the schools and labs I had been interested in working with, hers was at the top of my list; I am forever grateful for this opportunity to not only further my education, but to be afforded the many opportunities to challenge and expand myself in multiple directions. Her limitless patience, energy, and guidance has been astounding. With every freak out in her office, every long ranting email, and every frenetic series of texts, she not only takes the madness all in stride, but can turn it all into a workable and calm order. Additionally, many thanks to my awesome advisory committee: Dr. Ganyu Gu, Dr. Robert Williams, and Dr. Steve Rideout. Without Dr. Gu's exacting guidance, ideas, patience, advice, and suggestions, this project would never have taken off nor seen the light of day in its full form; Dr. Williams as a fantastic supporter and fellow Catbug enthusiast, I always felt there was someone to turn to whom would always be there to back me up; and, Dr. Rideout from whom this project began with. Thank you all so much for all your help, knowledge, and amazingness.

In the lab, I would like to say, "thank you" to my undergraduate researchers, Millie C. Clarke and Minh Duong, without whom none of the lab work would have ever been accomplished in a timely manner. I would especially like to thank Millie for her consistent support, time, and dedication to this project; she has worked with me through the hardest, most gut-wrenching portions of this project, kept the cool head around the lab, while still managing to make me laugh. Thank you to Kim Waterman for being there

through thick and thin: from dealing with my many spur-of-the-moment-and-desperate orders, to my many crazy bags of BioHazard waste, to letting me vent my frustrations. What a saint! I would also like to thank Dr. Wang for helping me solve the unsolvable!

Most especially, this big hug of love and gratitude goes to my fellow graduate students: Courtney Crist, Liyun Ye, Katheryn Goodrich, Stephanie Pollard, Ellen Ewell, Anthony Lukas, Jason Levy, Matthew Schroeder, Jian Wu, and roommate Matthew Longobardi. All of your constant support, encouragement, food, hugs, and drinks have not only made this homesick California girl feel like part of a family, but has made the transition and transformation through graduate school and away from the teetering edges of insanity, possible. Without you guys, I would be so lost and confused. Without you guys, I would have never found the balance. I could never tell you how much I cherish and appreciate the time we have together.

Last but not least, I would like to thank my family for their support throughout this entire process: from me moving away for college, then staying away to work for a few years, to an even further move to graduate school. One day, I will hopefully achieve and learn everything you hope for me to know; however, things will happen as they happen. Thank you to my best friends in California: Linnea Motts, Christine Lee, Crystal Yang, Sanh Luong, Eric Low, Chris Lee, Tim Chen, Jason Bacon, Jason Pang, Kalynda Stone, and Martin Cheng for always understanding, caring, talking, and waking me up every morning so I won't miss a deadline, miss class, or miss life. Thank you, Earl A. Campaigne III; meeting you has been quite the adventure which I hope will continue for a long time to come. Finally, I would like to thank Jacob Joseph Cameron Miller for teaching me the value and importance of letting go and chasing my dreams.

TABLE OF CONTENTS

List of Figures

Chapter 3

Page

List of Tables

Chapter 3

Chapter 1: Introduction

There are 9.4 million identifiable cases of foodborne illnesses estimated to occur annually in the United States (Scallan et al., 2011). *Salmonella enterica* is associated with the leading number of bacterial foodborne cases. Salmonellosis accounts for 1.2 million bacterial foodborne illnesses each year (Scallan et al., 2011). In recent years, there has been an increasing trend of *S. enterica* contaminations associated with fresh produce (Lynch et al., 2009). The four most common *S. enterica* serovars associated with fresh produce outbreaks are: Enteritidis, Typhimurium, Newport, and Javiana (CDC, 2009). This project focuses on *S.* Typhimuirum and *S.* Newport serovars because of their associations with tomato outbreaks in the United States. (Zheng et al., 2013).

Approximately 14 million tons of tomatoes are produced annually in the United States, making *Solanum lycopersicum* the second highest value market vegetable crop (Boriss et al., 2005). The American public consumes approximately two million tons of fresh tomatoes per year. In recent years, tomatoes have been identified in a high number of *S. enterica* outbreaks. *Salmonella* linked contamination of raw tomatoes has been attributed to 15 multistate outbreaks between 1973 through 2010; 12 outbreaks having occurred since 2000 (FDA, 2013). Typhimurium and Newport are two of the most common *S. enterica* serovars associated with tomato outbreaks.

Produce can become contaminated with foodborne pathogens through many pathways. Contamination may occur from soil, water, human contact, processing procedures, and even inherited from each flowering generation (Zheng et al., 2013). The association of foodborne pathogens with tomatoes and tomato plants is a very complex process influenced by temperature,

pH, soil type, tomato cultivar, tomato maturity, and even the contaminating serovar of *S. enterica* (Zhuang et al., 1995; Barak et al., 2008; Zheng et al., 2013). Understanding the complexities of produce food safety issues is challenging. In recent years, due to increased numbers of (multi-drug) antibiotic resistant *S. enterica serovars*, the need to identify and understand *S. enterica* invasion and colonization of fresh produce from farm-to-table has become more pronounced. Due to the virulent nature of these pathogens, research must be confined to a controlled Biosafety Level 2 research laboratory. This limitation makes it difficult to transfer discovery to real world situations in the field or in the processing plant unless otherwise quarantined. One approach for overcoming this obstacle, when working in real world situations, is to use indicator or surrogate organisms instead of the virulent pathogen. A surrogate organism is a type of indicator organism that may be used "to imitate the survival of microbial pathogens in fresh produce" (James et al., 2006). This project examines attenuated isolates of *S. enterica* developed from pathogenic *S. enterica* serovars Typhimurium and Newport. Attenuation has been accomplished by introducing point mutations to knock out one or more genes associated with virulence (Noel et al., 2010). The null hypothesis is that these attenuated strains will not demonstrate significant differences in growth ability, competitiveness or persistence when compared against the pathogenic wild types; hence, attenuated strains of pathogenic organisms may be used as surrogate organisms for testing in field settings (Noel et al., 2010). The proposed research will focus on investigating the effects of virulence and attachment/motility genes on *Salmonella* survival, and comparing attenuated *S. enterica* Typhimurium and Newport strains to corresponding wild-type strains to identify specific strains for candidacy as promising surrogates to be used for further studies on tomatoes grown in the field.

Specific Objectives

(1) Compare the growth rates of attenuated *S. enterica* Typhimurium and *S. enterica* Newport strains against clinical, virulent strains of *S.* Typhimurium and *S.* Newport in optimum and minimal growth media.

(2) Evaluate the growth, survival, and persistence of attenuated *S. enterica Typhimurium* and *S. enterica Newport* strains against clinical, virulent strains of *S.* Typhimurium and *S.* Newport on and within imported, commercial, red ripe tomatoes from the grocery store.

Hypothesis

H_0: The growth, survival, and persistence of attenuated strains of *S. enterica* Typhimurium and *S. enterica* Newport are not significantly different from virulent, wild-type strains of *S. enterica* Typhimurium and *S. enterica* Newport, *in vivo* and *in vitro*.

H_a: The growth, survival, and persistence of attenuated strains of *S. enterica* Typhimurium and *S. enterica* Newport are significantly different from virulent, wild-type strains of *S. enterica* Typhimurium and *S. enterica* Newport, *in vivo* and *in vitro.*

Chapter 2: Literature Review

Food-borne Outbreaks

Produce-related foodborne illnesses

There are an estimated 48 million cases of foodborne illnesses annually in the United States; of these, 128,000 cases result in hospitalizations and 3,000 cases result in death. (Scallan et al., 2011). While foodborne illnesses can be caused by a variety of sources, the severity of illness varies among individuals depending on age, health, demographic, and even location. Previously, foodborne outbreaks were primarily associated with animal products; however, recent trends have shown an increasing association with fresh produce (Meng and Doyle, 2002; Lynch et al., 2009). In the 1970's, less than 1% of reported outbreaks were associated with fresh produce; but by the 1990's, over 6% were. Similarly, the number of foodborne illness cases associated with produce outbreaks rose from <1% to 12% (Svapalasingam et al., 2004). Between 1998 – 2008, produce accounted for 46% of illnesses and 1.1 million individuals fell ill to fruits associated with foodborne pathogen contamination (Painter et al., 2013).

The increasing number of reported outbreaks can be attributed to several trends including the consumers' demand for fresh produce year-round to more sophisticated tools for identifying and tracking outbreaks. Furthermore, increased demand and increased land use bring crop areas closer with areas of animal production, animal waste, and animal byproducts; thereby, increasing the likelihood of fresh produce becoming contaminated. Produce handling has changed to include longer transportation for minimally processed fresh produce to reach the customer. Additionally, in the past two decades, because of increased consumer demand for fresh fruits and vegetables, fresh produce imports have increased dramatically (Huang and Huang, 2007).

Increased transportation times and distances provide a greater opportunity for bacteria to quickly take residence and multiply upon improperly processed and handled produce (Lynch et al., 2009).

Pathogen Contamination of Produce

Pathogens can contaminate a food product at any point throughout the food's production cycle. Fruits and vegetables can become contaminated during the pre-harvest environment directly from the soil, through the root system, and even through abrasions, cuts or openings on the surface. Pathogen uptake from contaminated water has also been demonstrated through leaf stomata and root systems (Gu et al., 2011). Post-harvest contamination can occur through cross contamination in the packinghouse or during handling. Pathogens can also easily enter produce that has been cut or bruised during its life cycle or during processing through natural openings like the stem scar or the calyx; as well as invade plant seeds early during the reproductive cycle of the plant and become internalized in the next generation (Charkowski et al., 2002; Gu et al., 2011). Once the pathogen has been internalized, it is not likely to be removed or killed through surface washing or submersion in disinfectant. As a result, prevention of contamination is paramount in fresh produce production.

S. enterica Contamination in Food

Salmonella enterica is the second leading cause of foodborne illnesses (Scallan et al., 2011) with more than 1.2 million cases identified annually (CDC, 2011). The cases account for 35% of yearly hospitalizations and are the leading cause of foodborne illness associated deaths

with 450 deaths per year (Scallen et al., 2011). Since 2006, *S. enterica* spp. foodborne outbreaks of salmonellosis have been linked to such common foods as tomatoes, peanut butter, ground beef and cantaloupes (CDC, 2013). The four *S. enterica* serotypes with the highest isolation rates are *S.* Enteritidis, *S.* Typhimurium, *S.* Newport and *S.* Javiana (CDC, 2009).

Despite its desirability, fresh market tomatoes have also been linked to numerous outbreaks of salmonellosis. Between 1998 and 2006, 12 of 71 produce related outbreaks were associated with tomatoes (CSPI, 2007; Berstein, 2007). While there have not been many import associated outbreaks, scares with related import produce like the 2008 multistate outbreak of *S. enterica* serovar Saintpaul discovered in jalapenos, peppers, and thought to have also been in tomatoes, has increased vigilance and awareness (Klontz et al., 2010).

Both *S.* Typhimurium and *S.* Newport have consistently been linked to recurring tomato-associated salmonellosis. Of twelve *S. enterica* tomato-related outbreaks, at least four outbreaks originated from the same pulse-type of *S.* Newport that was traceable back to Virginia's Eastern Shore where they were grown, harvested, and then packed for shipment. A pulse-type is the recognizable Pulse Field Gel Electrophoresis [PFGE] pattern established by each strain. In one specific outbreak, it was determined that the outbreak isolate was derived from environmentally contaminated pond water used for irrigation and pesticide application and whose paths had cross-connections with the water pipes irrigating the crops (Greene et al., 2008). In a recent study, multiple *S. enterica* serovars were introduced to Virginia's Eastern Shore's sandy loam soil and inoculated onto tomato blossoms. Serovars Newport and Javiana were found to thrive within Virginia's sandy loam soil. *S.* Newport had the highest recovery rate in the tomato's root and water system and persisted very well on the tomato fruit; whereas, *S.* Typhimurium did not compete well and had a low recovery from those locations (Zheng et al., 2013). Zheng et al.

(2013) concluded that some serovars of *S. enterica* (Javiana and Newport) "are more adapted for survival and persistence in the tomato plant environment" while others (Typhimurium) may be more likely to contaminate tomatoes in the post-harvest environment. Furthermore, they demonstrated that the recovery of *S. enterica* was also dependent on the fruit ripeness and its field/harvest conditions.

Salmonella enterica is a hardy and adaptive pathogen. The bacterium is able to grow and persist on the tomato surface and within its pulp (Zhuang et al., 1995). Moreover, it can also become internalized within the tomato and persist through maturation and propagation (Guo et al., 2001). With the development of more complex processing procedures to increase commodity shelf-life, opportunities for *Salmonella* proliferation or cross-contamination also rise. (Lynch et al., 2009). Not only has salmonellosis been linked to pre-diced tomatoes held at room temperature; but, colonization, persistence and contamination by *S. enterica* on whole product can vary depending on tomato cultivar, tomato maturity, soil type and *Salmonella* serovar (Barak et al., 2008; Zheng et al., 2013).

Tomato Production

With a total production yield of more than $2 billion dollars a year, the tomato (*Solanum lycopersicum*) is now the second-highest value and "fourth most popular" market vegetable in the United States" (Boriss et al., 2005; USDA ERS, 2014). In 2005, a total of 14 million tons of tomatoes, 2 million for fresh consumption and 12 million for processing, were produced; that number has since increased to over 28.9 million tomatoes produced for fresh consumption in 2010 (Boriss and Brunke, 2011). From 1985 to 2008, the per capita consumption of raw

tomatoes has increased from 12.3 pounds to 18.5 pounds (Boriss and Burke, 2011). It is lauded not only as a delicious addition to one's meal, but also as an antioxidant rich, nutritious food.

The increased desire for tomatoes has led to an increase of production both within the United States and in other countries. California and Florida are the largest tomato producing states, followed closely by Virginia, Georgia, Ohio, and Tennessee (Boriss and Brunke, 2011; USDA ERS 2014). While these states produce the majority of tomatoes, the United States also imports about one-third of the tomatoes it consumes. Winter, spring, and fall demand is heavily met by imports from Mexico garnering about 1.5 million tons of tomatoes in 2010 (Boriss and Brunke, 2011). Increased demand for nationwide production and imports have been attributed to and driven by better growing conditions, protected-culture technologies, increased diversity of tomato availability, and consumer preferences (USDA ERS, 2014).

The Use of Surrogate Organisms

Addressing produce food safety issues is challenging. Due to the nature of the pathogens, work must be confined to a controlled Biosafety Level 2 Research Laboratory. This constraint accentuates the difficulties of transferring discovery to real world situations either in the field or in the processing plant. One approach to overcoming this obstacle in real world situations is to use indicator or surrogate organisms instead of the pathogen. An indicator organism is defined as "a microorganism or group of microorganisms that indicate a food has been exposed to conditions posing an increased risk that food may be contaminated with a pathogen or held under conditions conducive for pathogen growth" (FDA/CFSAN, 2001b). When present in food, indicators may signal the presence of target pathogen(s) in the food. One type of indicator organism recommended by the Food and Drug Administration [FDA] to be used in evaluating production processes is a surrogate organism. A surrogate organism should be an avirulent strain

of the pathogen used to "imitate the survival of microbial pathogens in fresh produce" (Busta, 2003; James, 2006) through similar growth and survival patterns as the target pathogens. For instance, as fears of *Listeria monocytogenes* contamination have increased in ready-to-eat products, the presence of non-pathogenic strains of *Listeria* (*L.* innocua and *L.* welshimeri) may not only indicate the presence of *L. monocytogenes* in the processing environment, but also serve as surrogate organisms to test the efficacy of processing methods during production (Busta, 2003).

First implemented in the low-acid canning industry, the surrogate organism *Bacillus stearothermophilus* was used to determine the presence or absence of a pathogen after antimicrobial treatment without having to introduce pathogenic *Clostiridum botulinum* spores into the food (FDA/CFSAN, 2001b; Gurtler, 2010). Identifying a surrogate organism to use in place of a pathogen would greatly benefit pre-harvest fresh produce microbiology. Researchers could inoculate the surrogate organism into the growing environment – either in the plant or in the field – in lieu of the pathogen and determine/extrapolate its growth and persistence. For a surrogate organism to be effective, it must meet six criteria (FDA/CFSAN, 2001b; James 2006): (1) It should be a nonpathogenic strain of the target organism; (2) It should demonstrate similar behavior as the target organisms in the environment study; (3) It should be easy to prepare and to remain stable; (4) It should exhibit enumeration, sensitivity, and inexpensive detection means; (5) It should attach to the produce surface at a similar rate as the target organism; (6) It should exhibit genetic stability to allow the test to be reproduced independently.

The development of attenuated microorganisms from virulent strains to be used as surrogate organisms is thought to be more applicable for produce to prevent the environmental release of virulent strains. One way to identify a surrogate is to "create" one through attenuation

of a previously pathogenic strain. While there has been speculation regarding the comparability of developed attenuated strains to virulent strains, it is oftentimes assumed that elimination of virulence genes does not impact growth, "stress tolerance, phenotypes, competitiveness" or persistence (James, 2006). Attenuated strain development has increased rapidly and simple screening tests have cursorily shown that pathogen and attenuated surrogate are equal (Noel et al., 2010); however, more in-depth research must be conducted to confirm the surrogate organism exhibiting similar characteristics to its pathogenic counterpart.

Salmonella enterica spp.

Salmonella is a facultative, anaerobic Gram negative, rod shaped, non-spore forming bacterium in the *Enterobacteriaceae* family. The majority of its members are motile and *Salmonella* serovars are often characterized by their somatic heat-stable lipopolysaccharide O-antigens, capsular surface virulence [Vi] antigen (found only in *S.* Typhi, Paratphyi C, and Dublin) and flagellated heat-labile H antigen proteins (Cooke et al., 2007; Saeed and Naji, 2007). There are two species of *Salmonella*, *S. enterica* and *S. bongori*, consisting of over 2600 serovars. The *S. enterica* species is further divided into seven subspecies characterized by DNA homology and hosts (Saeed and Naji, 2007). Subspecies 1, *Salmonella enterica* subspecies *enterica*, contains the many strains associated with human pathogenicity resulting in salmonellosis.

Salmonella is a highly adaptive microorganism; it can adapt well to extreme temperatures, extreme pH conditions, fluctuating water activities, varying salt concentrations, and can survive for years in frozen foods (Montville & Matthews, 2008). The optimal pH range

of *S. enterica* is between pH 6.5 – 7.5; but, the bacterium can also grow at pH levels as low as 4 and as high as 9.5. The organism's ability to persist at pH 4 contributes to its survival in tomatoes and other low-acid foods. When *Salmonella* is acid shocked, it will initiate an acid tolerance response to remain in stationary phase. Once adapted to adverse conditions (acid or increased temperatures), mutations within the microorganism will allow it to continue to persist and multiply while its other faculties for survival will also increase.

Salmonellosis

Salmonella enterica is characterized as typhoidal *Salmonella* or non-typhoidal *Salmonella* [NTS] (Saeed and Naji, 2007). Young children, elderly, and immuno-compromised individuals tend to be most susceptible to infection. The chemical composition of fattier foods also contributes to a lower infective dose (Montville & Matthews, 2008). Symptoms of infection with NTS usually occur within 8 – 72 hours of infection. Salmonellosis is usually self-limiting and will not persist into a carrier state. Usually, the illness presents as gastroenteritis distress resulting in nausea, vomiting, diarrhea, abdominal cramping, chills, and possible fever (Saeed and Naji, 2007). In extreme cases, NTS can result in systemic infections such as aseptic reactive arthritis, Reiter's Syndrome, endocarditis (in *S.* Typhimurium), and infantile/juvenile meningitis (Montville & Matthews, 2008; Saeed & Naji, 2007).

Basics of *Salmonella enterica* Pathogenicity and Virulence

The virulence and pathogenicity of *Salmonella* can be found in a collection of chromosomal pathogenicity islands [SPI], genomic islands [SGI], and virulence plasmids (Rhen et al., 2007). To incur salmonellosis, *Salmonella* must compete with host gut microflora by colonization through attachment and entering/invading intestinal epithelial cells using proteinaceous appendages (Montville & Matthews, 2008). While the sole effect of virulence genes is to initiate and sustain infections, the role is split into two types. Shared among most pathogens, general "housekeeping" genes regulate nutrient uptake, enact stress response, and ensure pathogen survival. On the other hand, SPIs typically contain the classical virulence genes conveying the pathogenesis for infection (Morgan, 2007). The genetic determinants for virulence and antibiotic resistance often occur on the *pSLT* virulence plasmid (Montville & Matthews, 2008). Other virulence and antibiotic resistance genes can be found in the bacterial genome. In this project, the specific genes that will be discussed are the *agfB*, *afgC*, *yihT, yihT-ompL, bcs*, *sirA* genes and the *pSLT* virulence plasmid in *S.* Typhimurium, and the antibiotic resistance *TolC* genes in *S.* Newport.

Salmonella enterica Pathogenicity Islands (SPI) & *sirA* gene

Unlike many pathogenic bacteria in which SPIs dictate the direct physical manifestations of pathogenicity, SPIs are differentiated between the many *Salmonella* serovars. *S. enterica* serovars Typhimurium and Newport contain complexly regulated virulence genes (Dorman et al., 2007). As previously mentioned, while many of these genes contribute to direct

pathogenicity, many also regulate "housekeeping" duties. *Salmonella* pathogenicity island 1 (SPI-1), a 38.8 kb segment of chromosome, is important in invading epithelial cells, initiating inflammatory responses by lysing macrophages, and initiating enteropathogenesis through the type-III secretion system (TTSS) found in mammalian virulence by transporting proteins across three biological membranes (Hueck, 1998). Additionally, SPI-1 facilitates iron (Fe2+) and magnesium (Mg2+) uptake; both being important in basic cellular function (Morgan, 2007). SPI-1 contains four genes coding for regulatory proteins: *hilA*, *hilC*, and *hilD*, and *hilF*. Proteins HilC and HilD both affect and regulate the *hilA* gene, which in turn regulates the *sirA* gene.

Established on multiple *Salmonella* pathogenicity islands (i.e. SPI-4 and SPI-5), the *sirA* (*Salmonella* invasion regulator) operon, coding for *sirA* gene expression, which dictates the "global regulator of genes mediat(ing) enteropathogenesis" is the start of an important cascading reaction. It is crucial in assisting attachment to abiotic surfaces while interacting with short chain fatty acids in the host organism through the BarA/SirA-Csr protein complex (Ahmer et al., 1999). The BarA/SirA-Csr two-component system indirectly affects flagellar genes by increasing virulence genes and decreasing expression of motility genes. (Teplitski et al., 2003; Dorman, 2007). Additionally, the *sirA* gene is necessary for the development of virulence factors, including encoding for a type-III export apparatus which injects *Salmonella* proteins into the host cell by bypassing two of its own membranes and invading the host's cell membrane and into its cytoplasm (Ahmer et al., 1999). It is also responsible for biofilm formation (Teplitski et al., 2006).

The SirA protein/BarA sensor kinase complex transmits environmental levels of short chain fatty acids which in turn reciprocally regulates SPI-1 and flagellar gene expression. In mammals *S. enterica* invasion occurs within the large intestine and SirA/BarA can distinguish

between the large and small intestines by detecting the presence of bile. Previous research by Noel et al. (2010) indicated that *S.* Typhimurium with mutations to *sirA* did not differ significantly in competitive fitness from the wild type *S.* Typhimurium. Because of its importance in virulence transmission, it is imperative to understand *sirA's* capabilities for growth and persistence.

Adhesin: Curli genes (afg operon)

Adhesion of *Salmonella*, using non-flagellar, fimbrial and non-fimbrial adhesins, to the host's cell wall (plant or mammal) is imperative for invasion and infection (Korhonen, 2007; Fronzes et al., 2008). Adhesins of the outer membrane consist of a high concentration of fimbriae, autotransporters, and outer membrane proteins that interact with host components. In mammals, expression of the fimbriae, filamentous surface organelles, contribute to intestinal persistence by attachment and infection (Tezcan-Merdol et al., 2007). They consist of a large portion of a cell's energy expense; but due to multiple variations of fimbrial structures all contributing to pathogenicity, the genes coding for fimbrial structures are important targets for gene regulation in cells (Korhonen, 2007). These structures not only mediate interactions between the bacterium, other bacteria, and the host, but also contribute to colonization, invasion, conjugation, and biofilm formation (Gibson, 2006). In mammals, fimbrial attachment occurs in ileal Peyer's patches to maximize *Salmonella enterica* growth in the intestines. Continued interaction with the host will result in colonization of damaged tissues and contribute to invasion through weaker spots in the tissue and possibly into the systemic system. Due to the combination of having the major subunit protein, the fimbrin, bound to smaller subunits, the fimbriae of both plants and mammals are able to interact and bind with host organism components and

carbohydrate receptors. Finally, fimbriae can form biofilms to protect themselves and continue persistence.

There are at least thirteen characteristic types of fimbrial operons in *Salmonella* Typhimurium; however, the one pertinent to this project is the thin and aggregative curli structure governed by the *agf* operon oft found in *S.* Typhi, *S. Typhimurium, S.* Newport and other NTS strains (Townsend et al., 2001; Zon et al., 2011). Curli production has been strongly attributed to both plant and mammalian adhesion and invasion through divergently expressed agfBAC and agfDEFG operon expressions (Fronzes et al., 2008). Additionally, the *agf* operon controlling curli manifestation is found in a common ancestor of both *Salmonella* and *Escherichia coli*. Not only does the *agf* operon control curli expression, it contains the pathogen-associated molecular patterns necessary to induce the characteristic response of salmonellosis. Curli presence upregulates a pro-inflammatory response in the host and can cause extensive bleeding and septic shock in the victim. This project specifically focuses on *S.* Typhimurium isolates with mutated *agf* genes because it is very difficult to delete *S.* Newport *agf* genes (Barak et al., 2005). *AgfB* and *agfC* genes are associated with the expression of fimbrial curli genes necessary for attachment and invasion of *Salmonella* in both plants and mammals. Additionally, both sets of genes positively contribute to pro-inflammatory responses and biofilm formation. *AgfB* is the main positive effector regulating surface attachment of bacteria; thus, mutations and/or deletions in the expression of this protein have demonstrated reduced attachment capabilities (Barak et al., 2011; Zaragoza, 2012).

AgfD complex on *bsc*, *yihT*, and *yihT-ompL* gene expressions

The *agf*D complex, mentioned briefly above, also controls thin aggregative fimbriae expression in response to environmental conditions (Römling, 1998). *AgfD* also affects the bacterial cellulose synthesis (*bcs*) operon which encodes for four proteins contributing to cellulose biosynthesis and attachment: *bscA, bscB, bscC,* and *bscD* (Zogaj et al., 2001). The *bsc* genes, not only increases the pathogen's tolerance to environmental stress, but forms a main component of the biofilm extracellular matrix while also acting as a blocking agent for flagella rotation motility (Gualdi, 2008). With increased production in *curli*, there may be decreased cellulose production (Anriany et al., 2006). When the *bcs* gene is mutated, cellulose production is lowered; however, these mutants were as competitively fit as the wild type strains and no difference in transcription was seen (Anriany et al., 2006; Noel et al., 2010).

Also positively affected by *agfD* genes, *yihT* and *yihT-ompL* gene expressions are associated with encoding aldolase in O-antigen capsule synthesis and translocation while positively affecting pathogen attachment onto plant tissue (Zaragoza et al., 2012). This set of genes is neither virulent nor expressed in mammalian cells (Zaragoza et al., 2012; Morvasi et al., 2013). In immature green tomatoes, the *agfD* genes are expressed and actively contribute to plant pathogenesis and persistence; however, in mature red tomatoes, while the genes are still expressed, the results do not affect competitive fitness when compared with wild-type growth (Zaragoza, 2012; Noel et al., 2011).

Virulence Plasmid: *pSLT*

S. Typhimurium contains a 90kb virulence plasmid (*pSLT)* with the ability to replicate on the same bacterial chromosome. The virulence plasmid codes for virulence genes that have been shown to mutate and to fuse to form drug resistance genes, thereby conferring antibiotic resistance to the pathogen (Dorman et al., 2007). Additionally, the plasmid has the ability to transmit and transfer itself *in vivo* to aid in systemic spread, rapid multiplication. and infection of other tissues in the host (Tezcan-Merdol et al., 2007). The virulence plasmid also codes for proteins that function to lyse macrophages, thereby initiating an inflammatory response. In rich growth medium, virulence plasmid gene expression is low; whereas in low pH minimal media, gene expression is high (Tezcan-Merdol et al., 2007). Experiments have shown that deletion of the *pSLT* plasmid did not seem to affect the competitive fitness of *Salmonella* presence in tomatoes; therefore even without the plasmid, mutated *S.* Typhimurium species appeared to colonize and persist at relative rates; thus, indicating that the plasmid's main effect is pathogenicity as opposed to basic growth (Noel et al., 2010).

Antibiotic Resistance and *TolC* receptors

Non-typhoidal *Salmonella* strains are widespread and have a multitude of hosts and reservoirs: humans, soil, intestinal tracts of animals, and water. There is increasing concern at the high numbers of animal-associated strains cultured from foodborne outbreaks or human illnesses, many of which demonstrate antibiotic resistance. Antibiotic resistance in bacteria is either intrinsic or acquired. Acquired resistance has been attributed to mutations and/or horizontal gene transfer events. Antibiotic resistance expresses itself through antibody-destroying enzymatic

production, to efflux systems preventing antibiotic activity within the cell, to modifications to the drug's target site, to production of bypass pathways to avoid the antibiotic completely (Bakry et al., 2014).

Discovered in the 1980's, *S.* Typhimurium Type 104 [DT 104] was the first established *Salmonella* serovar to develop multi-drug resistance. Furthermore, *Salmonella* Newport has demonstrated multi-drug resistances to multiple first and second line antibiotics (Parvathi, 2011). It seems that the multi-drug resistance of *S.* Newport is 18 times more highly associated in bovine origin than in human isolates; however, this serovar can still be confer pathogenicity into human hosts (Hoelzer, 2010). More alarmingly, the number antibiotic resistant *Salmonella* strains have increased significantly in the meat/poultry industry where antibiotics are oftentimes used to control endemic infections (Boonmar et al., 1998). The rise in antibiotic resistant bacteria is attributed to the widespread use of antibiotics in nonhuman niches, especially in its liberal application in food-producing animals, feeds, and lots (Martinez and Baquero, 2002). To provide an avenue for identification and tracking of drug resistant strains, the National Antimicrobial Resistance Monitory System [NARM] was developed in 1996 through the combined efforts of the FDA, CDC, and USDA (Montville & Matthews, 2008).

Present in both *S.* Typhimurium and *S.* Newport the *TolC* genes encode for an outer membrane channel. It is part of a set of multidrug efflux pumps, constituted as a transporter exit duct, which contribute to multidrug resistances by expelling antibacterial agents from within the pathogen. Thus, antibiotic resistance increases and antimicrobial agents introduced into the system are rendered useless (Stegmeier et al., 2006; Zgurskaya & Nikaido, 2000; Poole, 2002). The transporters not only exclude antibiotics, but also distribute waste products and infective proteins into the host (Horiyama et al., 2010). Studies conducted by Horiyama et al. (2010) have

demonstrated that mutated *TolC* genes can increase susceptibility of the organism to infection; this has yet to be repeated. While it has been extremely difficult to attenuate the virulence of any strain of *S.* Newport, the antibiotic resistant *TolC* genes and corresponding receptors has been successfully altered and attenuated (Barak et al. 2011).

Ultimately, the genes chosen for this project are considered imperative for bacterial pathogenesis. By demonstrating that with these attenuations, bacterial growth within the host can still be mimicked, then these attenuated strains may possibly be used in the field to further imitate and demonstrate *Salmonella* persistence and pathogenesis from soil to plant to table.

References

Ahmer, B. M., J. van Reeuwijk, P. R. Watson, T. S. Wallis, and F. Heffron. 1999. *Salmonella* SirA is a global regulator of genes mediating enteropathogenesis. *Mol. Microbiol.* 31:971–82.

Anriany, Y., S. N. Sahu, K. R. Wessels, L. M. McCann, and S. W. Joseph. 2006. Alteration of the rugose phenotype in waaG and ddhC mutants of *Salmonella enterica* serovar Typhimurium DT104 is associated with inverse production of curli and cellulose. *Appl. Environ. Microbiol.* 72:5002–12.

Bakry, M. A., A. S. Hakim, S. A. Nagwa, and M. S. Zaki. 2014. Role played by gene factor in initiation of bacterial antibiotic resistance. *Life Sci. J.* 11:154–160.

Barak, J. D., L. Gorski, P. Naraghi-arani, and A. O. Charkowski. 2005. *Salmonella enterica* virulence genes are required for bacterial attachment to plant tissue. *Appl. Environ. Microbiol.* 71:5685–5691.

Barak, J. D., L. C. Kramer, and L. Hao. 2011. Colonization of tomato plants by *Salmonella enterica* is cultivar dependent, and type 1 trichomes are preferred colonization sites. *Appl. Environ. Microbiol.* 77:498–504.

Barak, J. D., and A. S. Liang. 2008. Role of soil, crop debris, and a plant pathogen in *Salmonella enterica* contamination of tomato plants. *PLoS One* 3:e1657.

Bernstein, N., S. Sela, and S. Neder-Lavon. 2007. Assessment of contamination potential of lettuce by *Salmonella enterica* serovar Newport added to the plant growing medium. *J. Food Prot.* 70:1717–22.

Boonmar, S., a Bangtrakulnonth, S. Pornruangwong, S. Samosornsuk, K. Kaneko, and M. Ogawa. 1998. Significant increase in antibiotic resistance of *Salmonella* isolates from human beings and chicken meat in Thailand. *Vet. Microbiol.* 62:73–80.

Boriss, B. H., and H. Brunke. 2014. Fresh Tomatoes Profile 1–4.

Boriss, H., and H. Brunke. 2005. Commodity profile : tomatoes, fresh market. *Agric. Mark. Resour. Cent.*

Busta, F. F., T. V. Suslow, M. E. Parish, L. R. Beuchat, J. N. Farber, E. H. Garrett, and L. J. Harris. 2003. The use of indicators and surrogate microorganisms for the evaluation of pathogens in fresh and fresh-cut produce. *Compr. Rev. Food Sci. Food Saf.* 2:179–185.

CDC. 2009. National Enteric Disease Surveillance : *Salmonella* annual summary, 2009 2009–2012.

CDC. 2011. National Enteric Disease Surveillance : *Salmonella* surveillance overview 1–12.

CDC. 2013. Reports of selected salmonella outbreak investigations 2013 13–15.

Center for Science in the Public Interest. 2009. Outbreak alert! analyzing foodborne outbreaks 1998 to 2007.

Cooke, F. J., E. J. Threlfall, and J. Wain. 2007. Current trends in the spread and occurence of human salmonellosis: molecular typing and emerging antibiotic resistance, p. 1 – 31. *In* M. Rhen, D. Maskell, P. Pastroeni, and J. Threlfall (eds.), Salmonella: Molecular Biology and Pathogenesis1st ed. horizon bioscience, Norfolk.

Dorman, C. J., T. O. Croinin, and M. W. Mangan. 2007. Virulence gene regulation in salmonella enterica serovar Typhimurium, p. 105 – 126. *In* M. Rhen, D. Maskell, P. Masroeni, and J. Threllfall (eds.), Salmonella: Molecular Biology and Pathogenesis, 1st ed. horizon bioscience, Norfolk.

ERS, and USDA. 2014. United States Department of Agriculture 2–5.

ERSA, USDA, and N. A. F. Market. 2014. United States Department of Agriculture North American fresh tomato market 1–5.

FDA. 2013. FDA's team tomato fights contamination.

FDA/CFSAN. 2001. Analysis and evaluation of preventitive control measures for the control and reduction/elimination of microbial Hazards on Fresh and Fresh-Cut Produce.

Fronzes, R., H. Remaut, and G. Waksman. 2008. Architectures and biogenesis of non-flagellar protein appendages in Gram-negative bacteria. *EMBO J.* 27:2271–80.

Gibson, D. L., a P. White, S. D. Snyder, S. Martin, C. Heiss, P. Azadi, M. Surette, and W. W. Kay. 2006. *Salmonella* produces an O-antigen capsule regulated by gfD and important for environmental persistence. *J. Bacteriol.* 188:7722–30.

Greene, S. K., E. R. Daly, E. a Talbot, L. J. Demma, S. Holzbauer, N. J. Patel, T. a Hill, M. O. Walderhaug, R. M. Hoekstra, M. F. Lynch, and J. a Painter. 2008. Recurrent multistate outbreak of *Salmonella* Newport associated with tomatoes from contaminated fields, 2005. *Epidemiol. Infect.* 136:157–65.

Gu, G., J. M. Cevallos-Cevallos, and A. H. C. van Bruggen. 2013. Ingress of *Salmonella enterica* Typhimurium into tomato leaves through hydathodes. *PLoS One* 8:e53470.

Gu, G., J. Hu, J. M. Cevallos-Cevallos, S. M. Richardson, J. a Bartz, and A. H. C. van Bruggen. 2011. Internal colonization of *Salmonella enterica* serovar Typhimurium in tomato plants. *PLoS One* 6:e27340.

Gualdi, L., L. Tagliabue, S. Bertagnoli, T. Ieranò, C. De Castro, and P. Landini. 2008. Cellulose modulates biofilm formation by counteracting curli-mediated colonization of solid surfaces in Escherichia coli. *Microbiology* 154:2017–24.

Guo, X., J. Chen, R. E. Brackett, and R. Larry. 2001. Survival of Salmonellae on and in tomato plants from the time of inoculation at flowering and early stages of fruit development through fruit ripening survival of salmonellae on and in tomato plants from the time of inoculation at flowering and early stage. *Appl. Environ. Microbiol.* 67:4760 – 4764.

Gurtler, J. B., R. B. Rivera, H. Q. Zhang, and D. J. Geveke. 2010. Selection of surrogate bacteria in place of E. coli O157:H7 and *Salmonella* Typhimurium for pulsed electric field treatment of orange juice. *Int. J. Food Microbiol.* 139:1–8.

Hadjok, C., G. S. Mittal, and K. Warriner. 2008. Inactivation of human pathogens and spoilage bacteria on the surface and internalized within fresh produce by using a combination of ultraviolet light and hydrogen peroxide. *J. Appl. Microbiol.* 104:1014–24.

Hoelzer, K., Y. Soyer, L. D. Rodriguez-Rivera, K. J. Cummings, P. L. McDonough, D. J. Schoonmaker-Bopp, T. P. Root, N. B. Dumas, L. D. Warnick, Y. T. Gröhn, M. Wiedmann, K. N. K. Baker, T. E. Besser, D. D. Hancock, and M. a Davis. 2010. The prevalence of multidrug resistance is higher among bovine than human *Salmonella enterica* serotype Newport, Typhimurium, and 4,5,12:i:- isolates in the United States but differs by serotype and geographic region. *Appl. Environ. Microbiol.* 76:5947–59.

Horiyama, T., A. Yamaguchi, and K. Nishino. 2010. TolC dependency of multidrug efflux systems in *Salmonella enterica* serovar Typhimurium. *J. Antimicrob. Chemother.* 65:1372–6.

Hueck, C. J. 1998. Type III protein secretion systems in bacterial pathogens of animals and plants. *Microbiol Mol Biol Rev.* 62:379–433.

James, J. 2006. Chapter 1: Overview of microbial hazards in fresh fruit and vegetables operations, p. 1–36. *In* Microbial Hazard Identification in Fresh Fruit and Vegetables. John Wiley & Sons, Inc.

Klontz, K. C., J. C. Klontz, R. K. Mody, and R. M. Hoekstra. 2010. Analysis of tomato and jalapeño and serrano pepper imports into the United States from Mexico before and during a national outbreak of *Salmonella* serotype Saintpaul infections in 2008. *J. Food Prot.* 73:1967–74.

Korhonen, T. K. 2007. Adhesins of salmonella and their putative roles in infection. *In* M. Rhen, D. Maskell, P. Mastroeni, and J. Threlfall (eds.), Salmonella: Molecular Biology and Pathogenesis, 1st ed. horizon bioscience, Norfolk.

Lang, M. M. 2004. Evaluation of inoculation method and inoculum drying time. The University of Georgia.

Lynch, M. F., R. V Tauxe, and C. W. Hedberg. 2009. The growing burden of foodborne outbreaks due to contaminated fresh produce: risks and opportunities. *Epidemiol. Infect.* 137:307–15.

Mahmoud, B. S. M. 2010. The effects of x-ray radiation on *Escherichia coli O157:H7*, *Listeria monocytogenes*, *Salmonella enterica* and *Shigella flexneri* inoculated on whole Roma tomatoes. *Food Microbiol.* 27:1057–63.

Martínez, J. L., and F. Baquero. 2002. Interactions among strategies associated with bacterial infection : pathogenicity , epidemicity , and antibiotic resistance interactions among strategies associated with bacterial infection : Pathogenicity , Epidemicity , and Antibiotic Resistance † 15.

Marvasi, M., C. E. Cox, Y. Xu, J. T. Noel, J. J. Giovannoni, and M. Teplitski. Differential regulation of *Salmonella* Typhimurium genes involved in O-antigen capsule production and their role in persistence within tomatoes 1–25.

Meng, J., and M. P. Doyle. 2002. Introduction to Microbiological Food Safety, p. 395 – 397. *In* Introduction. Microbiological Food Safety.

Montville, T. J., and K. R. Matthews. 2008. Food microbiology - an introduction, 2nd ed. American Society for Microbiology.

Morgan, E. 2007. Salmonella pathogenicity islands, p. 67 – 88. *In* M. Rhen, D. Maskell, P. Mastroeni, and J. Threlfall (eds.), Salmonella: Molecular Biology and Pathogenesis, 2nd ed. horizon bioscience, Norfolk, UK.

Network, S., U. S. Sites, S. M. Crim, M. Iwamoto, J. Y. Huang, P. M. Griffin, A. B. Cronquist, M. Cartter, M. T. Angelo, D. Blythe, K. Smith, S. Lathrop, S. Zansky, P. R. Cieslak, J. Dunn, K. G. Holt, S. Lance, R. Tauxe, O. L. Henao, and P. Author. 2014. Incidence and trends of infection with pathogens transmitted commonly through food – foodborne diseases active 2013:2006–2013.

Noel, J. T., N. Arrach, A. Alagely, M. McClelland, and M. Teplitski. 2010. Specific responses of Salmonella enterica to tomato varieties and fruit ripeness identified by in vivo expression technology. *PLoS One* 5:e12406.

Painter, J. A., R. M. Hoekstra, T. Ayers, R. V Tauxe, C. R. Braden, F. J. Angulo, and P. M. Griffin. 2013. Attribution of foodborne illnesses , hospitalizations , and deaths to food commodities by using outbreak 19:407–415.

Parvathi, A., J. Vijayan, G. Murali, and P. Chandran. 2011. Comparative virulence genotyping and antimicrobial susceptibility profiling of environmental and clinical Salmonella enterica from Cochin, India. *Curr. Microbiol.* 62:21–6.

Poole, K. 2002. Mechanisms of bacterial biocide and antibiotic resistance. *Symp. Ser. Soc. Appl. Microbiol.* 55S–64S.

Rhen, M., D. Maskell, P. Mastroeni, and J. Threlfall. 2007. Salmonella: Molecular Biology and Pathogensis, 2nd ed. horizon bioscience, Norfolk, UK.

Römling, U., W. D. Sierralta, K. Eriksson, and S. Normark. 1998. Multicellular and aggregative behaviour of Salmonella Typhimurium strains is controlled by mutations in the agfD promoter. *Mol. Microbiol.* 28:249–64.

Saeed, A., and R. Naji. 2007. Infectious agents, p. 18 – 39. *In* T. Lask (ed.), Epidemiological Principles and Food Safety. Oxford University.

Scallan, E., R. M. Hoekstra, F. J. Angulo, R. V. Tauxe, M.-A. Widdowson, S. L. Roy, J. L. Jones, and P. M. Griffin. 2011. Foodborne illness acquired in the United States—major pathogens. *Emerg. Infect. Dis.* 17:7–15.

Seale, J. L., L. Zhang, and M. R. Traboulsi. 2013. U . S . import demand and supply response for fresh tomatoes, cantaloupes, onions, oranges, and spinach 3:435–452.

Sivapalasingam, S., C. R. Friedman, L. Cohen, and R. V. Tauxe. 2004. Fresh produce: a growing cause of outbraeks of foodborne illness in the United States, 1973 through 1997. *J. Food Prot.* 67:2342.

Stegmeier, J. F., G. Polleichtner, N. Brandes, C. Hotz, and C. Andersen. 2006. Importance of the adaptor (membrane fusion) protein hairpin domain for the functionality of multidrug efflux pumps. *Biochemistry* 45:10303–12.

Teplitski, M., A. Al-Agely, and B. M. M. Ahmer. 2006. Contribution of the SirA regulon to biofilm formation in Salmonella enterica serovar Typhimurium. *Microbiology* 152:3411–24.

Teplitski, M., R. I. Goodier, and B. M. M. Ahmer. 2003. Pathways leading from BarA / SirA to Motility and virulence gene expression in Salmonella. *J. Bacteriol.* 185:7257–7265.

Tezcan-Merdol, D., S. E. Ygberg, and M. W. Mangan. 2007. The *Salmonella enterica* virulence plasmid and the spv gene cluster, p. 89 – 104. *In* M. Rhen, D. Maskell, P. Mastroeni, and E.J. Threlfall (eds.), Salmonella: Molecular Biology and Pathogenesis, 2nd ed. horizon bioscience, Norfolk, UK.

Townsend, S. M., N. E. Kramer, R. Edwards, S. Baker, N. Hamlin, M. Simmonds, K. I. M. Stevens, S. Maloy, J. Parkhill, and G. Dougan. 2001. *Salmonella enterica* serovar Typhi possesses a unique repertoire of fimbrial gene sequences 69:2894–2901.

Wang, H., V. S. Gill, K. a. Irvin, M. Byrd, C. M. Bolger, J. Zheng, E. E. Dickey, R. E. Duvall, A. P. Jacobson, and T. S. Hammack. 2012. Recovery of *Salmonella* from internally and

externally Contaminated whole tomatoes using several different sample preparation procedures. *J. AOAC Int.* 95:1452–1456.

Zaragoza, W. J., J. T. Noel, and M. Teplitski. 2012. Spontaneous non-rdar mutations increase fitness of Salmonella in plants. *Environ. Microbiol. Rep.* 4:453–458.

Zgurskaya, H. I., and H. Nikaido. 2000. Multidrug resistance mechanisms: drug efflux across two membranes. *Mol. Microbiol.* 37:219–25.

Zheng, J., S. Allard, S. Reynolds, P. Millner, G. Arce, R. J. Blodgett, and E. W. Brown. 2013. Colonization and internalization of *Salmonella enterica* in tomato plants. *Appl. Environ. Microbiol.* 79:2494–502.

Zhuang, R. Y., L. R. Beuchat, and F. J. Angulo. 1995. Fate of *Salmonella* Montevideo on and in raw tomatoes as affected by temperature and treatment with chlorine. *Appl. Environ. Microbiol.* 61:2127–31.

Zogaj, X., M. Nimtz, M. Rohde, W. Bokranz, and U. Römling. 2001. The multicellular morphotypes of *Salmonella* Typhimurium and *Escherichia coli* produce cellulose as the second component of the extracellular matrix. *Mol. Microbiol.* 39:1452–63.

Chapter 3

Survival and Growth of Attenuated *Salmonella enterica* Serovars Newport and Typhimurium in Growth Media and Tomatoes

L. Yang[1], R. R. Boyer[1], G. Gu[2], R. C. Williams[1], M. Clarke[1], S. Rideout[2]

[1]Virginia Polytechnic Institute and State University, Department of Food Science and Technology, 22 Duck Pond Drive, Blacksburg, VA 24061 USA

[2] Virginia Polytechnic Institute and State University, Eastern Shore Agricultural Research and Extension, Department of Plant Pathology, Physiology, and Weed Sciences, 33446 Research Drive, Painter, VA 23420 USA

Keywords: *Salmonella enterica*, Typhimurium, Newport, tomato(es), attenuated, media

ABSTRACT

Fresh market tomatoes have been associated with 15 multistate *Salmonella* outbreaks between 1973 and 2010. While, *S. enterica* survival has been studied in tomato plants, field studies have been limited. To understand pathogen growth and survival, in crop fields, surrogate or attenuated organisms must be developed and validated. The purpose of this study was to compare the growth and survival of seven attenuated *S. enterica* Typhimurium and Newport strains against virulent strains *S.* Typhimurium ATCC14028 and *S.* Newport J1892 in optimum (TSB and TSB+kan) and minimal M9 growth media, and in commercial, red ripe tomatoes. Bacterial growth in media was assessed via BioScreen. Tomatoes were separately inoculated with 7 Log CFU/g of each isolate via vacuum infiltration, surface spot inoculation, or diced inoculation. Populations of each strain were determined on Days 0, 1, 3, and 5. In media, there were few differences in overall growth and growth rates between mutant isolates and wild-type ($P<0.05$). Growth in M9 was less ($P<0.01$), while growth rates were higher ($P<0.01$) than in TSB. In tomatoes (per treatment), there were no significant differences between growth rates of each isolate compared to WT ($P>0.05$); however, S*almonella* strains in diced tomatoes had a higher growth rate than that in spot treated tomatoes ($P>0.05$). The growths of all the isolates in tomatoes indicated that under the tested conditions, isolates acted similarly to their WT counterparts. Thus, these strains may be able to be used as surrogate organisms in field studies.

INTRODUCTION

There are an estimated 9.4 million cases of foodborne illnesses annually in the United States. *Salmonella enterica* is the leading bacterium associated with these illnesses, accounting for 1.2 million bacterial foodborne illnesses each year (Scallan et al., 2011). Recently, there has been an increasing trend of *Salmonella enterica spp.* outbreaks associated with fresh produce (CDC, 2009; Lynch et al., 2009). *Salmonella* linked contamination of raw tomatoes has been attributed to 15 multistate outbreaks between 1973 through 2010; 12 outbreaks having occurred since 2000 (FDA, 2013). Tomatoes are the second-highest value market vegetable, and the "fourth most popular fresh-market vegetable" (Bernstein, 2007; Boriss et al., 2005; CSPI, 2007; USDA ERS, 2014). Many tomato recalls are still due to the possibility of *Salmonella enterica* contamination (FDA, 2014). Two of the most common *S. enterica* serovars associated with tomato outbreaks are Typhimurium and Newport, (Zheng et al., 2013).

Produce can become contaminated through many pathways. Contamination may occur from soil, water, handling, processing, or may even be inherited from each flowering generation (Zheng, 2013). The association of foodborne pathogens, including *Salmonella*, with tomatoes and tomato plants is a very complex process influenced by a multitude of factors including temperature, pH, soil type, plant cultivar, plant maturity, and even serovar type (Zhuang et al, 1995; Barak et al., 2008; Gu et al., 2013; Gu et al., 2011; Gu et al., 2008; Zheng et al., 2013). In recent years, partially due to increased numbers of (multi-drug) antibiotic resistant *Salmonella* serovars, the need to identify and understand *Salmonella* invasion and colonization of fresh produce from farm-to-table has become more pronounced.

Due to the virulent nature of these pathogens, research must be confined to a controlled Biosafety Level 2 research laboratory. This limitation makes it difficult to transfer discovery to real world situations in the field or in the processing plant unless otherwise quarantined. One approach for overcoming this obstacle is to use indicator or non-virulent, attenuated surrogate organisms "to imitate the survival of microbial pathogens in fresh produce" instead of the virulent pathogen (James et al., 2006).

Attenuated isolates of the clinically virulent *S.* Typhimurium ATCC 14028 and field-isolated virulent *S.* Newport J1892 have been produced through point mutations or deletions of virulence-associated genes and have been previously described (Noel et al., 2010). The virulence pathogenicity of *Salmonella* can be found in a collection of chromosomal pathogenicity islands, genomic islands, and virulence plasmids (Rhen et al., 2007). The *pSLT* virulence plasmid is often associated with virulence and antibiotic resistance (Montville & Matthews, 2008). The agf operons found in a common ancestor of both *Salmonella* and *Escherichia coli* (Fronzes et al., 2008; Townsend et al., 2001) is also extensively studied with two divergent operons, the agfBAC complex and the agfD complex. The *agfB* and *agfC* genes, controlled by the agfBAC operon, contribute specifically to fimbrial curli production, inflammation response within the host, and biofilm formation. Curli's presence up-regulates the pro-inflammatory response in mammalian hosts leading to extensive bleeding and septic shock. Conversely, the *agfD* complex gives rise to the *bcs* genes functioning in attachment, cellulose production, and biofilm formation to increase persistence of *Salmonella* within its host, and increase the pathogen's tolerance to environmental stresses (Zogaj et al., 2001; Gualdi, 2008). The *agfD* complex also positively affects the *yih* operons leading to *yihT* and *yihT-ompL* gene expressions associated with encoding aldolase in O-antigen capsule synthesis and translocation, while positively affecting pathogen attachment onto

plant tissue with aggregative fimbriae (Gibson et al., 2006; Zaragoza et al., 2012; Marvasi et al., 2013). It has been discovered that the *TolC* genes encode for an outer membrane channel contributing to antibiotic resistance as multidrug efflux pumps by simply excluding and displacing antibiotics, antimicrobials, and proteins from within the cell (Horiyama et al., 2010; Barak et al., 2011).

The previously described attenuated isolates will be examined further in this study. The attenuated strains created in Noel et al. (2010) will be evaluated to determine whether any would be a suitable surrogate for use in field studies. The purpose of this study was to specifically assess whether the growth and persistence of these attenuated *Salmonella* isolates are comparable to wild-type *Salmonella* Typhimurium and Newport *in vitro (*in nutrient-rich and minimal media), and *in vivo* (in imported red tomatoes). By demonstrating that with these attenuations, bacterial growth within the host can still be mimicked, these attenuated strains may possibly be used in the field to further imitate and demonstrate *Salmonella* persistence and pathogenesis from soil to plant to table.

MATERIALS AND METHODS

Bacterial Strains

Nine strains of *Salmonella enterica* serovars Typhimurium and Newport were used for this experiment. All isolates are described in Table 1. Briefly, two control strains, *S.* Typhimurium wild-type (WT) (ATCC 14028) and *S.* Newport WT (J1892; isolated from tomato-related outbreak) were used. Six mutated *S.* Typhimurium (ATCC 14028) strains (described by Noel et al., 2010); BA770 (*14028 pSLT-*); JSG1748 (*10428Δbcs::kan*); TIM2260 (*14028ΔagfB36::kan*); TIM2261 (*14028ΔagfC37::kan*); TIM2262 (*14028ΔompL19::kan*); TIM2263 (*14028ΔyihT27::kan*) (obtained from Dr. Max Teplitski from the University of Florida); and one attenuated *S.* Newport strain #17 (SeN*ΔtolC::kan*) obtained from Dr. Jie Zheng at the FDA. *S.* Newport strain #17 (*ΔtolC::kan*) were used. Previously, 50 parts per million kanamycin (kan) resistance was conferred onto strains JSG1748, TI2260, TIM2261, TIM2262, TM2263, and SeN #17.

Stored cultures were activated at 36ºC in Tryptic Soy Broth (TSB; BD and Company, Sparks, MD) or TSB + 50ppm kan (TSB+kan; Sigma-Aldrich, Saint Louis, MO) if kanamycin resistant. Each isolate was transferred consecutively into TSB or TSB+kan and also t-streaked three times onto Tryptic Soy Agar (TSA; BD and Company, Sparks, MD) or TSA + 50ppm kan (TSA+kan). Colonies from TSA or TSA+kan were then t-streaked and incubated at 36ºC onto Xylose-Lysine-Tergitol 4 (XLT-4; Remel Products, Lenexa, KS) to presumptively confirm genus. Presumptive positive colonies from XLT-4 plates (and from TSA+kan for JSG1748) were then confirmed to be *S. enterica* via biochemical testing with an API 20E Test Kit (bioMérieux, Inc., Durham, NC). Confirmed culture stocks were stored at 4°C and transferred weekly or bi-weekly for the remainder of the experiments. Prior to each experiment, each culture was

activated by transferring into 10 mL TSB or TSB+kan and incubated for 24 hr at 35°C three times consecutively to achieve an actively growing set of cultures

Bioscreen Growth Curve

The growth of each strain was evaluated separately in two types of growth media: nutrient-rich media (TSB and TSB+kan) and minimal media (M9). Activated cultures were centrifuged at 6000 x g RPM for 10 minutes at 5°C, the supernatant was decanted, and cells were resuspended in 0.1% PW. Cells were washed twice more and resuspended in 0.1% PW. Washed cells were then serially diluted 1:10 in 0.1% PW. An aliquot of washed cells were then inoculated into fresh TSB, TSB+kan or M9 in Bioscreen honeycomb microwell plate (Bioscreen, Growth Curves, Piscataway, NJ) to achieve a final inoculation of 100 cells/well (200 μL final volume/well). Five wells were inoculated with each treatment (strain/media combination). Positive controls were the growth of both WT strains, and negative controls were wells of growth medium that were uninoculated,

The plates were read in the Bioscreen C (Growth Curves USA, Piscataway, NJ) at 420 – 540 nm wavelengths with readings taken every 15 minutes, with a 10 second shaking period prior to read for 48 hours (TSB and TSB+kan) or 72 hours (M9) at 36°C. Growth curve data was generated by the EZExperiment Software (Growth Curves, Piscataway, NJ). Three repetitions were conducted (n=15).

Inoculation and Treatment of Tomatoes

Whole tomatoes (cultivar Red Beef tomatoes imported from Mexico; 256.83 ± 0.96 g; pH 4.38±0.05) were obtained no more than 24 hrs prior to use from a Kroger grocery store in Blacksburg, VA. Tomatoes were stored at room temperature 22°C (±3°C) overnight. Directly prior to treatment on the same day, tomatoes were sprayed with a 70% ethanol solution to reduce natural microbial load. Tomatoes were then thoroughly rinsed with sterile deionized water to remove any remaining ethanol and dirt particles. Tomatoes were placed into a laminar flow hood and allowed to air dry (t = 30 minutes).

Each *Salmonella enterica* strain was inoculated in two types of tomatoes (diced or whole). Whole tomatoes were either spot or vacuum inoculated. Therefore, three treatments were examined (1. diced, 2. whole spot, or 3. whole vacuum). For each treatment, tomatoes were inoculated with 100 µL of 8 log CFU/mL inoculum. The positive controls were tomatoes inoculated with *S.* Typhimurium ATCC #14028 and tomatoes inoculated with *S.* Newport J1892. The negative controls were tomatoes inoculated with sterile 0.1% PW.

Whole Vacuum Inoculated Tomatoes

Each tomato was inoculated at the stem scar with 100 µL of 8 log CFU/mL *S. enterica* strain (one tomato per strain). Tomatoes were immediately placed in the vacuum chamber and subjected to 0.01MPa of pressure for two minutes. The pressure was released to allow equilibration to atmospheric pressure. The vacuum-release cycle was repeated twice more for a total of three consecutive exposures to vacuum (t = 2 min per exposure). Finally, the tomatoes

were placed into individual sterile Whirl-Pak filtered stomacher bags (Whirl-Pak, Nasco, Ft. Atkinson, WI), sealed, weighed, then incubated at 22°C (±3°C) to mimic counter-top storage.

Whole Spot Inoculated Tomatoes

Tomatoes were spot inoculated by dispensing 100 µL of 8 log CFU/mL *S. enterica* strain (one tomato per strain) in small drops within a 3 cm^2 area around the blossom scar (10 – 15 drops). Inoculum was allowed to dry (t = 20 minutes) before tomatoes were placed into individual sterile Whirl-Pak filtered stomacher bags, sealed, weighed, then incubated at 22°C (±3°) to mimic counter-top storage.

Diced Tomatoes

Tomatoes were diced with a 70% ethanol cleaned knife into 1 x 1 x 1 cm cubes. 150 grams were weighed and placed into separate Whirl-Pak bags. Inoculum was added directly to the bag by dispensing 100 µL of 8 log CFU/mL *S. enterica* strain (one strain per bag). Bags were sealed, and tomatoes were stored at 5°C (±0.5°C) to mimic refrigerator storage of cut tomatoes.

The populations of each strain on the tomatoes were evaluated on Days 0, 1, 3, and 5. Three tomatoes per strain inoculated per day were tested on each day. The spot inoculation and vacuum inoculation treatments were both repeated three times each (n=9), and the diced tomato inoculation treatment was repeated four times (n=12).

Microbial Analysis of Tomatoes Samples

On each sampling day, tomatoes were weighed, manually crushed, and homogenized in a stomacher (Easy Mix, AES Laboratories, Princeton) for two minutes. Tomato homogenates were diluted into 0.1%PW, and samples were spread plated in duplicate onto XLT-4, XLT-4+kan, TSA, and/or TSA+kan plates. *S.* Typhimurium ATCC 14028, *S.* Newport J1892, and *S.* Typhimurium BA770 were plated on XLT-4. All kanamycin resistant strains – excluding *S.* Newport #17 isolate – were plated on XLT-4+kan; SeN#17 was plated on TSA+kan because its attenuation had disabled growth on XLT-4. Negative control samples were plated in duplicate on all four types of plates. All plates were incubated for 48 hr at 35°C. The growth of the nine *Salmonella* strains in tomatoes for the three *in vivo* treatments was performed in triplicate on four separate days. XLT-4 and XLT-4+kan plates were counted for *Salmonella* colony formation characterized by black colonies. Due to the addition of kanamycin antibiotic to TSA, white-yellow colonies growing on TSA+kan plates were assumed to be SeN#17 colonies.

Statistical Analysis

The growth of the nine *Salmonella* strains in nutrient rich and minimal media was performed in duplicate and triplicate over 48 – 72 hours. Overall growth of strains was calculated using the trapezoidal rule of approximating definite integrals for the area under the curve (AUC). Data collected from the BioScreenC growth curve was analyzed with One-Way and Two-Way ANOVAs, with Tukey's HSD ($P<0.05$) in JMP 10 (SAS Institute Inc., Cary, NC).

Due to varying tomato weights, the tomato weights were normalized to maintain consistency of data among treatments. The linear growth rate (Δ) and exponential growth rate

(μ) constants of all nine *Salmonella* isolates in media were determined from the exponential growth phases of each isolate using growth rate constant equations found in Christian et al. (1982).

Data from inoculation treatments was analyzed using One-Way and Two-Way ANOVAs, with Tukey's HSD ($P<0.05$) in JMP 10. (both one-way and two-way). Growth rates and y-intercepts of population densities were calculated by fitting a linear regression line to averaged growth curve data in Microsoft Excel 2010 (Microsoft, Seattle, WA). Correlation coefficients were determined with Pearson's parametric or Spearman's non-parametric correlation coefficients in JMP 10 (SAS, Cary, NC).

RESULTS

Confirmation of Bacterial Cultures

All *Salmonella* strains were presumptively confirmed on XLD agar (TSA+kan for *S.* Newport isolate SeN#17) and with biochemical API 20E tests. Presumptive confirmation of *Salmonella* on XLD agar was determined by growth of black colonies resulting from H2S utilization. Thereafter, *Salmonella* strains were routinely grown and confirmed on XLT-4, XLT-4+kan, and TSA+kan agar plates. XLT-4 agar plates were used for presumptive confirmation of isolates ATCC 14028, BA770, and SeN J1892. XLT-4+kan plates were used for presumptive confirmation of *S.* Typhimurium strains TIM2260, TIM2261, TIM2262, TIM2263, JSG1748. Positive *Salmonella* growth was indicated by black colonies, sometimes with opaque zones of clearing. Confirmation of the SeN#17 isolate was determined on TSA+kan plates due to its inability to grow normally on XLT-4 agar; growth was indicated by uniform white-yellow colonies. Other bacterial growth was suppressed by the 50 ug/mL kanamycin in TSA. Uninoculated tomato homogenates were plated on TSA, TSA+kan, XLT-4, and XLT-4+kan to assess any background microflora growth on XLT-4 and in the presence of kanamycin; XLT-4 plates and all plates supplemented with kanamycin had no growth, confirming that there were no kanamycin resistant bacterial strains endemic in tomatoes. All *Salmonella* isolates were confirmed in Steve Rideout's lab via PCR amplification of the *invA* gene.

Growth Curve and Survival of Strains in Media (in vitro)

Each *S. enterica* strain was grown separately in three types of media: nutrient rich media (TSB and TSB+kan) and minimal media (M9). The areas under the curve [AUC] and growth

rates (linear and exponential) were calculated for each strain in each media. Figure 1 shows the growth curve of all *Salmonella enterica* strains in (a) TSB, (b) M9, and (c) TSB+kan. The AUC of all *S. enterica* strains in each growth medium (TSB, TSB+kan, and M9) is compared in Table 2, and also shown in Figure 2: (a) TSB, (b) M9, and (c) TSB+kan.

Two-Way ANOVA was performed to analyze the effects of strains and media types (nutrient-rich TSB and minimal media M9) on bacterial growth as well as interactions amongst main factors. The interaction between strain and media type is significantly different ($P<0.01$). Growth between the two media are significantly different from one another ($P<0.01$), with overall growth in TSB greater than M9; however, according to Pearson's correlation coefficient, there is no correlation between TSB media growth and M9 media growth ($r=0.17$; P-value = 0.12) implying that results obtained from both media types would not be comparable.

The strains grew well in nutrient-rich TSB media (Figure 2a). As seen in Table 2, growth of both *Salmonella* wild-type [WT] strains was not significantly different from one another ($P=0.69$). Growths of *S.* Typhimurium mutant isolates BA770, JSG1748, TIM2260, TIM2261, TIM2262, and TIM2263 were not significantly different from the WT ATCC14028 ($P>0.05$). Strains TIM2260, TIM2261, TIM2263, BA770 all grew significantly better than TIM2262 ($P<0.05$), which had the least growth of the *S.* Typhimurium strains (AUC = 3030.12); while JSG1748 and TIM2262 grew similarly ($P=0.93$). TIM2262's growth, however, was not significantly different from the WT ($P>0.05$). Growth of both isolates of *S.* Newport, SeN J1892 and SeN#17 were not significantly different from one another ($P>0.05$).

In minimal M9 media, growth (Figure 2b) in all strains decreased dramatically compared to TSB (AUC = approx. 3000 to approx. 1500). Both *Salmonella* WT strains had significantly different AUCs ($P=0.0026$), with WT isolate ATCC14028 growing significantly better than

isolate SeN J1892 (P<0.10) (Table 2). TIM2263 grew significantly better than the mutants JSG1748, TIM2260, TIM2261, TIM2262 (P<0.01) and ATCC14028 (P<0. 01) isolates. Contrary to growth in TSB, TIM2260 and TIM2261 grew worse than TIM2262 (P=0.10) in M9 media. Unlike in TSB or TSB+kan, SeN#17 grew significantly better than *S.* Typhimurium TIM2261 (P<0.05) in M9. SeN#17 (AUC = 1692.24) also grew significantly better than its wild-type counterpart SeN J1892 (AUC = 1307.96; P<0.01), demonstrating that there is a difference in growth between mutant strains and wild-type strains. All other isolates of *S.* Typhimurium were not significantly different from wild-type ATCC14028, except for TIM2263 (P<0.01).

Two-Way ANOVA was also performed on overall strains in two similar media types (TSB and TSB+kan) to compare specific differences between kanamycin-resistant specific strains. The interactions between the media on the strains were significantly different (P<0.01). Growth of JSG1748, TIM2260, TIM2261, and TIM2263 in both media was similar (P=1.00). In media, these strains grew significantly better than isolate TIM2262 (P<0.01). SeN#17 grew similarly to JSG1748 (P=0.58) and TIM2262 (P=0.14); but grew significantly less than TIM2263 (P<0.01), TIM2260 (P<0.01), TIM2261 (P<0.01). The analysis indicated there was a slight correlation between both AUCs (r = 0.2105; P=0.02).

In nutrient-rich TSB+kan media (Figure 2c), none of the WT isolates nor BA770 grew which was expected since none were kanamycin resistant; AUCs of all WT isolates were not significantly different from each other (P=1.00). Comparison in TSB+kan media was between kanamycin resistant strains. Again, *S.* Typhimurium strain TIM2262 (AUC=3020.19) grew significantly less than all the other *S.* Typhimurium strains, including TIM2260, TIM2261, TIM2263, and JSG1748. (P<0.05). Growing very similarly, isolates TIM2260, TIM2261, and TIM2263 had the highest levels of growth. On the other hand, as with TSB media, SeN#17

(AUC = 3066.66) and TIM2262 (AUC = 3020.19) grew similarly; however, both grew significantly less than other *Salmonella* strains ($P<0.01$).

Linear and Exponential Growth Rates of Strains in Media (in vitro)

The linear growth rate (Δ) and exponential growth rate (μ) of all nine *Salmonella* isolates in media were determined from the exponential growth phases of each isolate using equations found in Christian et al., 1982. Growth rates are depicted in Table 3: A) Linear Growth Rate, B) Exponential Growth Rate.

Two-Way ANOVA was performed to analyze the effects of strains and media types (nutrient-rich TSB and minimal media M9) on growth rates as well as interaction amongst main factors. The interaction between strain and media type for linear ($P<0.03$) and exponential ($P<0.02$) growth rates are significantly different. The linear growth rate between the two media was not significantly different ($P=0.11$); whereas, the exponential growth rate between the two media was significant ($P<0.01$), with growth rate in M9 greater than in TSB. According to Pearson's correlation coefficient, there are no correlations between TSB media linear and exponential growth rates, and M9 media linear and exponential growth rates (linear: $r = -0.26$; P-value = 0.31; exponential: $r = -0.33$; P-value = 0.19) implying that results obtained from both media types would not be comparable.

The linear and exponential growth rates of *Salmonella* strains in TSB were noticeable. As seen in Table 3, the linear and exponential growth rates of both *Salmonella* wild-type [WT] strains were not significantly differently from one another ($P = 0.6$; $P = 0.10$). The linear growth rate of *S.* Typhimurium mutant isolates BA770, JSG1748, TIM2260, TIM2261, and TIM2263 was not significantly different from WT ATCC14028 ($P>0.05$) (Table 3a). TIM2260 had the

greatest linear growth rate (Δ = 0.50±0.03), while TIM2262 had the slowest growth (Δ = 0.30±0.03); the two isolates had significantly different linear growth rates ($P<0.01$). Similarly, seen in Table 3b, *S.* Typhimurium mutant isolates BA770, JSG1748, TIM2260, TIM2261, and TIM2263 grew at similar exponential rates as ATCC14028, which grew the fastest (μ = 0.16±0.01; $P>0.1$). As with the linear growth rate, TIM2262 also had the lowest exponential growth rate (μ = 0.09±0.01), and grew at a significantly lower rate than WT ATCC14028 ($P<0.01$). Its growth rate was similar to JSG1748 (μ = 0.11±0.01), TIM2261 (μ = 0.12±0.01), TIM2263 (μ = 0.12±0.01), and SeN#17 (μ = 0.10±0.01) (Table 3b, $P>0.11$). Linear and exponential growth rates of *S.* Newport isolates SeN J1892 (Δ = 0.45±0.03; μ = 0.13±0.01) and SeN#17 (Δ = 0.39±0.03; μ = 0.11±0.01) were not significantly different ($P=0.76$; $P=0.31$).

Growth rates in minimal M9 media, observed in Table 3, were greater than in TSB for both linear growth and exponential growth. Linear growth rate was higher in M9 media at $\Delta \approx$ 0.47±0.02, compared to TSB's linear growth rate at $\Delta \approx$ 0.43±0.01. The exponential growth rate increased significantly from $\mu \approx$ 0.12±0.01 (TSB) to $\mu \approx$ 0.18±0.01 (M9) ($P<0.01$). However, linear and exponential growth rates of *Salmonella* strains in media were not significantly different from each other nor the WT isolates ($P>0.12$). *S.* Typhimurium isolate growth rates were not significantly different from ATCC14028 (Δ = 0.54±0.09; μ = 0.21±0.03; $P>0.21$). In M9 media, TIM2261 had the lowest overall growth; this also corresponded with the lowest growth rates (Δ = 0.24±0.09; μ = 0.08±0.03). SeN J1892 (Δ = 0.47±0.09; μ = 0.20±0.03) and SeN#17 (Δ = 0.46±0.09; μ = 0.18±0.03) also had similar growth rates and were not significantly different ($P<0.10$).

Two-Way ANOVA of growth rates was also performed on strains in two similar media types (TSB and TSB+kan) to compare specific differences between kanamycin resistant specific

strains. The interactions between the media on the growth rates of the strains were significantly different ($P<0.01$). Similar to the overall growth of strains, in nutrient-rich TSB+kan media, none of the WT isolates nor BA770 grew. Growth rates of all WT isolates were not significantly different from each other ($P=1.00$). Comparison in TSB+kan media was between ability to grow of kan-resistant strains, and thereby determine the growth rate of strains. Linear and exponential growth rates of TIM2260, TIM2261, and TIM2263 in both media were not significantly different ($P>0.05$). The fastest growing kanamycin-resistant isolates were TIM2263 ($\Delta = 0.50\pm0.02$; $\mu = 0.14\pm0.00$) and TIM2260 ($\Delta = 0.44\pm0.02$; $\mu = 0.10\pm0.00$), followed by SeN#17 ($\Delta = 0.40\pm0.02$; $\mu = 0.11\pm0.00$). In both instances, TIM2262 not only had the lowest growth rate, but also was statistically different from both TIM2263 ($P<0.01$) and TIM2260 ($\Delta = 0.31\pm0.02$; $\mu = 0.09\pm0.00$; $P<0.01$). SeN#17 had a comparable linear growth rate to all *S.* Typhimurium isolates, including TIM2261 ($P=0.99$), JSG1748 ($P=0.88$), and TIM2262 (0.07). SeN#17 had a significantly lower exponential growth rate than TIM2263 ($P<0.01$), but was otherwise similar to JSG1748 ($P=0.97$), TIM2260 ($P=0.49$), TIM2261 ($P=1.00$), and TIM2262 ($P=0.09$). There are no correlations between TSB and TSB+kan media linear and exponential growth rates. (Linear: $r = -0.12$; P-value = 0.57; Exponential: $r = -0.38$; P-value = 0.06) implying that results obtained from both media types would not be comparable.

Growth Rate of Salmonella Strains in Tomato

The survival of each strain in three types of tomatoes was evaluated: diced inoculation, vacuum inoculation, and spot inoculation. The growth rates and y-intercepts of *Salmonella* isolates were calculated and compared (Table 4). Figure 3 shows the growth curves of all *Salmonella enterica* isolates in tomatoes during various treatments (a) diced inoculation, (b) spot

inoculation, and (c) vacuum inoculation. Growth rates and y-intercepts of all *Salmonella* isolates – except SeN#17 – are comparable because enumeration was performed on XLT-4 or XLT-4+kan plates; SeN#17, however, was enumerated on TSA+kan plates. Due to this plate difference, the recovery rate of SeN#17 may actually be very different than isolates grown on XLT-4 and XLT-4+kan plates. Despite the differences, for this project, all isolates will be treated equally.

A two-way ANOVA to determine the interactions among the growth rates for the three treatments and nine strains was analyzed. There were no interactions between strain and inoculation methods ($n=17$; $P>0.88$). However, there was a difference in the growth rates among treatments ($n=3$; $P=0.0058$). The diced treatment growth rate ($n=34$, 0.82 ± 0.07) was significantly higher than spot treatment growth rate ($n=27$; 0.49 ± 0.62; $P>0.05$). Neither was significantly different from the vacuum treatment growth rate ($n=27$; 0.62 ± 0.62; $P=0.13$ against diced, and $P=0.42$ against spot). Shown in Table 4, there were no significant differences among the growth rate of the strains in each treatment ($n=9$; $P>0.23$). However, in vacuum treatment, and spot treatment, TIM2261 did have lower growth rates than the other strains (vacuum: 0.53 ± 0.08; spot: 0.33 ± 0.20).

Another two-way ANOVA was used to determine the y-intercept interactions of the strains and treatments to ascertain that all strains were not only inoculated similarly, but to determine their initial growth. There was no significant interaction between the strain and inoculation method ($n=17$; $P=0.47$). Differences in y-intercepts among the strains were observed ($n=9$; $P=0.01$). In fact, SeN#17 had the highest y-intercept of all the strains ($b=3.93$); which is significantly higher than its wild type strain SeN J1892 ($b=3.93\pm0.19$; $P=0.01$). In fact, this difference is significantly observable in a greater y-intercept of diced inoculation SeN#17

(b=4.64±0.28; P=0.04). Mostly, the y-intercepts for all other *S.* Typhimurium isolates between strains were not significantly different from ATCC14028. In diced tomatoes, isolate TIM2260 (2.81±0.28) was significantly different from TIM2262 (4.20±0.28; $P<0.05$). The y-intercepts were also significantly different between diced, spot, and vacuum inoculation treatments (n=3; $P<0.0001$). Vacuum treatment saw the greatest y-intercept (n=27; b=4.01±0.11) difference and was significantly greater than both diced treatment (n=35; 3.43±0.10; P=0.0015), and spot treatment (n=27; 1.89±0.12; $P<0.01$). The y-intercept for diced inoculation was also significantly higher than spot inoculation ($P<0.01$).

Spearman's non-parametric correlation was conducted to determine correlation of growth rates, and y-intercepts among different inoculation treatments. Spearman's non-parametric correlation (ρ) of growth rate indicated that there were no correlations between any of the growth rates and treatments: between spot inoculation and diced inoculation ($\rho = 0.30$; P=0.14); vacuum inoculation and diced inoculation ($\rho = -0.08$; P=0.71); and vacuum inoculation and spot inoculation ($\rho = -0.0037$; P=0.99). Additionally, no correlations were found between y-intercepts and inoculation methods, either: spot treatment and diced treatment ($\rho = -0.23$; $P = 0.26$); vacuum treatment and diced treatment ($\rho = -0.1289$; P=0.5302); and vacuum treatment and spot treatment ($\rho = 0.08$; P=0.69).

DISCUSSION

Prior to this study, much of the research pertaining to *Salmonella* contamination of tomatoes, with wild-type isolates and also mutant strains, had been conducted on the tomato fruit or the tomato plant. This project is the first to measure and observe the differences of specific attenuated *Salmonella* isolates in different growth media in different growth media. Additionally, this project contributes and affirms results previously obtained regarding these strains and other tomato-related projects (Gu et al., 2011; Gu et al., 2013; Noel at al., 2010; Zheng et al., 2013).

Salmonella isolates were grown in different media to determine their ability to grow since the media could be cursory representatives of substrate. Nutrient-rich TSB promotes the growth of *Salmonella*, while minimal media M9 mimics the real-life scenarios of nutrient depravation, or the natural environmental reservoirs that *Salmonella* could be found in. Significant interactions between TSB and M9 media on strains, demonstrates the fitness and utilization of media by isolates. The lack of correlation between both growth and strain type, and growth rate and strain type, was not expected because there is correlation between the two types of nutrient-rich broths. The results may have been due to human error and may need to be examined further.

As cursorily demonstrated by Noel et al. (2010) in tomatoes, bacterial growth in nutrient rich TSB was not significantly different between the various *S.* Typhimurium mutant isolates evaluated in this study and wild-type ATCC14028. In addition, the growth rate constants of the strains were not too dissimilar from one another and the wild-type *S.* Typhimurium. In fact, isolates TIM2260, TIM2261, and TIM2263, while demonstrating the most significant growth, also had the fastest growths and highest growth rate constants. This reinforces previous studies establishing that the genes responsible for virulence are specifically interactive within a tomato host and the genes are independent of *Salmonella* growth and survival (Noel et al., 2010; Barak

et al, 2005). However, TIM2262 (Δ*ompL*), while not significantly different from the WT, had a lower overall growth than the other mutant strains. With its overall lower growth, TIM2262 also demonstrated a much lower linear growth constant. In a previous study, TIM2262, while not significantly different from WT ATCC14028, also displayed a decreased competitive fitness in nutrient-rich media (Noel et al., 2010). SeN#17's overall growth and growth rate was similar to *S.* Newport J1892 in TSB, which could indicate that the isolate's lack of efflux pumps did not significantly impact how well the bacteria grew. Additionally, it is likely that growth rate can positively predict the overall growth of *Salmonella*.

Overall growth in minimal media was significantly lower than for growth in nutrient rich media. An earlier study looking at the effects of minimal media utilization with varying glucose concentrations of *Salmonella* ATCC14028 demonstrated higher levels of growth and increased growth rates with the addition of more nutrients (Burge et al., 1987). As observed in Figure 1B, not only is the lag phase longer in minimal media, the exponential phase, too, is considerably shorter (Burge et al., 1987). Despite Burge et al.'s study demonstrating a positive correlation between higher nutrient concentrations with higher growth rates, exponential growth rate constants in minimal M9 media were significantly higher than in nutrient-rich TSB, while linear growth rate constants were similar to nutrient-rich TSB. This may be due to the organism's ability to quickly adapt to a natural environment given the constraints; additionally, this further supports the theory that the mutated genes do not affect the ability of *Salmonella spp.* to growth and persist. Conversely, this difference should be studied further.

Of the *S.* Typhimurium isolates, the AUC value of TIM2263 in M9 is significantly higher than that of JSG1748, TIM2260, TIM2261, TIM2262, and wild-type ATCC14028. Although the *agfD* genes control both TIM2262 and TIM2263, TIM2262 may have experienced diminished

growth because mutating the entire *ompL* gene may have triggered a larger survival affect than mutating only the TIM2263 *yihT* gene, which is located upstream. The lower AUC values of TIM2262 may also indicate that the *ompL* gene does play a greater role in basic survival, and mutated *yihT-ompL Salmonella* isolates may be slightly disadvantaged in normal environmental reservoirs. As demonstrated in this study and in other tomato studies, mutants "lacking the entire *yihT-ompL* operon persisted similarly to WT inside red ripe tomatoes"; however, it's expression in green tomatoes results in pathogenesis and persistence (Noel et al., 2010; Teplitski et al., 2012; Zaragoza et al., 2012). As such, this is only pertinent to tomato persistence through aggregative fimbriae and capsule biosynthesis capabilities for plant surfaces, but may be affected in media growth. Additionally, the AUC and growth rates of isolate TIM2261 in M9 media were observably lower than in nutrient-rich TSB. Previous research data has also indicated that while the competitive fitness is lowered in *agfB* and *agfC* mutated genes in nutrient rich media, it was, as seen, not significantly different (Noel et al., 2010). However, if places in an environmental reservoir, it is likely that these genes which would normally code for biofilm formation, curli production, and inflammation has a diminished function, and that these genes are also necessary for survival in stressful conditions.

SeN#17's ability to survive significantly better in media than WT SeN J1892 may be affected by its mutation. SeN#17's mutation occurs in the deletion of its efflux pumps, perhaps due to the inability of its pumps, the isolate is instead able to utilize all the nutrients available within the M9 media, versus its WT SeN J1892 that would be able to discriminate between nutrients available.

Finally, comparison of fitness for attenuated strains is imperative to observe minute differences. The interactions between both types of media and strains were significantly different

due to many of the strains not having kanamycin resistance. As indicated previously in kanamycin resistant isolates, JSG1748, TIM2260, TIM2261, and TIM2263 not only had good overall growth and higher growth rates, but isolate TIM2262's decreased growth and lower growth rate exemplified positive relationships between growth rate and overall growth, and further demonstrated gene *yihT-ompL's* significance in *S. enterica* growth and survival in different substrates. Finally, mutant SeN#17's lowered overall growth in TSB+kan, which was not observed in either non-kanamycin supplemented media types may be a testament of its reaction to the antibiotic. It is likely that there are other conditions affecting its ability to discern antibiotic from nutrients.

The survival and persistence of *Salmonella* within tomatoes and tomato plants has been extensively studied (within the leaves, internalization through stomata, on the surface of tomato, between *Salmonella* serovars, and between tomato cultivars) (Barak et al., 2005; Barak et al., 2011; Gu et al., 2011; Gu et al., 2013; Noel et al., 2010; Zaragoza et al., 2012; Zheng et al., 2013). Indeed, the initial study by Noel el al. (2010) demonstrated that the isolates used in this study did not demonstrate significant differences in growth and persistence (fitness) compared to the wild-type strains when inoculated on "superficially wound[ed] surfaces" of ripe-red tomato fruits. This study confirms this, as the growth rates of the mutant strains were not significantly different from their wild-type counterparts (ATCC14028 or SeN1892) between the different treatments. Indeed, even at the lowered pH of 4.38, *Salmonella* survival has demonstrated remarkable recovery with its initial inoculation levels (Gu et al., 2011).

However, as seen in Table 4, initial concentrations (y-intercept) among different treatments were observable ($P < 0.05$). It is possible that the strains do not colonize on the

tomatoes at the same starting concentrations. The strains inoculated in diced tomatoes had a significantly higher growth rate than spot treatment. *Salmonella*'s psychrotrophic abilities to continue to persist in colder temperatures allow for its continued persistence; however, acidity has been shown to enhance and contribute to *Salmonella* survival, especially at lower temperatures (Adelia et al., 1987; D'Aoust, 1991). In this case, diced tomatoes (pH ~ 4), were inoculated with *Salmonella*, then stored at 4°C and tested over 5 days. In other refrigerated substrates, *Salmonella* concentrations have been shown to increase by one-log within 24 hours (D'Aoust, 1991) as is comparable in this study with a one-log increase within 24 hours but no significant increases until Day 3. As expressed in D'Aoust's (1991) and Adelia et al.'s (1987) studies, this increases concerns about the safety of refrigerated foods, especially in low-acid conditions with great nutrient availabilities (as seen in diced tomatoes). Prior to the beginning of the study, all isolates were confirmed to grow at 10^9 c/mL every day. All isolates were diluted and inoculated into the samples at same concentration.

Both diced and spot treatments had similar growth rates as the vacuum inoculation treatment. Vacuum inoculation is used to mimic internalization of *Salmonella spp.* without the need of inoculating tomato plants at inception. As seen in Gu et al. (2011), internalized *Salmonella spp.* persisted and had higher recovery values than surface inoculated tomatoes. While the tomatoes were stored at 24°C or room temperature which is lower than the ideal *Salmonella* optimal growing temperature, Maitland et al. (2011) and Pollard et al.'s (2013) previous studies with vacuum inoculation to internalize *S. enterica* into tomatoes, demonstrated comparable recovery rates from within tomatoes. This study further confirmed Pollard's findings of 5 Log CFU/g average recovery from SeN J1892 vacuum inoculated tomatoes. Additionally, internalized *Salmonella* survives better than surface colonized *Salmonella* even when

contamination occurs prior to fruit production (Gu et al., 2011). All the strains grew at the same rates in vacuum inoculated tomatoes, thus allowing them all to be potential candidates for further studies.

The average growth rate of *Salmonella* strains in spot inoculated tomatoes was significantly lower than that in diced inoculated treatment ($P<0.01$). Methodologies of spot inoculations vary between studies: some studies inoculate isolates on the stem scar (Maitland et al., 2013), while other studies (Lang et al., 2004) inoculate on the blossom scar. Inoculation on the stem scar would be similar to inoculation via wounded surface (Noel et al., 2010), which with these specific strains demonstrated no significant differences in growth rate. On the other hand, this study inoculated on the blossom scar (stem scar down) thus mimicking a supposedly impenetrable, waxy surface that is not ideal for *Salmonella* persistence. The inability to distinguish between recovery from the surface versus what may have become internalized or penetrated into the tomato was a limitation in this study and could be explored / addressed for the future. Separation of peel from fruit, and enumeration of isolates, will further explore the internalization capabilities of certain strains via expressed genes.

Not only is growth rate important, but also the y-intercept (inoculation concentration) was observed; and as mentioned before, there were differences in the initial inoculation concentration at Day 0 between strains. As had been cursorily reported before in Noel et al. (2010), for *S.* Typhimurium isolates, none were significantly different from ATCC14028. SeN#17 had the highest initial concentration of all isolates, even when compared with wild-type SeN J1892. TIM2261 also had lowered growth rate values than the other *S.* Typhimurium isolates; indeed, mutating the *agfC* gene may be detrimental to tomato survival, even if not significantly (Noel et al., 2013; Zaragoza, 2012; Barak et al., 2011). Theoretically speaking,

SeN#17 should not have had a higher initial concentration than all other isolates. Because the *tolC* gene is a multi-drug efflux pump that also pumps out any non-nutritive materials, perhaps since mutant SeN#17 is lacking the pumps; it instead, is able to utilize most components of the tomato upon interaction. Additionally, since SeN J1892 is perpetually indicted as a tomato-related infectious agent, it is possible that a mutant strain would have similar if not worse colonization properties than the wild-type strain (Horiyama, 2011). In fact, deleting the *TolC* gene may be generally detrimental to basic *Salmonella* survival *in vivo*.

As observed, and unexplainable, there were no correlations between the growth rates and the inoculation treatments; nor was there any correlation between y-intercepts and inoculation treatments. Another confounding and unexpected factor is that while there wasn't any correlation between nutrient-rich media, minimal media, and growth; there was correlation between the two types of nutrient-rich media.

In conclusion, while the strains mostly grew and persisted at the same rate, due to slight variations, some strains should be studied further to determine positive and/or negative interactions before being further tested, including SeN#17, TIM 2261, and TIM 2262. SeN#17 persisted quite variably *in vivo* and *in vitro*, while TIM2261 persisted differently in minimal media versus nutrient rich media, and TIM2262 continually demonstrated decreased fitness when compared to the other strains.

References

Allard, S., A. Enurah, E. Strain, P. Millner, S. L. Rideout, E. W. Brown, and J. Zheng. 2014. In situ evaluation of *Paenibacillus alvei* in reducing carriage of *Salmonella* Newport on whole tomato plants. *Appl. Environ. Microbiol.*

Barak, J. D., L. Gorski, P. Naraghi-arani, and A. O. Charkowski. 2005. Salmonella enterica virulence genes are required for bacterial attachment to plant tissue. *Appl. Environ. Microbiol.* 71:5685–5691.

Barak, J. D., L. C. Kramer, and L. Hao. 2011. Colonization of tomato plants by Salmonella enterica is cultivar dependent, and type 1 trichomes are preferred colonization sites. *Appl. Environ. Microbiol.* 77:498–504.

Bernstein, N., S. Sela, and S. Neder-Lavon. 2007. Assessment of contamination potential of lettuce by Salmonella enterica serovar Newport added to the plant growing medium. *J. Food Prot.* 70:1717–22.

Boriss, B. H., and H. Brunke. 2014. Fresh tomatoes profile 1–4.

Boriss, H., and H. Brunke. 2005. Commodity profile : tomatoes , fresh market. *Agric. Mark. Resour. Cent.*

Burge, W. D. 1987. Substrate (Salmonella regrowth in compost) 243–253.

Center for Science in the Public Interest. 2009. Outbreak alert! analyzing foodborne outbreaks 1998 to 2007.

Christian, R. R., R. B. Hanson, and S. Y. Newell. 1982. Comparison of methods for measurement of bacterial growth rates in mixed batch cultures. *Appl. Environ. Microbiol.* 43:1160-1165.

FDA/CFSAN. 2001. Analysis of preventative control measures for the control and reduction/elimination of microbial hazards on fresh and fresh-cut produce.

Fronzes, R., H. Remaut, and G. Waksman. 2008. Architectures and biogenesis of non-flagellar protein appendages in Gram-negative bacteria. *EMBO J.* 27:2271–80.

Gibson, D. L., a P. White, S. D. Snyder, S. Martin, C. Heiss, P. Azadi, M. Surette, and W. W. Kay. 2006. Salmonella produces an O-antigen capsule regulated by AgfD and important for environmental persistence. *J. Bacteriol.* 188:7722–30.

Gu, G., J. M. Cevallos-Cevallos, and A. H. C. van Bruggen. 2013. Ingress of Salmonella enterica Typhimurium into tomato leaves through hydathodes. *PLoS One* 8:e53470.

Gu, G., J. Hu, J. M. Cevallos-Cevallos, S. M. Richardson, J. a Bartz, and A. H. C. van Bruggen. 2011. Internal colonization of Salmonella enterica serovar Typhimurium in tomato plants. *PLoS One* 6:e27340.

Gualdi, L., L. Tagliabue, S. Bertagnoli, T. Ieranò, C. De Castro, and P. Landini. 2008. Cellulose modulates biofilm formation by counteracting curli-mediated colonization of solid surfaces in Escherichia coli. *Microbiology* 154:2017–24.

Hadjok, C., G. S. Mittal, and K. Warriner. 2008. Inactivation of human pathogens and spoilage bacteria on the surface and internalized within fresh produce by using a combination of ultraviolet light and hydrogen peroxide. *J. Appl. Microbiol.* 104:1014–24.

Horiyama, T., A. Yamaguchi, and K. Nishino. 2010. TolC dependency of multidrug efflux systems in Salmonella enterica serovar Typhimurium. *J. Antimicrob. Chemother.* 65:1372–6.

James, J. 2006. Chapter 1: Overview of microbial hazards in fresh fruit and vegetables operations, p. 1–36. *In* Microbial Hazard Identification in Fresh Fruit and Vegetables. John Wiley & Sons, Inc.

Lang, M. M. 2004. Evaluation of inoculation method and inoculum drying time. The University of Georgia.

Lynch, M. F., R. V Tauxe, and C. W. Hedberg. 2009. The growing burden of foodborne outbreaks due to contaminated fresh produce: risks and opportunities. *Epidemiol. Infect.* 137:307–15.

Maitland, J. E., R. R. Boyer, J. D. Eifert, and R. C. Williams. 2011. High hydrostatic pressure processing reduces Salmonella enterica serovars in diced and whole tomatoes. *Int. J. Food Microbiol.* 149:113–7.

Marvasi, M., C. E. Cox, Y. Xu, J. T. Noel, J. J. Giovannoni, and M. Teplitski. Differential regulation of Salmonella Typhimurium genes involved in O-antigen capsule production and their role in persistence within tomatoes 1–25.

Montville, T. J., and K. R. Matthews. 2008. Food microbiology - an introduction, 2nd ed. American Society for Microbiology.

Noel, J. T., N. Arrach, A. Alagely, M. McClelland, and M. Teplitski. 2010. Specific responses of Salmonella enterica to tomato varieties and fruit ripeness identified by in vivo expression technology. *PLoS One* 5:e12406.

Pollard, S. 2013. Survival of *Salmonella enterica* Newport in Ralstonia solanacearum - infected tomato. International Association for Food Protection. International Association for Food Protection, Charlotte, North Carolina.

Rhen, M., D. Maskell, P. Mastroeni, and J. Threlfall. 2007. Salmonella: molecular biology and pathogensis, 2nd ed. horizon bioscience, Norfolk, UK.

Scallan, E., R. M. Hoekstra, F. J. Angulo, R. V. Tauxe, M.-A. Widdowson, S. L. Roy, J. L. Jones, and P. M. Griffin. 2011. Foodborne illness acquired in the United States—major pathogens. *Emerg. Infect. Dis.* 17:7–15.

Teplitski, M., J. T. Noel, A. Alagely, and M. D. Danyluk. 2012. Functional genomics studies shed light on the nutrition and gene expression of non-typhoidal Salmonella and enterovirulent E. coli in produce. *Food Res. Int.* 45:576–586.

Townsend, S. M., N. E. Kramer, R. Edwards, S. Baker, N. Hamlin, M. Simmonds, K. I. M. Stevens, S. Maloy, J. Parkhill, and G. Dougan. 2001. Salmonella enterica Serovar Typhi possesses a unique repertoire of fimbrial gene sequences 69:2894–2901.

Zaragoza, W. J., J. T. Noel, and M. Teplitski. 2012. Spontaneous non-rdar mutations increase fitness of Salmonella in plants. *Environ. Microbiol. Rep.* 4:453–458.

Zheng, J., S. Allard, S. Reynolds, P. Millner, G. Arce, R. J. Blodgett, and E. W. Brown. 2013. Colonization and internalization of Salmonella enterica in tomato plants. *Appl. Environ. Microbiol.* 79:2494–502.

Zhuang, R. Y., L. R. Beuchat, and F. J. Angulo. 1995. Fate of Salmonella montevideo on and in raw tomatoes as affected by temperature and treatment with chlorine. *Appl. Environ. Microbiol.* 61:2127–31.

TABLES & FIGURES

Table 1. *Salmonella* isolates used in experiment.

	Strain	Origination	Function
***S.* Typhimurium (SeT)**			
ATCC 14028	**Virulent wild-type strain**		
BA770	14028 ***pSLT-***		Virulence plasmid
JSG 1748	14028 Δ***bcs::kan***		Cellulose and biofilm secretion
TIM2260	14028 Δ***agfB36::kan***		(a) Curli production
TIM2261	14028 Δ***agfC37::kan***		(b) Inflammation (c) Biofilm formation
TIM2262	14028 Δ***ompL19::kan***		(a) O-antigen capsule
TIM2263	14028 Δ***yihT27::kan***		(b) attachment
***S.* Newport (SeN)**			
SeN J1892	**Virulent clinical strain**		
SeN #17	SeN Δ***tolC::kan***		Multi-drug efflux pumps

Table 2. Area under the curve [AUC] for *Salmonella* strains grown in TSB, TSB+kan, and M9 media.

	Strain	TSB[1,2]	TSB+kan[1,3]	M9[1,4]
***S.* Typhimurium**				
ATCC 14028	**WT**	3141.92 ± *413.91*^{abc}	445.15 ± *29.21*^{d}	1539.51 ± *197.61*^{bcd}
BA770	**Δ*pSLT-***	3271.90 ± *171.09*^{a}	466.09 ± *51.52*^{d}	1620.21 ± *154.16*^{abc}
JSG 1748	**Δ*bcs***	3236.12 ± *137.50*^{ab}	3194.29 ± *193.54*^{b}	1403.16 ± *196.88*^{de}
TIM2260	**Δ*agfB***	3369.76 ± *148.00*^{a}	3247.65 ± *95.03*^{ab}	1478.68 ± *103.53*^{cde}
TIM2261	**Δ*agfC***	3346.43 ± *78.98*^{a}	3257.59 ± *116.46*^{ab}	1418.77 ± *147.62*^{cde}
TIM2262	**Δ*ompL***	3030.12 ± *228.75*^{bc}	3020.19 ± *111.57*^{c}	1480.49 ± *114.45*^{cde}
TIM2263	**Δ*yihT***	3313.58 ± *110.79*^{a}	3331.80 ± *99.75*^{a}	1788.35 ± *39.99*^{a}
***S.* Newport**				
J1892	**WT**	3005.25 ± *193.33*^{c}	444.86 ± *38.74*^{d}	1307.96 ± *120.68*^{e}
SeN #17	**Δ*tolC***	3206.01 ± *105.47*^{abc}	3066.66 ± *90.43*^{c}	1692.24 ± *106.84*^{ab}
	Media (Control)	476.73 ± *28.52*^{d}	463.64 ± *21.16*^{d}	317.85 ± *8.99*^{f}

Columns with different superscript letters are significantly different.

[1] One-way ANOVA; Tukey's HSD; $p<0.05$

[2] Number of replicates: ATCC 14028 (n = 10), media (n = 14), all other strains (n = 15)

[3] Number of replicates: SeN#17 (n = 11), media (n = 14), all other strains (n = 15)

[4] Number of replicates: all strains and media (n = 10)

Table 3. Growth Rates for *Salmonella* strains grown in TSB, TSB+kan, and M9 media: (A) Linear Growth Rate; (B) Exponential Growth Rate.

(A) Linear Growth Rate (Δ)

	Strain	TSB[1,2]	TSB+kan[1,2]	M9[1,2]
***S.* Typhimurium**				
ATCC 14028	**WT**	0.50 ± *0.03*[a]	5.55e-17±*0.02*[d]	0.54 ± *0.09*[a]
BA770	**Δ*pSLT-***	0.42 ± *0.26*[ab]	0.00±*0.02*[d]	0.56 ± *0.09*[a]
JSG 1748	**Δ*bcs***	0.42 ± *0.26*[ab]	0.36 ± *0.21*[bc]	0.32 ± *0.09*[a]
TIM2260	**Δ*agfB***	0.50 ± *0.26*[a]	0.44 ± *0.02*[ab]	0.56 ± *0.09*[a]
TIM2261	**Δ*agfC***	0.43 ± *0.26*[ab]	0.39 ± *0.02*[bc]	0.24 ± *0.09*[a]
TIM2262	**Δ*ompL***	0.30 ± *0.26*[b]	0.31 ± *0.02*[c]	0.52 ± *0.09*[a]
TIM2263	**Δ*yihT***	0.43 ± *0.26*[a]	0.5 ± *0.02*[a]	0.56 ± *0.09*[a]
***S.* Newport**				
J1892	**WT**	0.45 ± *0.26*[a]	5.55e-17 ±*0.02*[d]	0.47 ± *0.09*[a]
SeN #17	**Δ*tolC***	0.39 ± *0.26*[ab]	0.40 ± *0.02*[abc]	0.46 ± *0.09*[a]

Columns with different superscript letters are significantly different.

[1] One-way ANOVA; Tukey's HSD; $p<0.05$

[2] TSB and TSB+kan (n = 3), M9 (n = 2)

(B) Exponential Growth Rate (μ)

	Strain	TSB[1,2]	TSB+kan[1,2]	M9[1,2]
***S.* Typhimurium**				
ATCC 14028	**WT**	0.16 ± *0.01*[a]	4.16e-17±*0.01*[d]	0.21 ± *0.03*[a]
BA770	**Δ*pSLT-***	0.13 ± *0.01*[ab]	1.39e-17±*0.00*[d]	0.23 ± *0.03*[a]
JSG 1748	**Δ*bcs***	0.12 ± *0.01*[ab]	0.10 ± *0.00*[bc]	0.15 ± *0.03*[a]
TIM2260	**Δ*agfB***	0.14 ± *0.01*[ab]	0.12 ± *0.00*[ab]	0.21 ± *0.03*[a]
TIM2261	**Δ*agfC***	0.12 ± *0.01*[ab]	0.11 ± *0.00*[bc]	0.08 ± *0.03*[a]
TIM2262	**Δ*ompL***	0.09 ± *0.01*[ab]	0.09 ± *0.00*[c]	0.21 ± *0.03*[a]
TIM2263	**Δ*yihT***	0.12 ± *0.01*[ab]	0.14 ± *0.00*[a]	0.21 ± *0.03*[a]
***S.* Newport**				
J1892	**WT**	0.13± *0.01*[ab]	0.00±*0.00*[d]	0.20 ± *0.03*[a]
SeN #17	**Δ*tolC***	0.11 ± *0.01*[ab]	0.11 ± *0.00*[bc]	0.18 ± *0.03*[a]

Columns with different superscript letters are significantly different.

[1] One-way ANOVA; Tukey's HSD; $p<0.05$

[2] TSB and TSB+kan (n = 3), M9 (n = 2)

Table 4. Statistical analysis of parameter estimates for the growth of *Salmonella* strains in treatments

Treatment	Strain	Slope ((log CFU/g)*day-1)	Intercept (log (CFU/g))
		(P=0.3715)	(P>0.0009)
	***S.* Typhimurium**		
	ATCC 14028	0.77 ± *0.29*[a]	3.15 ± *0.33*[bc]
	BA770	1.01 ± *0.25*[a]	2.98 ± *0.28*[bc]
	JSG 1748	1.03 ± *0.25*[a]	3.14 ± *0.28*[bc]
Diced Treatment[1,2]	**TIM2260**	1.04 ± *0.25*[a]	2.81 ± *0.28*[bc]
	TIM2261	1.18 ± *0.29*[a]	2.88 ± *0.33*[bc]
	TIM2262	0.32 ± *0.25*[a]	4.20 ± *0.28*[ab]
	TIM2263	0.73 ± *0.25*[a]	3.84 ± *0.28*[abc]
	***S.* Newport**		
	J1892	0.74 ± *0.25*[a]	3.26 ± *0.28*[bc]
	SeN #17	0.52 ± *0.25*[a]	4.64 ± *0.28*[a]
		(P=0.3818)	(P=0.128)
	***S.* Typhimurium**		
	ATCC 14028	0.68±*0.08*[a]	3.75±*0.24*[a]
	BA770	0.75±*0.08*[a]	3.62±*0.24*[a]
	JSG 1748	0.61±*0.08*[a]	3.97±*0.24*[a]
Vacuum Treatment[1,3]	**TIM2260**	0.73±*0.08*[a]	3.73±*0.24*[a]
	TIM2261	0.53±*0.08*[a]	4.19±*0.24*[a]
	TIM2262	0.63±*0.08*[a]	4.38±*0.24*[a]
	TIM2263	0.50±*0.08*[a]	4.01±*0.24*[a]
	***S.* Newport**		
	J1892	0.64±*0.08*[a]	3.81±*0.24*[a]
	SeN #17	0.52±*0.08*[a]	4.62±*0.24*[a]
		(P=0.6793)	(P=0.5976)
	***S.* Typhimurium**		
	ATCC 14028	0.61±*0.20*[a]	1.59±*0.46*[a]
	BA770	0.62±*0.20*[a]	1.51±*0.46*[a]
	JSG 1748	0.54±*0.20*[a]	1.63±*0.46*[a]
	TIM2260	0.63±*0.20*[a]	1.84±*0.46*[a]
Spot Inoculation[1,3]	**TIM2261**	0.33±*0.20*[a]	2.63±*0.46*[a]
	TIM2262	0.52±*0.20*[a]	1.74±*0.46*[a]
	TIM2263	0.40±*0.20*[a]	1.93±*0.46*[a]
	***S.* Newport**		
	J1892	0.63±*0.20*[a]	1.63±*0.46*[a]
	SeN #17	0.13±*0.20*[a]	2.52±*0.46*[a]

Columns (by treatment) with different superscript letters are significantly different.

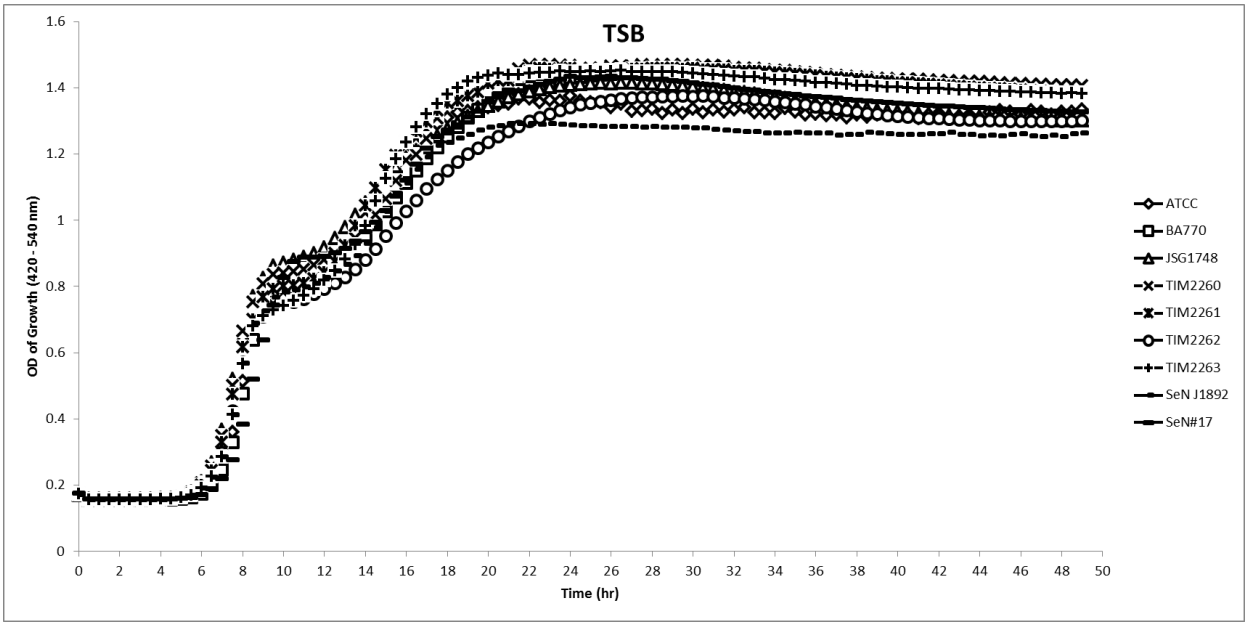
[1] One-way ANOVA; Tukey's HSD; $p<0.05$

[2] ATCC14028 (n = 3), all other strains (n = 4)

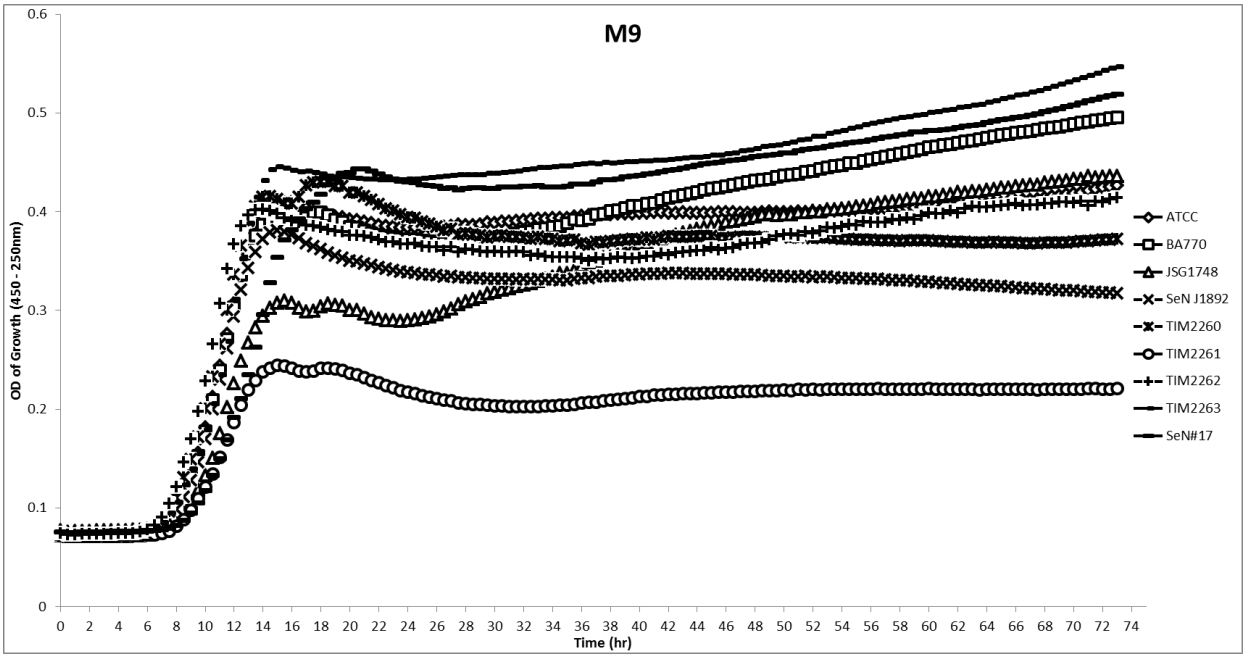
[3] n = 3

Figure 1. Growth Curves of *Salmonella* strains in various media: (A) TSB; (B) M9; (C) TSB+kan

(A) TSB



(B) M9



(C) TSB+kan

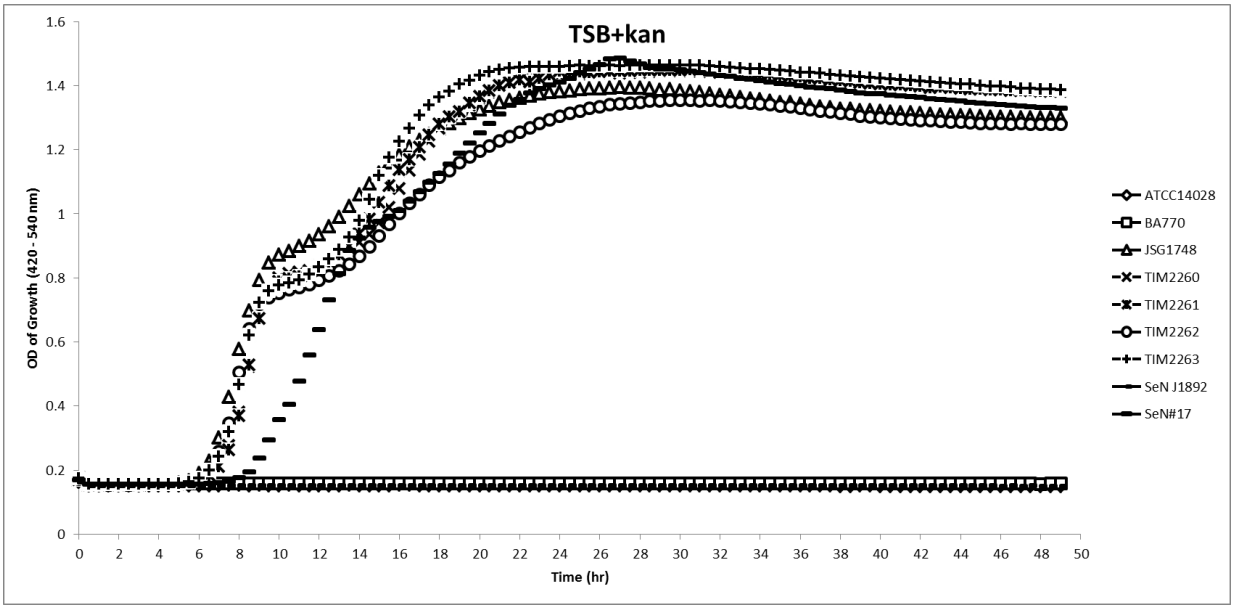
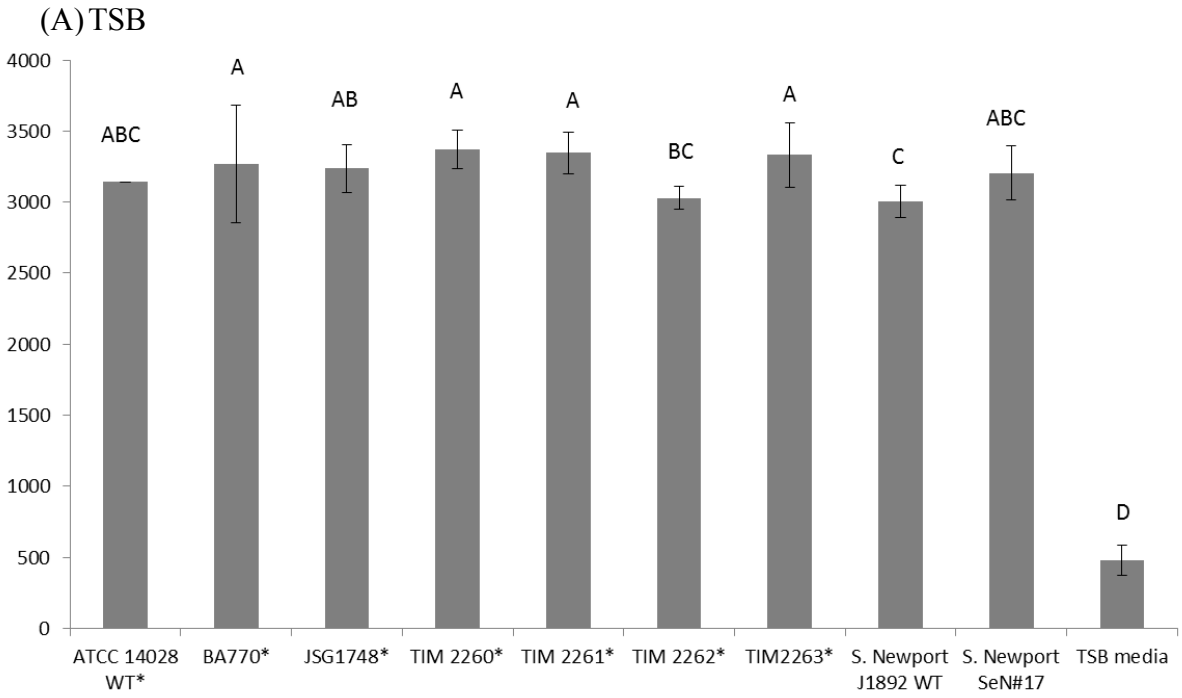
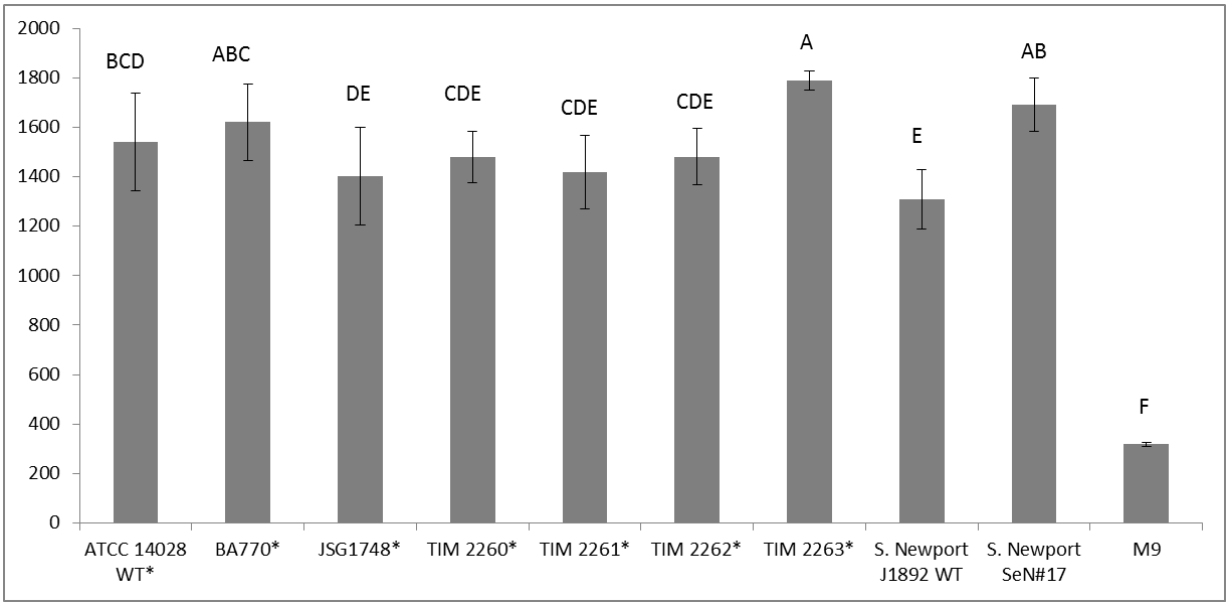

TSB+kan
OD of Growth (420 - 540 nm)
Time (hr)
ATCC14028
BA770
JSG1748
TIM2260
TIM2261
TIM2262
TIM2263
SeN J1892
SeN#17

Figure 2. AUC of *Salmonella* isolates in each type of media: (A) TSB; (B) M9; (C) TSB+kan.



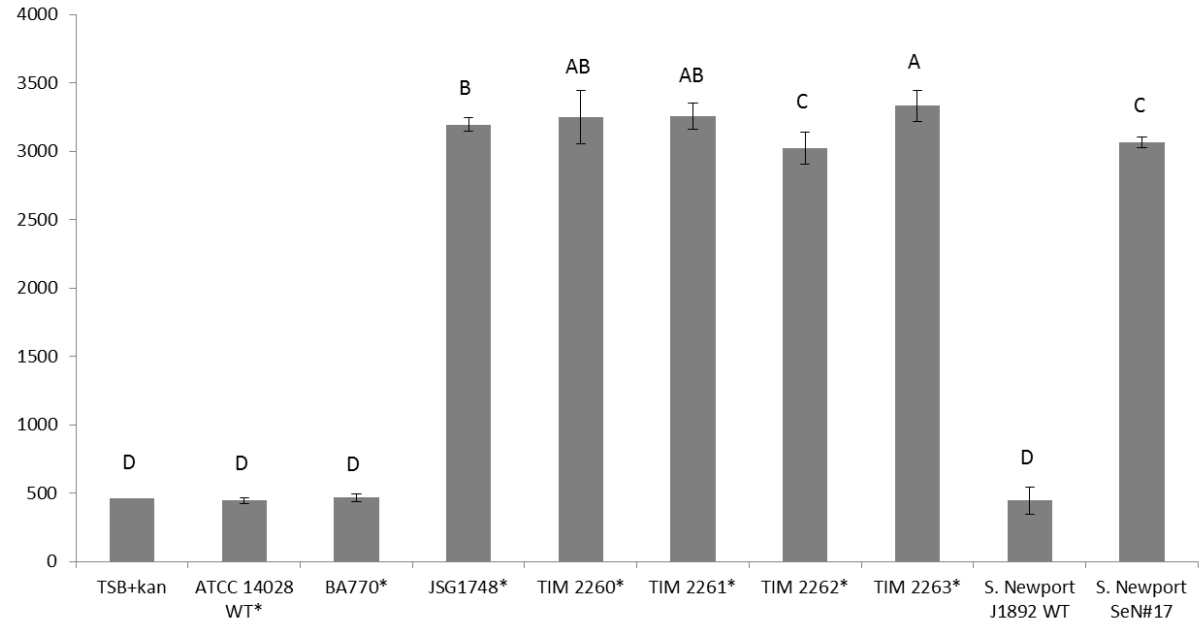
Isolates with different superscript letters are significantly different; One-way ANOVA; Tukey's HSD; $p<0.05$

(B) M9



Isolates with different superscript letters are significantly different; One-way ANOVA; Tukey's HSD; $p<0.05$

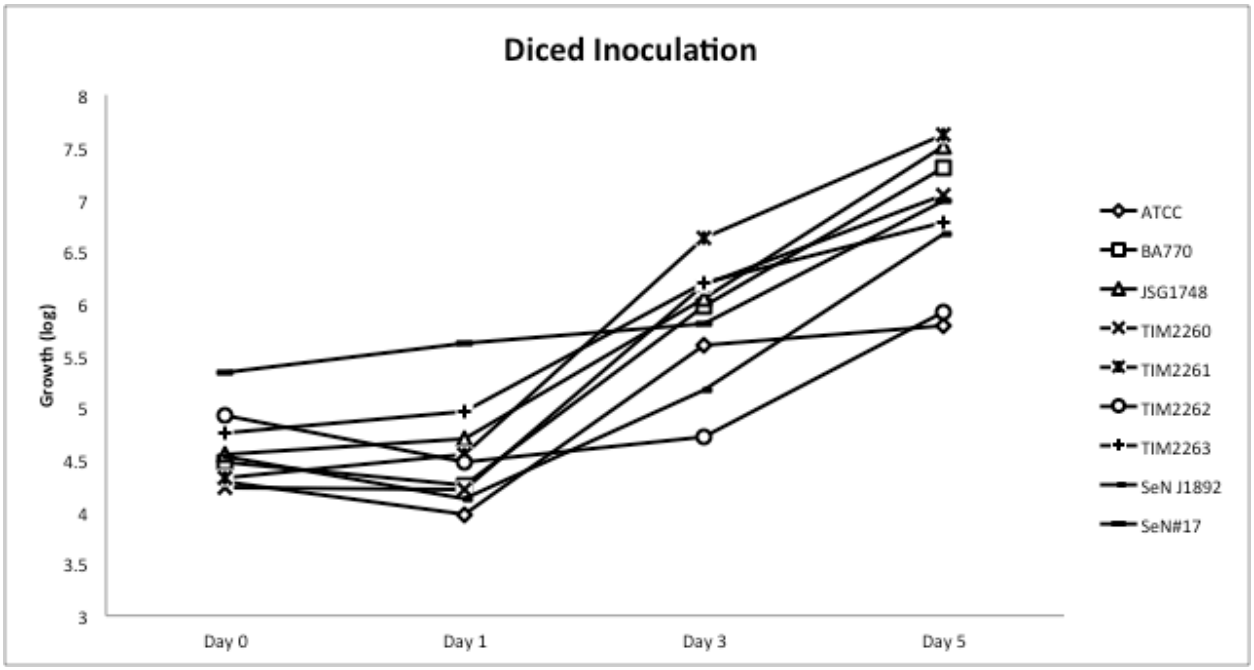
(C) TSB+kan



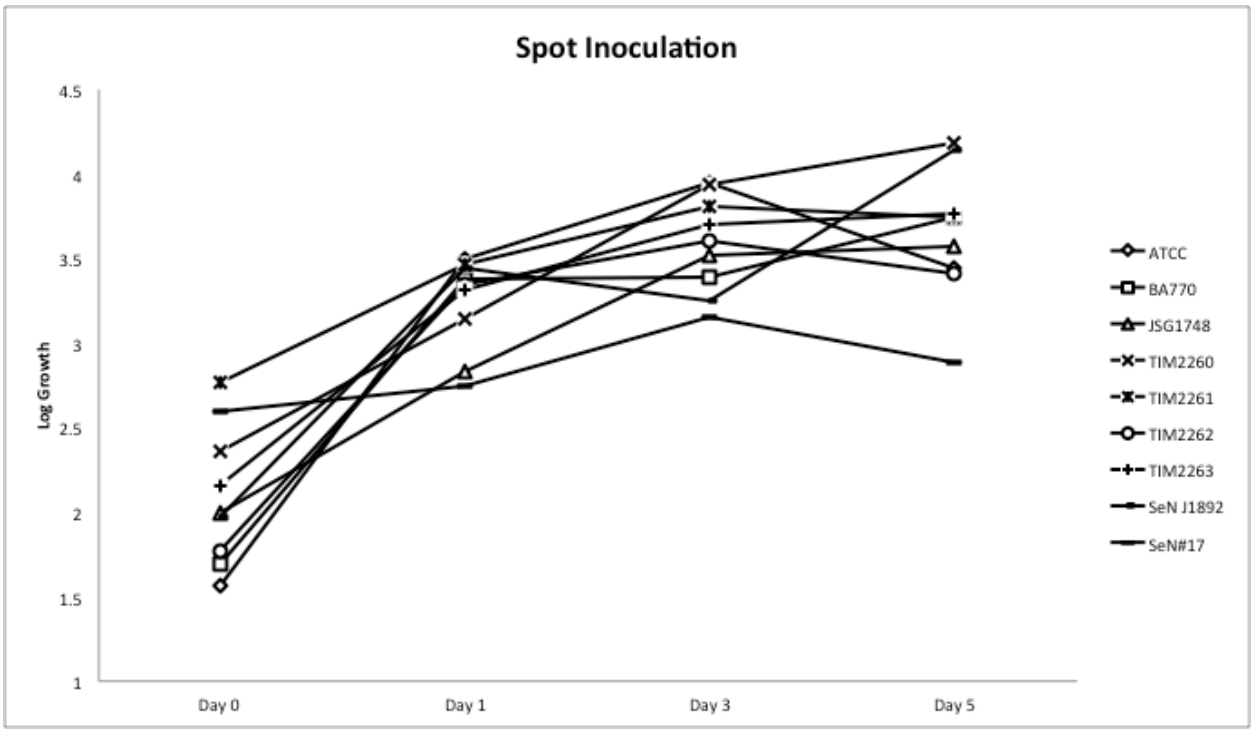
Isolates with different superscript letters are significantly different; One-way ANOVA; Tukey's HSD; $p<0.05$

Figure 3. Growth Curves of *Salmonella* strains in tomatoes under various treatments: (A) Diced Inoculation; (B) Spot Inoculation; (C) Vacuum Inoculation.

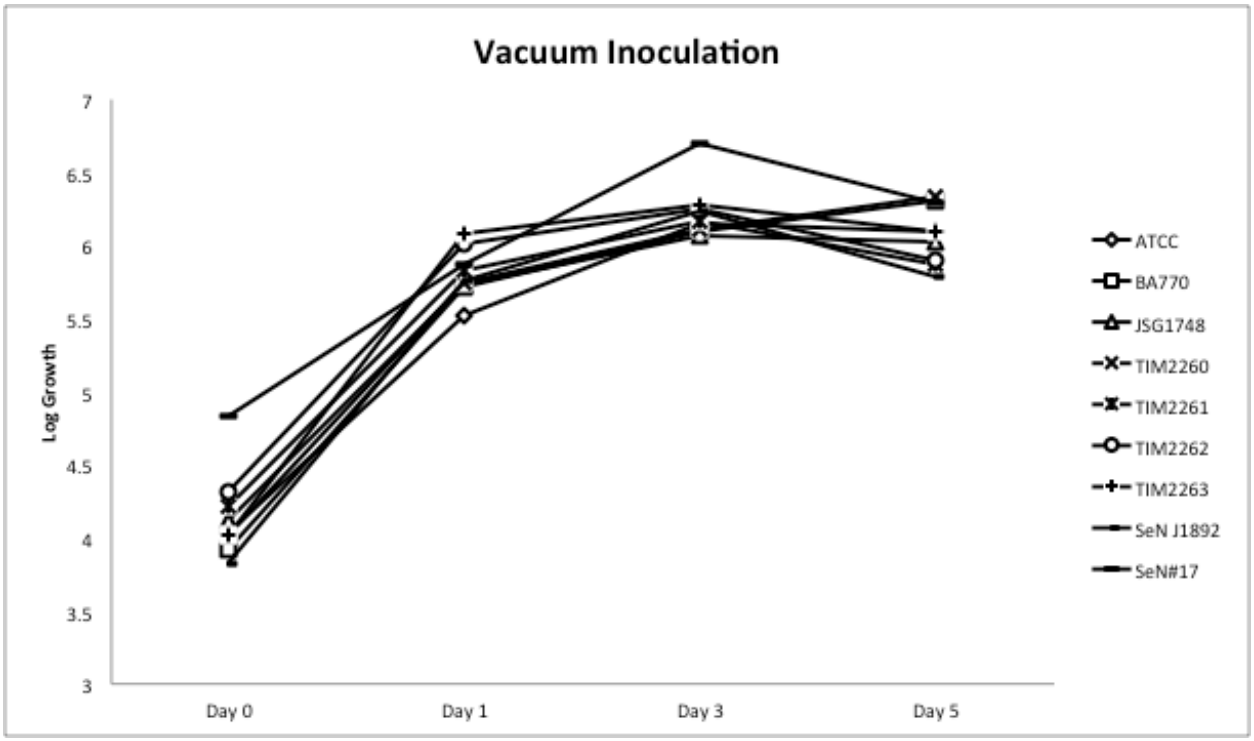
(A) Diced Inoculation



(B) Spot Inoculation



(C) Vacuum Inoculation



Chapter 4: Conclusion

Ultimately, the goal of this study is to determine if the growth and persistence of *Salmonella* serovars Typhimurium and Newport mutated isolates was similar to their WT counterparts. For the most part, many of the isolates do grow comparably to their WT strain. If only the growth rate of isolates within the different tomatoes treatments were considered, all isolates all grew very similarly. However, *Salmonella* strains are very condition dependent. Some strains will remain in certain phases of growth at varying times. When the growths of strains were compared in nutrient rich and minimal media, there was not much difference in overall growth; however, considerations must be taken depending on the media type. Minimal media, while mimicking an environmental reservoir, affected the growth and growth rates of TIM2261 and SeN#17 more than other isolates; additionally, *S.* Typhimurium isolates TIM2261 and TIM2262 had lower growth than the other isolates, which when translated into an environmental function may actually show decreased recovery rates.

A few limits of the study should also be taken into account. It might be beneficial to compare growth and persistence at two different temperatures, rather than the optimal growth temperatures that we chose in this study. Additionally, instead of inoculating at 7 Log CFU/mL may not have mimic inoculation levels that would be seen in a field setting. An additional set of trials should be considered where the initial inoculation level is lower (4 Log CFU/mL).

The inoculation methods that were chosen for this study were to mimic the most replicated methods from current research. Additional inoculation methods could have

been explored such as observing the differences in the location of the spot inoculation treatments (blossom scar versus stem scar). Regardless of inoculation method, all tomatoes were analyzed using the same method. For spot inoculated fruit, it may have been beneficial to separate the flesh from the tomato fruit and enumerate *Salmonella* from it separately. This approach may have provided more information related to detection and recovery of *Salmonella* isolates. This could very well show how much *Salmonella* can be internalized with regards to the various strain mutations, too.

The future of this project is quite promising. As has been demonstrated, the growth and persistence of all the isolates in tomatoes (both in this study and in previous studies) indicate that the isolates are very similar to their WT. To ensure that strains are indeed attenuated, with no significant virulence, an animal study with zoonotic pigs could be performed. From there, strains that are truly identified as attenuated and having their virulence negated can be used as surrogate organisms in field studies. Future fieldwork with *Salmonella* strains can consist of full fieldwork (instead of in selected fields only) to determine *Salmonella* distribution, colonization, and persistence in tomato fields, in plants, and in the fruit itself.