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Investigation of microbial aspects related to *Salmonella* as a food pathogen:

bioluminescent reporting system and mechanisms for host invasion

By

Kevin Matthew Howe

A Dissertation
Submitted to the Faculty of
Mississippi State University
in Partial Fulfillment of the Requirements
for the Degree of Doctorate of Philosophy
in Veterinary Medical Science
in the College of Veterinary Medicine

Mississippi State, Mississippi

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Investigation of microbial aspects related to *Salmonella* as a food pathogen:

bioluminescent reporting system and mechanisms for host invasion

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Salmonella can reside in healthy animals without the manifestation of any adverse effects on the carrier. If raw products of animal origin are not handled properly during processing or cooked to a proper temperature during preparation, salmonellosis can occur. In this research, microbial aspects related to *Salmonella* as a food pathogen are investigated. A bioluminescent reporting system was developed for *Salmonella* to monitor the attachment and growth of the pathogen on food products. Twelve and eleven *Salmonella* strains from the broiler production continuum were tagged with bioluminescence by plasmid and integration of the *lux* operon into the chromosome, respectively. To assess the usefulness of bioluminescent *Salmonella* strains in food safety studies, an attachment model using chicken skin was developed. Variables including washing and temperature were tested in the attachment model to determine the effects on attachment of *Salmonella* strains to chicken skin, a characteristic that enhances persistence during processing. Additionally, the invasion process for two serovars of *Salmonella* with differing host tropism was examined with emphasis on the initial

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establishment of the bacterium in the host. The major facilitator for invasion, type III secretion system, was inactivated through deletion mutation to evaluate invasion of human epithelial cell line by additional means. The difference in host tropism between the two subspecies of *Salmonella* was also taken into account when evaluating invasion. Results showed that invasion of human epithelial cells can be initiated despite inactivation of the type III secretion system. A serovar of *Salmonella* that is not typically associated with human illness was also shown to initiate invasion of human epithelial cells, a result that carries public health implication as this serovar has recently been shown to be multi-drug resistant.

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DEDICATION

This manuscript is dedicated to the family of Kevin Howe that provided unconditional love and support through the completion of his research.

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CHAPTER I

INTRODUCTION

Enteritis salmonellosis is a continuing foodborne infectious disease for humans stemming from the commercial food-animal industry in the United States. The causative agent, non-typhoidal *Salmonella,* consists of more than 2,500 serotypes and is able to reside within warm-blooded and cold-blooded animals, and in the environment. However, only a small proportion of the serotypes (approximately 20) causes most human infections and are usually associated with specific animal reservoirs. A significant reservoir of this bacterium in the commercial food-animal industry is poultry. *Salmonella* is able to colonize the gastrointestinal tracts of poultry without causing the host detriment or to display symptoms of infection. However, if the bacterium is able to reach certain numbers within the host, it can cross the pathogenic threshold and cause illness. Typically, moribund animals are culled before they reach slaughter. More often than not though, *Salmonella* is introduced into the processing plant in healthy, asymptomatic animals. During the slaughter process, there is increased risk of cross-contamination occurring between contaminated carcasses and previously uncontaminated carcasses. A significant increase between the number of *Salmonella* positive broilers entering and then exiting the scalding water immersion tank and exiting the crop extractor demonstrate how in-plant cross-contamination can occur [1, 2].

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The health risk of *Salmonella* to humans is that the pathogen is zoonotic and in typical instances, the bacterium is transferred from non-human to human through ingestion of contaminated food or water. Each serotype associates with its specific host which causes the design of a uniform strategy to control this pathogen nearly impossible. According to FoodNet 2011 data, minimal progress has been made in reducing infections caused by *Salmonella* [3]. In 2011, non-typhoidal *Salmonella* was the second leading cause of domestically acquired foodborne illness in the United States, yet resulted in the highest incidence of hospitalizations and illness resulting in death [4]. This current study presents a novel reporter system in field-isolated *Salmonella* that extrapolates on existing bioluminescent techniques. In response to limitations of the initial design of this reporter system, the design underwent development and the re-engineering is outlined in this study. Application of the reporter system as a screening assay in food-animal processing is demonstrated in real-time evaluation of mitigation strategies against *Salmonella*-contaminated poultry skin. Further analysis in this study focused on invasion mechanisms of *Salmonella enterica* serovar Typhimurium and serovar Kentucky, an internationally emerging serotype with multi-drug resistant (MDR) consequence.

Background of the Problem

When analyzing the efficacy of various antimicrobial therapies, traditional methodology to collect data involved conventional microbiological cultural techniques to detect and enumerate pathogens. In this manner, after the pathogen-host relationship is established, treatment is applied, the product is sampled, microbiological culturing protocols are followed, and 48 to 72 hours later the results are known. In certain instances, analysis of pathogen survival requires cell removal, and often lysis, to detect

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the pathogen post-treatment. Collection of biological information in the context of intact host cells is difficult to obtain without destroying the host matrix. The destruction of host cells inhibits successive temporal data readings on a single sample, necessitating a larger number of samples. This limitation may become problematic in experimental designs in which resources and time are limited.

Bioluminescence is a naturally occurring enzymatic reaction that occurs in a number of organisms from a variety of different genera [5, 6]. In the bacterium *Photorhabdus luminescens*, the luciferase enzyme catalyzes the reduction of its substrate (commonly known as luciferin) in the presence of oxygen to produce light [7]. Bioluminescent photons emitted can be detected and quantified externally using sensitive photon detectors, like a bioluminometer. Due to the requirement of reducing power for the reaction to occur, this biological process is exclusive to only viable bacteria. The unique properties of this reaction are extremely attractive for the design of a nondestructive, real-time assay to detect pathogens and monitor their survival, and as a tool with the potential to impact antimicrobial therapy studies.

The type III secretion system (T3SS) is a specialized multi-protein complex that functions to facilitate the transfer of proteins, termed effectors, from the cytosol of the bacterium into the cytoplasm of the host cell [8]. Upon delivery into the host cell, effectors manipulate host cell biology, such as cell signaling, secretory trafficking, cytoskeletal dynamics, and the inflammatory response. The T3SS is characterized as having three main structural components: a basal body, a multi-ring system, and needle-like apparatus; all have been similarly identified in Gram-negative bacteria, including *Salmonella*, *Escherichia*, *Shigella*, *Yersinia*, and *Pseudomonas* [8]. In the context of

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Salmonella pathology, the T3SS is reported to aide bacterium translocation across the gastrointestinal epithelial barrier by inducing phagocytosis in non-phagocytic cells [9]. Recent scientific literature suggests a shift in the paradigm concerning the essentiality of the T3SS function to transcending the gastrointestinal epithelial barrier [10-12], alluding to the possibility of *Salmonella* to initiate phagocytosis independently of T3SS. As internalization in non-phagocytic cells is central to the pathogenesis of *Salmonella*, especially in relation to the maturation of infection, it is certainly viable that redundant mechanisms exist with an overlapping function to preserve the invasion process. Further experimentation would provide valuable information about these invasion mechanisms. An increasingly comprehensive understanding of such mechanisms would be extremely beneficial to the development of next-generation antimicrobial therapies; a direction of research that is gaining attention as the incidence of foodborne illnesses associated with MDR pathogens rises.

Statement of the Problem

A need exists for the development of reporter system technology in *S. enterica* serotypes associated with poultry contamination that is able to circumvent the limitations of traditional culture methodologies, while simultaneously expressing efficient and precise data in a timely manner. In this instance, an ideal system would be able to generate data in real-time without causing destruction of the host.

The invasion of the gastrointestinal tract is central to the pathology of *Salmonella*. Recent scientific literature suggests invasion of non-phagocytic cells can occur independently of T3SS. A gap in the knowledge exists describing the mechanisms facilitating invasion in this manner. Additionally, insufficient attention has been aimed to

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elucidate the molecular differences existing between nonpathogenic and pathogenic serotypes of *Salmonella* that account for discrepancy of invasion rates of humans

Purpose of the Study

The purpose of this study is to establish and characterize a bioluminescence reporting system in *S. enterica* using serotypes isolated from the poultry processing continuum. The sensitivity of the reporting system will be determined by estimating the theoretical photon emission from a single bacterium using plate counts and a bioluminometer. Characterization of the reporting system will also take into account the stability of the *lux* construct within each serotype.

A quantitative study will be performed with *S. enterica* serovars Kentucky and Typhimurium and corresponding strains carrying a knockout mutation of the T3SS complex. Each serotype and strain will be evaluated for their ability to invade a human colorectal adenocarcinoma cell line and two types of comparisons will be conducted: 1) between wild-type and the corresponding mutant strain and 2) between serovars Kentucky and Typhimurium.

Significance of the Study

This research is focused in the direction of enhancing food security, contributing to the ability of the food animal industry to meet regulations and standards thus maintaining integrity in the food supply. *Salmonella* is a problematic pathogen for the poultry industry and the Centers for Disease Control (CDC) estimates that *Salmonella* contamination of poultry causes illness in 1.4 million people each year in the United States with about 400 deaths [4]. U.S. Department of Agriculture's (USDA) Food Safety

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and Inspection Service (FSIS) report "Analysis of ALLRTE and RTE001 Sampling Results for *Salmonella* Species, Calendar Years 2005 through 2008" indicated that prevalence of *Salmonella*-positive samples from the ALLRTE and RTE001 sampling programs were < 1% for the random testing program (ALLRTE) and < 1% for the risk-based testing program (RTE001) [13].

This research is of economic importance to the state of Mississippi. The poultry industry is the largest income-producing agricultural commodity in Mississippi's economy, as the poultry sector exceeded $2.7 billion in sales at the farm gate in 2012 and total sales of poultry products by Mississippi processors exceeded $2.8 billion in 2010 [14] and employing greater than 28,000 people directly [14].

The rationale is to develop a reporting system capable of precise, quick pathogen detection that can potentially screen mitigation strategies focused against food-animal pathogens with minimal time constraints. Understanding the invasion mechanisms that enable the pathogen to enter the animal host is critical to our knowledge of the pathology of *Salmonella*, most certainly in the development of strategies to prevent and treat contamination.

Primary Research Questions

There are two primary research questions that were the motivation for this research. The first primary research question postulates whether bioluminescence can be established within *S. enterica* serotypes in a stable manner. Furthermore, will the bioluminescence be suitable for experimental use, and display sensitivity that will be able to identify significant changes in viable bacteria numbers.

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The second primary research question addressed facets of invasion by *Salmonella* and is divided into two components. The first phase was focused on invasion of non-phagocytic cells in a fashion independent of T3SS. The null hypothesis states there is no significant difference between invasion rates of the wild-type and the corresponding mutant strain. The alternative hypothesis states that a significant difference does exist between invasion rates of the wild-type and the corresponding mutant strain. The second phase of the primary research question examines the invasion ability of a nonpathogenic (*S. enterica* serovar Kentucky) and pathogenic (*S. enterica* serovar Typhimurium) serotype. The null hypothesis states there is no significant difference between invasion rates of nonpathogenic and pathogenic serotypes. The alternative hypothesis states there is a significant difference between invasion rates of nonpathogenic and pathogenic serotypes.

Research Design

A brief summary of the methodologies that were used in this research follows. *Salmonella* serotypes for this study were field-isolated serotypes from the poultry processing continuum. The instrumentation that was used to measure bioluminescence intensity was an IVIS Imaging System 100 series coupled with Living Image Software v2.50. Plasmid pAK*lux*1 harbors a construct of the *lux* operon and *Salmonella* serotypes were transformed with the plasmid using standard artificial competence and electroporation procedures. Separate experiments were conducted to characterize the *lux* operon in each serotype. The reporting system was evaluated for stability and the minimal detection limit. The development of an attachment assay with chicken skin was described to demonstrate the application of the bioluminescence reporting system.

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The next stage of this research explored the possible establishment of a permanently stable expression model for the *lux* construct. Plasmid pBEN276 encodes the unique properties of Tn7 transposon and the *lux* operon. *Salmonella* serotypes were transformed using pBEN276 by electroporation, and the *lux* operon were inserted into a specific noncoding region of genome by the Tn7 transposon. This version of the reporting system was characterized for stability and the minimum detection limit for each serotype. The performance of the reporting system was challenged in two assays simulating conditions and treatments similar to the poultry processing continuum.

The final phase of research was dedicated to the invasion process specific for *Salmonella*. This section focused on nonpathogenic *S. enterica* serovar Kentucky and pathogenic *S. enterica* serovar Typhimurium. Colorectal adenocarcinoma cell line HTB-37 was targeted for invasion and was maintained according to standard procedures. Mutations occurring within the T3SS operon in *Salmonella* Pathogenicity Island-1 (SPI1) were constructed using a PCR product designed to inactivate chromosomal genes. A standard gentamicin protection assay was used to determine invasion rates of the wild-type and the corresponding mutant strain.

Definition of Terms

In this study nonpathogenic refers to a serotype that is not traditionally known to infect humans.

Summary

In the introductory chapter, background information concerning *Salmonella* as a health-risk associated food borne pathogen was outlined. The research was introduced in

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the context of limitations associated with traditional cultivation technique for *S. enterica* species. The introduction of the research progressed to discuss invasion methods pertinent in the gastrointestinal tract. In the following chapter, key scientific concepts related to this research will be briefly explored in the literature review, further framing the scope of this study.

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CHAPTER II

LITERATURE REVIEW

Review of Bacterial Luciferase

A wide range of biological sensors has been used to monitor gene expression [15-17]. The application of these biosensors often involves an endpoint determination of the activity of a reporter enzyme, with samples taken at intervals for evaluation of activity in assays requiring additional reagents. Although these methods are often sensitive and reasonably rapid, limitations exist in that they are typically invasive and destructive and have to be performed off-line. As such, they do not act as real-time reporters. Bioluminescent reporters offer the ability to quantify gene expression at high sensitivity over a large dynamic range in real-time and non-destructively.

Bioluminescent organisms are widely distributed in nature and comprise a diverse set of species including bacteria, dinoflagellates, fungi, fish, insects, shrimp, and squid [18-20]. The enzymes that catalyze the bioluminescence reactions within these organisms are called luciferases. Significant differences exist between the bioluminescence reactions as well as the structures of the enzymes and substrates from different organisms [21]. Bacterial luciferases from *Photorhabdus luminescens*, and other species such as *Vibrio harveyi* and *Xenorhabdus luminescens*, have been cloned and their chemistries characterized [7]. These bacteria use luciferase to synthesize and degrade aldehydes

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along with the oxidation of flavin mononucleotide in the presence of oxygen that results in the subsequent emission of light. The reaction is as follows:

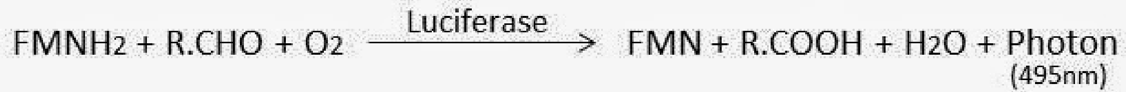

Figure 2.1 Luciferase reaction

The bioreaction catalyzed by luciferase resulting in the production of bioluminescent light.

The reaction is highly specific for $FMNH_2$. Modification of the flavin ring or removal of the phosphate group decreases the activity significantly [22]. Thus, the requirement of reducing power ($FMNH_2$) by the reaction implicates the measurement of *lux* gene activity as a means to monitoring metabolic activity.

The *lux* operon in *P. luminescens* consists of five genes, *lux*CDABE (Figure 2.2). Bacterial luciferase is a heterodimeric enzyme composed of α and β subunits which are encoded by *lux*A and *lux*B. Studies have shown that the α subunit almost exclusively controls substrate specificity and turnover rate of the enzyme [23]. The synthesis of aldehyde substrate for the bioluminescence reaction is catalyzed by a multi-enzyme fatty acid reductase complex containing three proteins, a reductase, a transferase, and a synthetase [24, 25]. These three polypeptides are encoded by *lux*C, *lux*D, and *lux*E, respectively. In brief, the transferase subunit catalyzes the transfer of activated fatty acyl group to water as well as other oxygen and thiol acceptors, creating the substrate for the following reaction catalyzed by the fatty acid reductase. The reductase and synthetase components make up the fatty acid reductase complex which catalyzes the reduction of

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fatty acids to aldehydes. The synthetase activates the fatty acid by creating a fatty acyl-AMP intermediate. In the presence of the reductase, the acyl group is reduced by NADPH to aldehyde [26]. All the components of the fatty acid reductase complex are specifically acylated during the enzyme reaction. Each subunit has been identified *in vivo* or in extracts by labeling with radioactive fatty acids [27].

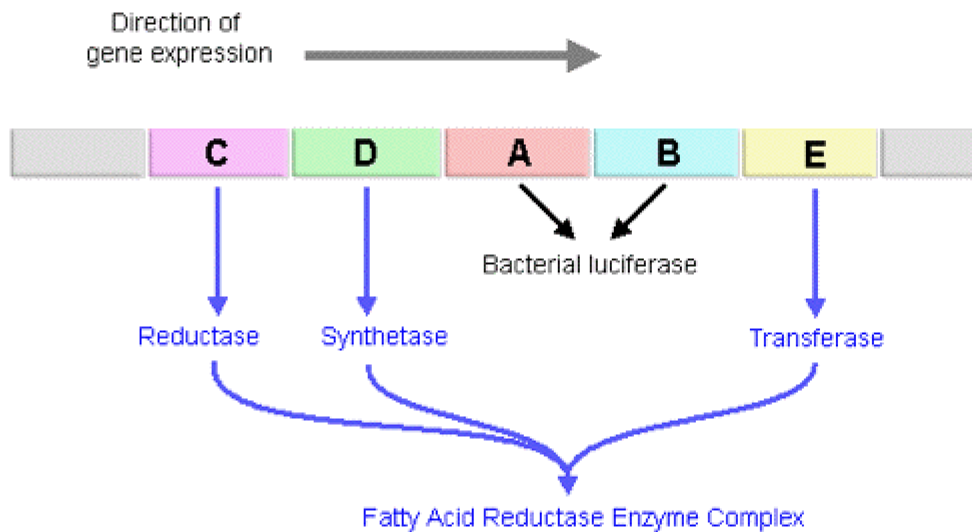

Figure 2.2 The *lux* operon.

The gene arrangements and direction of gene expression on the *lux*CDABE operon

The bioluminescence reaction does place an additional energy burden upon the cell and therefore may limit the scope of studies under certain environmental conditions, in which adequate energy reserves are not available. As is the case with sufficient energy reserves, sufficient O_2 is a requirement for the bioluminescence reaction to occur.

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Luciferase as a Rapid Screening Method

Luciferase has been shown to be a versatile tool to provide *in vivo* indication of cell localization and biological function. Emitted light from the luciferase reaction can be detected externally using sensitive photon detectors such as cooled integrating charge-coupled device (CCD) cameras to create bioluminescence images [28]. Light emission can then be measured with software designed specifically for *in vivo* applications. Bioluminescence imaging (BLI) has been applied to pathogen detection [29], tumor growth and response to therapy [30], patterns of gene regulation [31], and measurements of protein-protein interactions [32]. Since the initial demonstration of the expression of *lux* in *S.* Typhimurium [33], many bacterial strains have been engineered to track the course of an infection [34] or monitor the efficacy of antimicrobial therapies. The *lux* operon from *P. luminescens* has been transferred into indicator strains *S. aureus, E. coli,* and *S.* Typhimurium and used in an assay to demonstrate the well-documented effects of antimicrobial compounds tetracycline and nisin with EDTA on bacteria metabolism and viability [35]. The nature of tetracycline and nisin/EDTA is to disrupt protein synthesis and cell membrane integrity, respectively; causing destruction of the cell. For all indicator strains imaging indicated reduced bioluminescence, which paralleled reduced number of culturable cells. From these results, it was concluded that the effect of antimicrobial substances on the indicator strains can be detected earlier as reduced bioluminescence compared to standard plate counting and overnight incubation.

Additional applications of BLI include monitoring biological processes, such as the study of the biofilm forming-pathogen *S. aureus* in implanted medical devices [36], as well as identifying the unexpected extracellular replication of *L. monocytogenes* in the

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gall bladder lumen of mice [37]. In other instances, the *lux* operon was constructed to be induced under an appropriate promoter, such as iNOS (inducible nitrous oxide synthase gene) or NF-κB promoter, to monitor gene expression levels in animals [38-40]. Literature shows that traditional protocols can be supplemented with luciferase as a reporter gene in order to monitor gene expression or detect pathogens non-invasively through the course of infection, and in real time, to increase our understanding of the infectious disease processes, the underlying mechanisms of pathogenesis, and the host response to the disease. Currently, expression of the lux operon has been expressed in a narrow range of *Salmonella* species. This research aimed to expand the scope of luciferase reporter technology to include the most prevalent serotypes of *Salmonella* in the poultry processing continuum.

Review of Tn7 Transposon

Transposition is a unique process that involves the movement of genetic elements between non-homologous positions within genomes. Tn7 transposon is a rapid and reliable system for transposition that is distinguished by its ability to recognize and insert with high efficiency and unique orientation at specific site *att*Tn7 [41, 42]. This site of insertion is located just downstream of the coding region, in the transcriptional terminator, of the highly conserved chromosomal *glmS* gene [43]. The *glmS* gene encodes a glucosamine synthetase, which is required for cell wall synthesis [44]. Insertion does not disrupt the *glmS* open reading frame and causes no obvious fitness costs to the host [45]. Because the *glmS* gene is conserved among many bacteria, Tn7 is likely to have the same specific insertion site in many different bacteria [41, 46-53].

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Transposition is a process that is tightly regulated by the recognition of the target site and assembly of a TnsABC+D transposome from four transposon-encoded polypeptides: TnsA, TnsB, TnsC, and TnsD. Excision of Tn7 from the donor site does not occur unless *att*Tn7 and all the Tns proteins are present and site specific insertion does not occur without all four Tn7-encoded proteins [54]. Briefly, element-encoded transposases bind specifically to transposon ends and mediate the catalytic steps in transposition, cleaving the phosphodiester bonds that link the transposon to the donor site and joining the transposon ends to the insertion site [55-57]. TnsD binds to its 30-bp binding site at the end of the highly conserved *glmS* gene, which generates distortions in the DNA [54, 58]. TnsC is recruited to this distorted DNA [59]. The interaction of TnsC with TnsD-*att*Tn7 leads to the regulation of transposase activity via TnsC interactions with both TnsA and TnsB [60-63]. TnsA and TnsB form the transposase that carries out Tn7 DNA breakage and joining [64-66] and is controlled by interaction of TnsAB with TnsCD bound to *att*Tn7 [54].

Review of *Salmonella* Prevalence

Salmonella enterica infections are a significant public health concern globally, accounting for approximately 1 million cases, 19,000 hospitalizations, and nearly 400 deaths in the United States each year [4]. Human salmonellosis is typically associated with the consumption of contaminated foods, such as fresh and processed meat and poultry, eggs, and fresh produce [67]. The potential risk for exposure to *Salmonella* through contaminated food products is greater due to the increase in poultry consumption in the United States, and the subsequent growth of the commercial poultry sector in the animal agricultural industry. While strides have been made to limit the prevalence and

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frequency of *Salmonella* contamination in processed poultry, there is rising pressure on the commercial poultry industry to prevent and/or eliminate these pathogens during pre-harvest production. Pre-harvest is a critical stage for intervention due to the susceptibility of young birds to *Salmonella* colonization of the gastrointestinal tract by vertical transmission from infected parents or by horizontal transmission at the hatcheries during feeding, handling, and transportation [68, 69].

Review of *Salmonella* Pathology

Salmonella can colonize birds through fecal-oral transmission [70, 71]; however, in newly hatched chicks, colonization can also take place via the nose or cloaca [72]. These bacteria can pass through the gastrointestinal tract of the birds, in which *Salmonella* can express acid shock proteins (RpoS σ-factor, PhoPQ, and Fur) for survival at a low gastrointestinal pH and exposure to short-chain fatty acids in the poultry gut [73, 74]. *Salmonella* can colonize the intestinal area through several genetic changes, such as the expression of fimbria-associated proteins (Fim, Lpf, and Pef) [75-77] and genes encoding fimbrial types SEF17 and SEF21 [78] to facilitate adhesion of the bacteria to the host intestinal cell surface. *Salmonella* can then multiply and invade the intestinal mucosa, cecal tonsils, and Peyer's patches, survive and multiply in macrophages, spread to the liver and spleen via the bloodstream or lymphatic system, and eventually infect other organ systems (ovary, oviduct, gizzard, yolk sac, or lungs) [70, 74, 79]. The environment can become contaminated due to the excretion of the bacteria through feces. Vertical transmission of *Salmonella* has been reported in infected ovaries, oviducts, or infected eggs; these infections may be asymptomatic in adult birds [70].

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Review of *Salmonella*-Host Interaction

The interaction between *Salmonella* and host cells has been shaped by intimate coexistence, leading to a rather balanced interaction that allows bacterial replication while preventing excessive harm unless the host is compromised. The various serotypes of *S. enterica* cause different diseases, ranging from self-limiting gastroenteritis to a systemic illness such as typhoid fever. Central to the pathogenesis of *Salmonella* is the invasion of the gastrointestinal epithelium tissue. To accomplish this task, the bacteria have evolved virulence traits to adapt with their host that enables the organisms to induce their own internalization by non-phagocytic cells to successfully survive and replicate intracellularly [80]. The most extensively studied mechanism of invasion involves a multi-protein complex termed the type III secretion system (T3SS). Until recently, it was accepted that *Salmonella* invasion of eukaryotic cells required only T3SS encoded in the *Salmonella* Pathogenicity Island-1 (SPI1). However, recent studies have shown that *Salmonella* can cause infection in a manner independent of T3SS [11, 81]. This data sheds light on a new paradigm that slightly contrasts with traditional understanding, and lends itself to the possibility of unknown entry routes that may be specific to the serotype, the host, and the cell type considered, potentially affecting the outcome of different *Salmonella*-induced diseases.

Type III Secretion System

Many plant and animal bacterial pathogens assemble a complex needle-like nanomachine termed T3SS to translocate effector proteins from the cytoplasm of the bacteria into the cytosol of the host eukaryotic cell in order to initiate infection by activating or altering cellular processes [9]. The ability of bacteria to inject effectors into

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host cells is essential for infection, survival, and pathogenesis of many Gram-negative bacteria, including *Escherichia* [82], *Shigella* [83], *Yersinia* [84], *Pseudomonas* [85], and *Chlamydia* spp [86]. The structural genes, effector genes, and transcriptional regulators unique to *Salmonella* are clustered in one region of the genome, SPI1 (Figure 2.3). The gene cluster is theorized to have been acquired by one or more horizontal transfer events during its evolution [87]. Support for this theory stems from the deviating GC base content of the pathogenicity island from that of its chromosome and artifacts of transposable elements that flank the region [87].

Figure 2.3 Orientation of *Salmonella* Pathogenicity Island-1

The genes encoding structural proteins are denoted as grey, the genes encoding effectors are denoted as white, and the genes that encode transcriptional regulators are denoted as black.

Emergence of Alternate Mechanisms of Entry

Salmonella entry into host cells strictly depends on the function of the actin cytoskeleton as addition of drugs that interfere with actin dynamics effectively block bacterial internalization [88]. An emerging perspective on *Salmonella* speculates infection of gastrointestinal epithelium cells may occur through multiple pathways, which contrasts with the traditional essential role of T3SS to bacterial invasion of host cells. Although numerous studies demonstrate a role of type III secretion in host infection,

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recent studies using bovine, chicken, murine, and human models to determine the function of SPI1 suggests *Salmonella* is able to cause infection in a manner independent T3SS [10, 11, 81, 89]. Radtke et al. demonstrated *S. enterica* serovar Typhimurium was capable of active invasion of a 3-D model of human colonic epithelium cells without the expression of T3SS genes [10]. Desin et al. observed a *S. enterica* serovar Enteritidis strain mutated for SPI1 expression displayed invasion of polarized human intestinal epithelial cells (Caco-2) and chicken intestinal tissue explants in a reduced capacity in comparison to the wild-type strain [89]. The same study orally challenged 1-week-old chicks with the wild-type or SPI1 mutant strain and no difference in cecal colonization was observed, suggesting infection mechanisms correspond with chicken maturity and infection in this situation may not be exclusive to T3SS [89]. Similar findings were observed by Morgan et al., in which disruption of T3SS caused only minor reduction in *S. enterica* serovar Typhimurium colonization of chicks [90]. The type of interaction between *Salmonella* and its host leading to infection is sophisticated and dynamic, dependent on the serovar, cell-type considered, and host; and support from scientific literature suggests additional mechanisms may act in a complementary role to T3SS to facilitate and modify the initiation of eukaryotic cell entry [91, 92].

Emergence of *Salmonella* Kentucky

Spanning the last several decades, there have been significant shifts in *Salmonella* populations associated with poultry and human infections. Among the leading serotypes associated with human infections over the last quarter century, the widespread prevalence of *S. enterica* serovar Enteritidis among poultry developed into a significant problem in the commercial poultry industry [93, 94] until the National Poultry Improvement Plan

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(NPIP) began targeting *S*. Enteritidis for eradication in eggs 1989 and in meats in 1994 [95]. The ecological niche in poultry created by the reduction of *S*. Enteritidis may have been filled by *S. enterica* serovar Heidelberg and *S. enterica* serovar Kentucky due to differences in surface antigens [96]. Initially isolated from a chick in the U.S. in 1937 [97], *S*. Kentucky has a close association with poultry and poultry products, and recently has been the most frequently isolated serotype on broilers as they exit the chiller during processing [98-101]. The practice of antibiotic use in agriculture, especially in the United States, not only to treat and prevent disease but also to promote growth [102] may have contributed to the ascension and dissemination of *S*. Kentucky. Multidrug resistant variants of *S*. Kentucky have been identified at a high frequency from chickens in the United States [103] and in Ireland [104]. Additionally, multidrug resistant variants of *S*. Kentucky have been identified from human samples in France, England and Wales, Denmark, United States, and Canada with travel from northern Africa in common [105-108]

Summary

In the literature review chapter, key scientific concepts related to this study were discussed and placed in the context of the research community, highlighting the gap in the knowledge this current study aims to fill. Components of the *lux* operon were outlined, in addition to recent applications of the luciferase gene as a biosensor in microbiology. The details of the transposition reaction were explored as a molecular technique to be used in this study for gene transfer. A brief overview of the T3SS was outlined, highlighted with recent reports of possible entry methods for *Salmonella* that may supplement the function of T3SS. In the conclusion of this chapter, the emergence of

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S. Kentucky in the poultry industry and as an international MDR bacterial strain among travelers was reported in depth. The following chapter will encompass the research devoted to the establishment of the plasmid-based *lux* operon as a reporter system in field-isolated *Salmonella* serotypes.

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CHAPTER III

DEVELOPMENT OF PLASMID-BASED REPORTER SYSTEM FOR *SALMONELLA ENTERICA*

In this chapter, the aim was to develop twelve bioluminescent *S. enterica* serotypes that can be used for real-time monitoring of the pathogen's growth on food products. This study is unique in that it includes multiple *Salmonella* field-isolated serotypes from the broiler production continuum, including post hatchery, prior to harvest, arrival at the plant, pre-chill tank, and post-chill tank. This chapter also outlines the development of the protocol for a novel assay that can quickly and precisely quantify viable bacteria numbers present on chicken skin that has potential application as a tool to evaluate various mitigation strategies.

*The following chapter has been submitted for publication and is published in the open access journal BMC Microbiology.

Karsi A, Howe K, Kirkpatrick TB, Wills R, Bailey RH, Lawrence ML: **Development of bioluminescent *Salmonella* strains for use in food safety**. *BMC Microbiol* 2008, **8**:10.

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Methodology

Bacterial Serotypes and Growth Media

As part of a previous study, thousands of *Salmonella* specimens derived and catalogued from five different sites along the broiler production continuum: post-hatchery, prior to harvest, arrival at the plant, pre-chill tank and post-chill tank were collected, isolated, and serotyped [109]. *Salmonella* isolates were collected from 66 different flocks during the years 2003-2006. The catalogued information ascribed to each isolate includes: location within the production continuum, flock environmental and production parameters, and processing plant information. The twelve most commonly isolated *S. enterica* serotypes from the poultry production continuum were selected for the following study (*S.* Alachua, *S.* Braenderup, *S.* Enteritidis, *S.* Heidelberg, *S.* Kentucky, *S.* Mbandaka, *S.* Montevideo, *S.* Newport, *S.* Schwarzengrund, *S.* Senftenberg, *S.* Thompson, and *S.* Typhimurium). *S. enterica* serotypes were cultured in Luria-Bertani (LB) media and agar plates at 37°C. Plasmid selection was conducted in media supplemented with ampicillin (Amp) (100 µg/mL).

Bioluminescence Tagging of *Salmonella enterica* with Plasmid pAK*lux*1

Bioluminescent *Salmonella enterica* serotypes were established using broad host range plasmid pAK*lux*1 [110] (Figure 3.1). Plasmid pAK*lux*1 contains the *lux*CDABE operon cloned from *P. luminescens* (Meighen, 1991). Bacterial concentration was determined by optical density values at wavelength 600 nm (OD_{600}) using GENESYS 20 Visible Spectrophotometer (Thermo Fisher Scientific, Waltham, MA). *S. enterica* serotypes was grown to logarithmic phase (OD_{600} = 0.6-0.8) in preparation of washing with 10% v/v cold glycerol solution (4x) to create electrocompetent cells. Cells were

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stored at -80°C until transformation. Plasmid pAK*lux*1 was prepared from *Escherichia coli* DH5α strain using Qiagen QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA). Transformation of *Salmonella enterica* serotypes with plasmid pAK*lux*1 was conducted by electroporation using Gene Pulser II System with parameters 2.5 kV, 25 μF, and 400 Ω (Bio-Rad, Hercules, CA). Cells recovered in S.O.C. media (Invitrogen Corp., Carlsbad, CA) at 37°C for 1 h. Following recovery, bacteria suspensions were spread on LB + Amp agar to incubate at 37°C for approximately 16 h to select for successful transformations. Colonies displaying Amp resistance were tested for bioluminescence production using ChemiImager 5500 Imaging System integrated with AlphaEaseFC Software (Alpha Innotech, San Leandro, CA) or IVIS Imaging System 100 Series integrated with Living Image Software v2.50 (Xenogen Corp., Alameda, CA).

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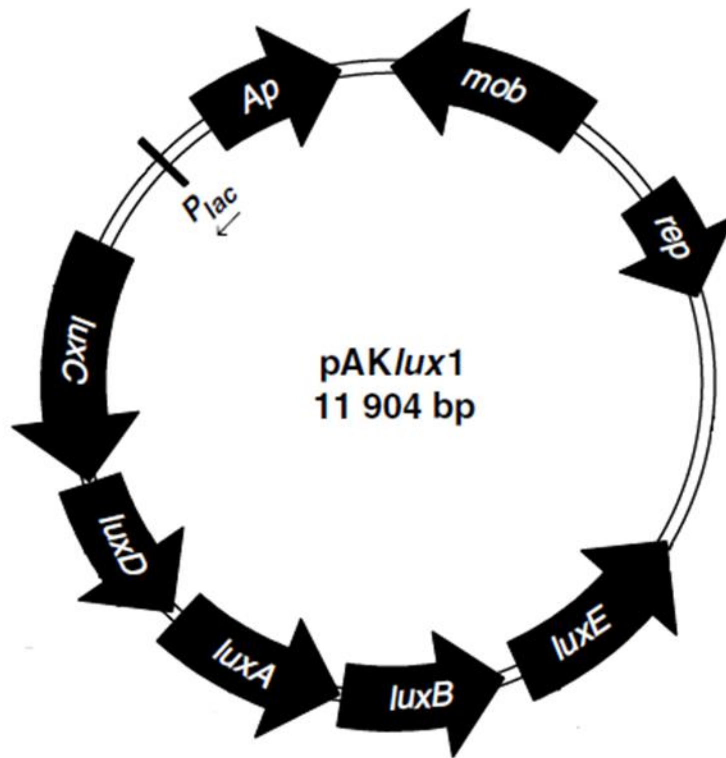

Figure 3.1 Physical map of the broad host range plasmid pAK*lux*1

A 6.9-kb *Eco*RI fragment carrying the *lux* operon cloned from *P. luminescens* is oriented such that the operon is transcribed from P_{lac}.

Characterizing Bioluminescence Properties of Plasmid-Based Luciferase Reporter System

Bioluminescent *S. enterica* serotypes maintaining plasmid pAK*lux*1 were grown to stationary phase and confirmed with OD_{600} values measured with Thermomax Spectrometer (Molecular Devices, Sunnyvale, USA). Serotypes were arranged as quadruplicate samples in black clear-bottom 96-well microtiter plate (Costar). A dilution series was prepared for each sample. Each series contained 2 x 10^{-3}, 4 x 10^{-3}, 8 x 10^{-3}, 1.6 x 10^{-4}, 3.2 x 10^{-4} 6.4 x 10^{-4}, 1.3 x 10^{-5}, and 2.6 x 10^{-5} dilutions. Bioluminescence was measured with IVIS Imaging System 100 Series with 5 s exposure at 37°C and quantified

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using Living Image Software v2.60. Bioluminescence was normalized by dividing total flux by OD_{600} readings. Minimum detectable bacterial densities were determined from the last two dilutions using plate counts on LB + Amp agar.

Linear correlation formulas were calculated for each serotype using bioluminescence values and bacteria population numbers determined by duplicate plate counts. The minimum detectible number for each serotype was identified using the number of bacteria present in the last dilution that displayed detectable bioluminescence above background. Colony counts aided in calculation of the theoretical amount of bioluminescence produced per colony forming-unit (CFU) for each serotype.

Stability of Plasmid pAK*lux*1 in *Salmonella* enterica

Stability of plasmid pAK*lux*1 in *S. enterica* serotypes was assessed by subculturing the twelve *S. enterica* serotypes with plasmid pAK*lux*1 in LB media with and without Amp for selection for a period of 15 d. For each passage, fresh 0.1 mL cultures were inoculated at a 40-fold dilution in black clear-bottom 96-well microtiter plate in quadruplicate from overnight cultures. Bacterial density (OD_{600}) and bioluminescence were determined for each of the overnight cultures prior to starting the next round of inoculations. Bioluminescence was measured using IVIS Imaging System with exposure time 5 s at 37°C and normalized by dividing total flux by OD_{600} readings. The average normalized bioluminescence for each serotype and passage was determined under non-selective and selective conditions. Plasmid stability was determined by calculating the ratio between normalized bioluminescence under non-selective conditions and selective conditions. This ratio revealed the half-life of plasmid pAK*lux*1 under non-selective conditions in each serotype.

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Development of Skin Attachment Model

The protocol for an experimental model to investigate bacterial attachment to chicken skin is outlined as follows. Chicken skin obtained from a Mississippi commercial poultry processing plant was submerged in 0.26% sodium hypochlorite solution for approximately 2 h and stored at 4°C until use. Circular sections, approximately 8 mm in diameter of chicken skin were made using a circular cutting blade and placed in black clear-bottom 24-well tissue plate (Wallac). In previous work to determine the optimal bacterial dose to prevent loss of experimental data due to image saturation, chicken skin was incubated with different numbers of bioluminescent *Salmonella* (1×10^8 - 2.5×10^4 CFU) in 1 mL of phosphate-buffered saline (PBS). Based on these results (data not shown), a bacterial concentration of 1×10^6 CFU/mL was used for subsequent experiments to avoid saturation.

Characterization of Skin Attachment Properties of *Salmonella enterica*

The skin attachment model was constructed with the bioluminescent *S. enterica* serotypes to develop a quick and precise method to determine differential attachment properties. Fresh bacterial culture was diluted to approximately 1×10^6 CFU/mL in sterilized distilled water, and 1 mL was inoculated on skin sections. Quadruplicates for each serotype were arranged in black clear-bottom 24-well tissue plate. After inoculation, cultures incubated at room temperature for 1 h to allow bacteria to attach to the skin. Following incubation superfluous bacterial suspension was vacuum removed and each well gently washed with sterilized distilled water. This process was repeated twice to extract unattached bacteria. Plates were immediately warmed to 37°C for 5 m and

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bioluminescence was quantified using IVIS Imaging System with exposure time 15 s. The experiment was repeated three times.

Total flux was calculated from the pseudo color images produced by the IVIS Imaging System. To normalize for bacterial density differences between serotypes, the total flux from each well was divided by OD_{600} of the initial bacterial suspension used to inoculate that well. Due to variance of bioluminescence among the different serotypes, theoretical numbers were calculated from bioluminescence using the linear correlation formulas calculated for each serotype. The data was transformed by taking the base 10 logarithm of the calculated bacterial numbers to improve normality. Statistics on the transformed data of the bacterial numbers were calculated by a two-way analysis of variance (ANOVA) using PROC GLM SAS 9.1 (SAS Institute Inc., Carey, NC). Variables for this model included replicate, serotype, and their interaction. Pairwise comparison of the means was conducted using Tukey procedure. A significance level of $P \leq 0.05$ was used for all statistical purposes. Data was then retransformed to percent mortality for interpretation.

Salmonella enterica Removal from Chicken Skin by Washing

During traditional poultry processing, a component in microbial removal from the carcasses includes chlorine hydration treatment in the forms of carcass cabinet washers, immersion chillers, or equipment sprays [111-113]. A simulation of these parameters was constructed using our skin attachment model in order to design a laboratory assay for various experimental treatments with quick and simple interpretation of results that operates under actual poultry processing conditions. Samples of our twelve bioluminescent *S. enterica* serotypes were incubated with chicken skin sections as

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described for the skin attachment model. The non-washed skin acted as the control group and the washed skin as the treatment group. Following the 1 h incubation period and wash and vacuum to extract superfluous bacteria, the assay plate was warmed to 37°C for 5 m in preparation for bioluminescence imaging. Each treatment well containing microbial contaminated poultry skin received 1 mL of sterilized distilled water. Assay plates were subjected to repeated series of incubations at room temperature (25°C) for 30 m with agitation at 200 rpm followed by water removal by vacuum. Bioluminescence was measured from the pseudo color images of the chicken skin to quantify bacterial presence. One mL of sterilized distilled water was added to the treatment group to begin the next round of washing until a total of 2 h washing had been completed. The groups were set up in quadruplicate and the results were collected from three separate trials. Total flux values were normalized as described previously in the skin attachment model.

The difference between the initial total flux of the samples before treatment (0 m) from the final total flux after treatment (120 m) was calculated to determine the accumulative effect all of the washes had on microbial removal from the surface of chicken skin. The difference value was averaged among the quadruplicates over the three trials to produce a mean difference value for each serotype. The mean difference for a serotype of the washed group was compared to its counterpoint in the non-washed group by ANOVA using PROC GLM SAS 9.1 to evaluate the effectiveness of washes to remove *Salmonella* from poultry skin. Variables in this model included replicate, treatment, and their interaction. The mean difference value was compared across serotypes with ANOVA using PROC GLM SAS 9.1 to determine the relevant degree of microbial removal due to washes. Variables for this model included replicate, serotype,

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and their interaction. Pairwise comparison of the means was also conducted using Tukey procedure. A significance level of $P \leq 0.05$ was applied in all statistical analyses.

Results and Discussion

Characterizing Bioluminescence Properties of Plasmid-Based Luciferase Reporter System

Plasmid pAK*lux*1 was transferred to twelve *S. enterica* serotypes that were isolated from the poultry production and processing continuum [109]. The serotypes represented the twelve most commonly isolated serotypes from the study. This result compared favorably with a previous report where transformation of a *lux* plasmid into *Salmonella* isolated from a poultry processing plant was only successful for one isolate out of seven attempted [114]. Expression of the *lux*CDABE operon, which encodes bacterial luciferase, was driven by the *lacZ* promoter on pAK*lux*1. Because *Salmonella* does not have $lacI^q$ in its chromosome, it constitutively expresses the *lacZ* promoter on pAK*lux*1 and hence produces continuous light while it is alive and metabolically active.

We showed that bacteria numbers and bioluminescence correlated well (R^2 = 0.99) in all serotypes used (Figure 3.2). The minimum detectable numbers for all twelve serotypes was less than 1500 CFU/mL and it was less than 300 CFU/mL for a majority of serotypes: *S*. Alachua, 334 CFU/mL; *S*. Braenderup, 217 CFU/mL; *S*. Enteritidis, 175 CFU/mL; *S*. Heidelberg, 169 CFU/mL; *S*. Kentucky, 229 CFU/mL; *S*. Mbandaka, 248 CFU/mL; *S*. Montevideo, 209 CFU/mL; *S*. Newport, 125 CFU/mL; *S*. Schwarzengrund, 1470 CFU/mL; *S*. Senftenberg, 1386 CFU/mL; *S*. Thompson, 1044 CFU/mL; and *S*. Typhimurium, 202 CFU/mL. This result was comparable to previous studies [33, 110, 115, 116].

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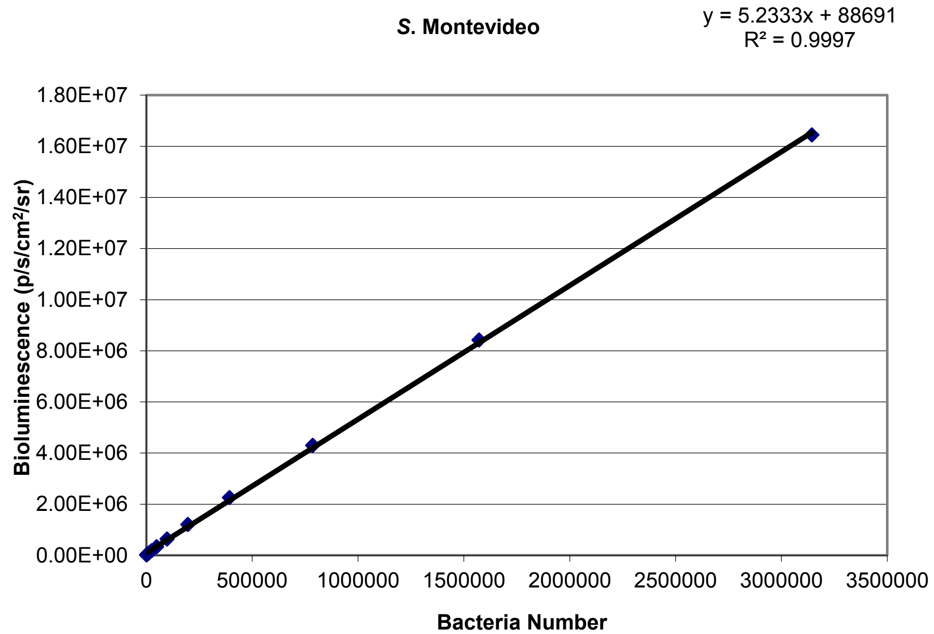

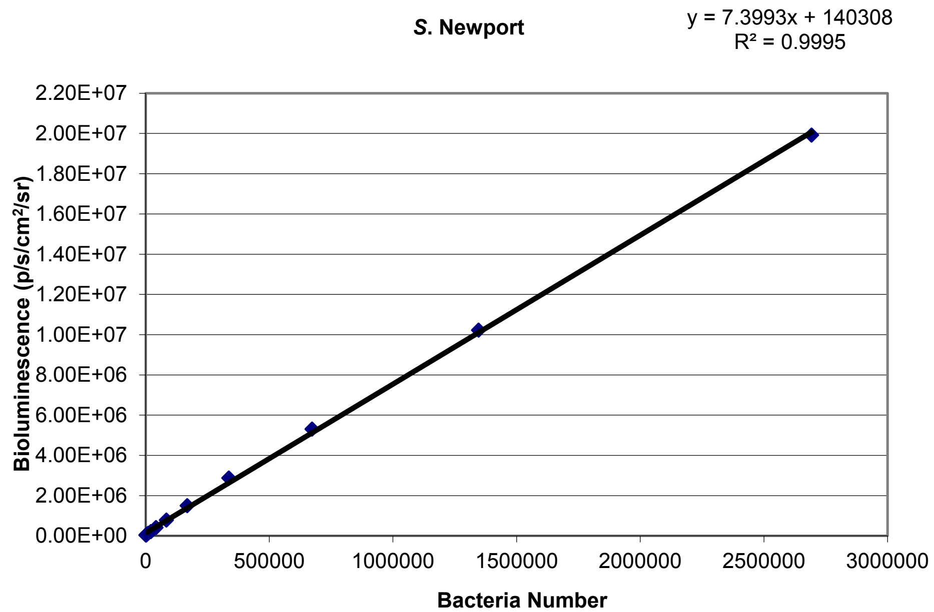

Figure 3.2 Correlation between bioluminescence and bacterial numbers

The correlation between bioluminescence signals and bacteria numbers for representative serovars *S.* Montevideo and *S.* Newport.

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The average theoretical light intensity per CFU was calculated for each serotype and revealed a greater than 10-fold difference between some serotypes (Figure 3.3). This difference was not due to lack of viability; bacterial plate counts showed that all the serotypes were viable at this stage and that viable bacterial densities for all the serotypes were within a 3 fold range. The difference in bioluminescence could reflect a difference between serotypes in efficiency of *lux*CDABE expression from the *lacZ* promoter, or it could reflect a difference between serotypes in the activity of bacterial luciferase within the bacteria. Alternatively, the difference could reflect differences in metabolic activity between the serotypes at this stage of growth (16 h). Bioluminescence is known to correlate well with bacterial metabolism [117, 118].

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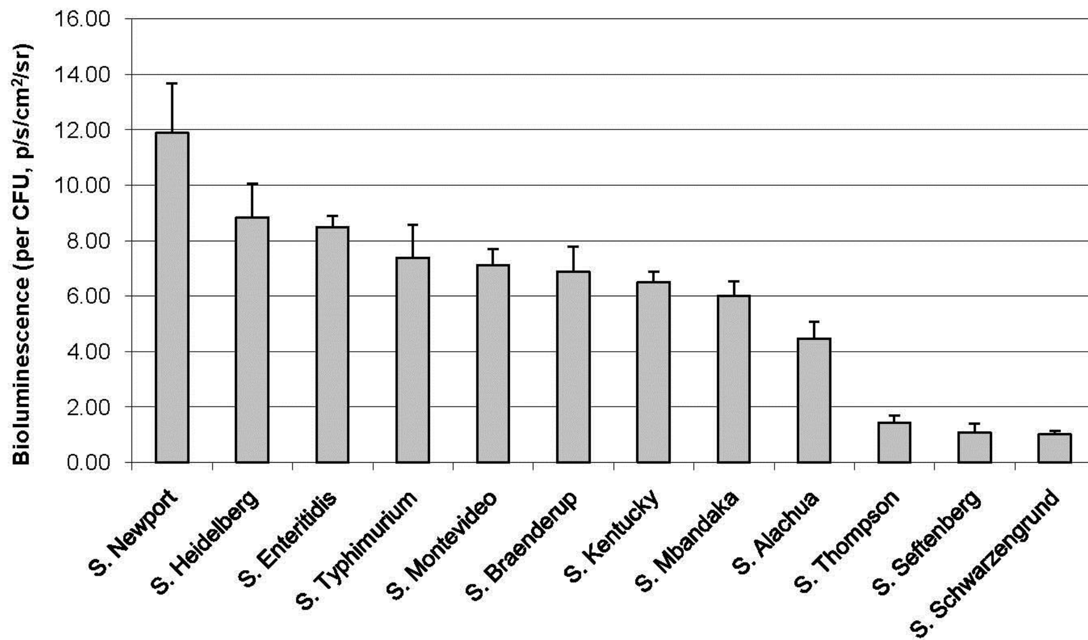

Figure 3.3 Expression of bioluminescence in *Salmonella enterica* serotypes

The theoretical amount of bioluminescence produced per CFU of each *S. enterica* serotype following 16 h of growth was calculated. Bioluminescence per CFU was calculated by dividing the background subtracted bioluminescence value (in p/s/cm^2/sr) in each well by the number of bacteria (determined by serial dilution and plate counts). The mean and standard error from four replicates were then determined for each serotype. Light emission was almost 12 fold higher in *S.* Newport as compared to *S.* Schwarzengrund

Stability of Plasmid pAKlux1 in *Salmonella enterica*

The stability of plasmid pAK*lux*1 was determined by subculturing eleven bioluminescent *S. enterica* serotypes in broth cultures under selective (Amp) and non-selective conditions for 15 d. Bioluminescence of *S. enterica* serotypes cultured under non-selective conditions was declining by day 2 and continued declining linearly (R^2 = 0.95) until the conclusion of the experiment (Figure 3.4). Based on the data, the average

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half-life of plasmid pAK*lux*1 in *S. enterica* was approximately 7 d under the described culture conditions. However, the stability varied between *S. enterica* serotypes. Among the *S. enterica* serotypes, plasmid stability was lower in *S.* Kentucky and *S.* Typhimurium as compared to the others. The half-life of plasmid pAK*lux*1 was about 4 d in *S.* Kentucky and about 5 d in *S.* Typhimurium (Figure 3.4). *S.* Newport, *S.* Alachua and *S.* Enteritidis maintained plasmid pAK*lux*1 longer than others, with a half-life of approximately 9 d.

Plasmid pAK*lux*1 is derived from the broad host range plasmid pBBR1, which is relatively stable in gram-negative bacteria in the absence of antibiotic selection [119-121]. Plasmid pAK*lux*1 was stable in the Gram-negative species *Edwardsiella ictaluri* for at least 10 d without antibiotic selection, and the plasmid caused no alterations in growth kinetics, native plasmids and pathogenicity as compared to the parent strain [110]. Data from the current study indicated that the plasmid pBBR1 replicon is not as stable in *Salmonella* as it is in other Gram-negative bacteria.

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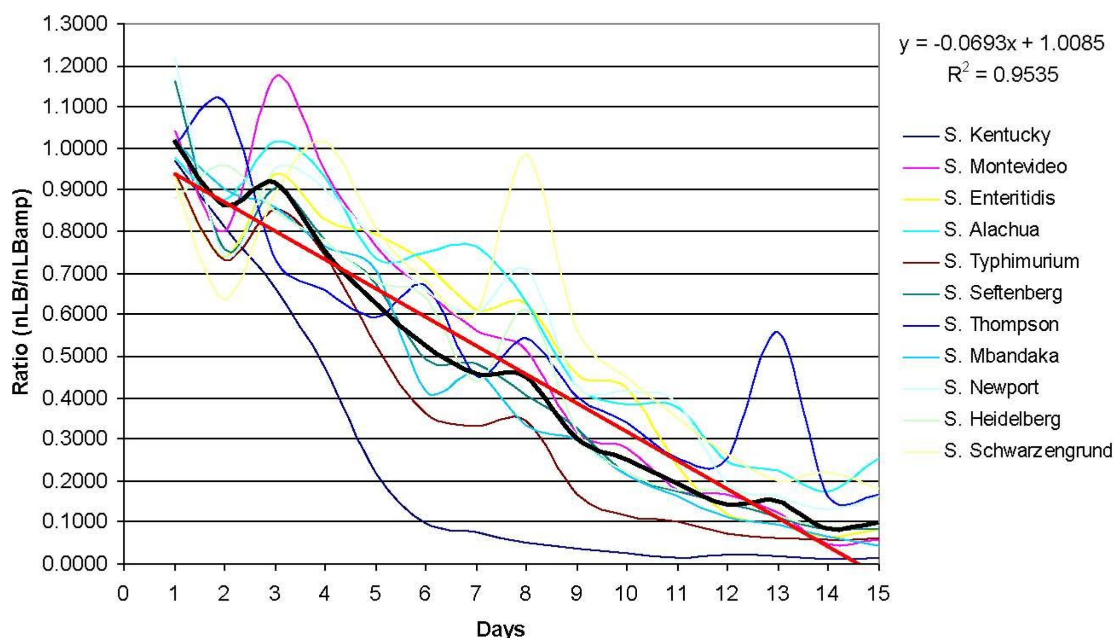

Figure 3.4 Stability of plasmid pAK*lux*1 in *Salmonella enterica*

S. enterica serotypes carrying pAK*lux*1 were subcultured under Amp selective and non-selective conditions for 15 d. At each passage, bioluminescence was measured and normalized for cell density (OD_{600}). The ratio between the normalized values under non-selective (nLB) and Amp selected conditions (nLBAmp) revealed the plasmid stability in different *S. enterica* serotypes. The black line represents the mean of eleven serotypes, and the red line is the linear trend line.

Characterization of Skin Attachment Properties of *Salmonella enterica*

We developed an *in vitro* skin attachment model for characterization of attachment properties of different *S. enterica* serotypes using bioluminescence imaging (BLI). Bioluminescence was successfully detected on chicken skin after being exposed to *S. enterica* serotypes expressing plasmid pAK*lux*1. Using this model, we were able to show that *S. enterica* serotypes vary in their ability to attach to chicken skin (Figure 3.5). Bacteria numbers in *S.* Senftenberg, *S.* Thompson and *S.* Schwarzengrund were significantly different from each other and from other serotypes ($P \leq 0.05$). The *S.* Heidelberg serotype had significantly less binding to chicken skin than all the other

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serotypes and was 79 fold lower than *S*. Senftenberg, which had the highest amount of binding (Figure 3.5). This result suggests that *S. enterica* serotypes vary in their ability to bind to chicken skin; this ability may be a discriminating factor determining whether *Salmonella* persist through processing or whether they are removed.

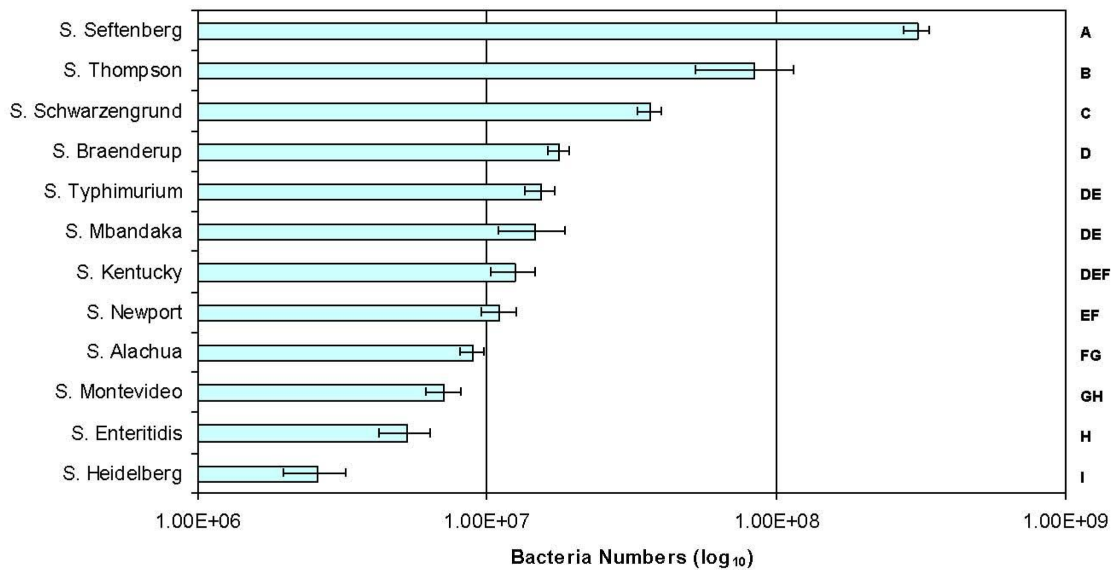

Figure 3.5 Attachment of *Salmonella enterica* to chicken skin

The amount of *Salmonella* attachment to chicken skin was determined by measuring the bioluminescence following 1 h incubation of bacterial suspension with quadruplicate skin samples. Bacteria numbers of each serotype were estimated from the bioluminescence values using each serotype's linear correlation formula.

Salmonella enterica Removal from Chicken Skin by Washing

Bioluminescence was an effective tool for measuring the effects of washing for removal of *S. enterica* from chicken skin using our model (Figure 3.6). Bioluminescent *S. enterica* have been previously utilized to monitor progress of infection, effect of heat and pH treatments, growth in food samples and toxicity [33, 114, 122, 123]. One study used a single bioluminescent *Salmonella enterica* serovar Hadar isolate to investigate the

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effectiveness of washing for removal of *Salmonella* from turkey skin [114]. The current study demonstrates to the extent of our knowledge, the first use of BLI to monitor twelve distinct *S. enterica* serotypes using a chicken skin model.

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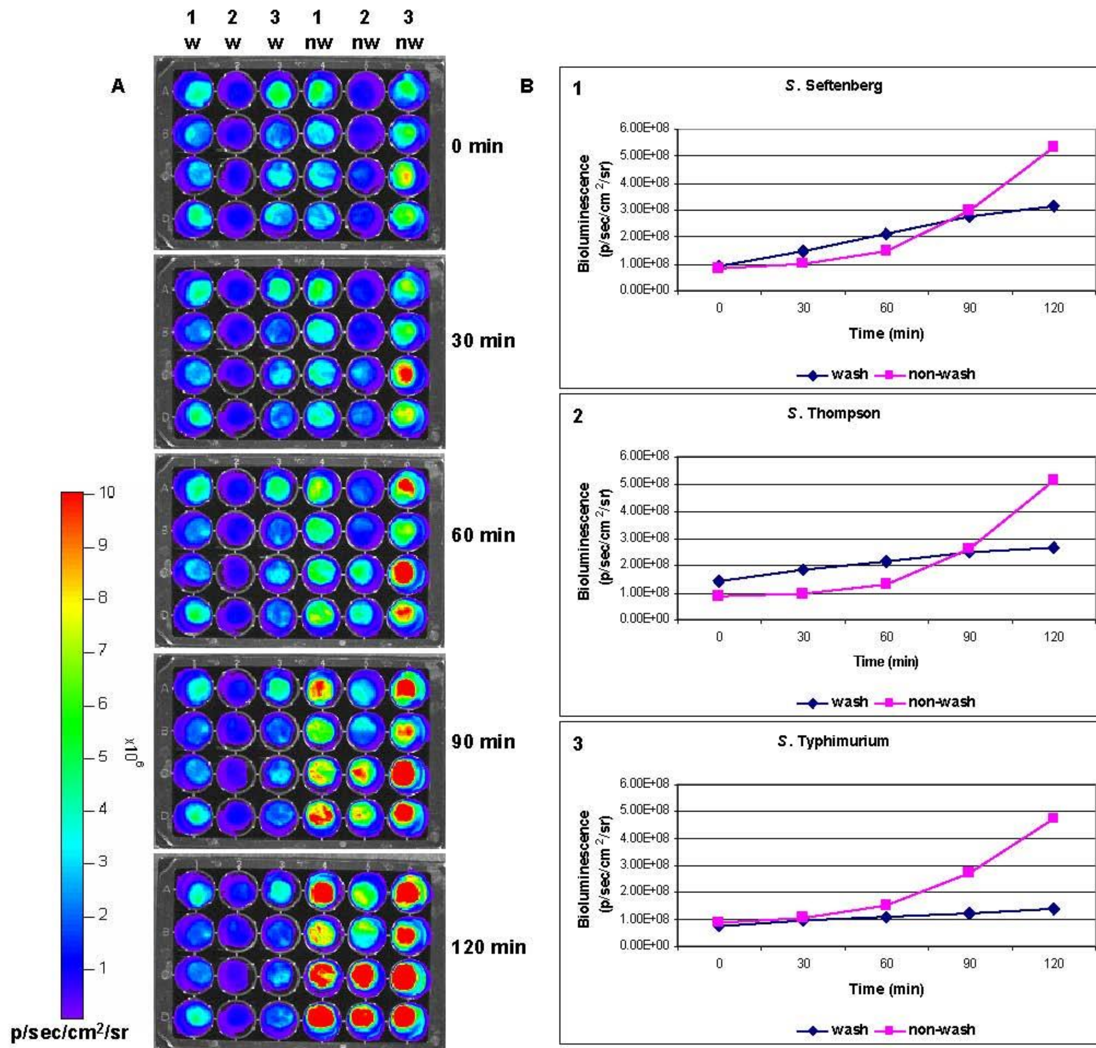

Figure 3.6 The effect of washing on removal of *Salmonella* from chicken skin

Bioluminescence was used to measure the ability of an agitated water bath to remove *S. enterica* serotypes from chicken skin. *Salmonella* was allowed to attach to chicken skin for 1 h, and then the amount of Salmonella present on skin that received four 30 m washes was compared to the amount of *Salmonella* present on non-washed skin. Bioluminescence was measured after each 30 m wash and the mean of four replicates from the three separate experiments was determined. A) Representative plate containing three *S. enterica* serotypes at five time points. Half of the plate included washed skin samples (w) and the other half included unwashed skin controls (nw). Column 1 is *S.* Senftenberg, column 2 is *S.* Thompson, and column 3 is *S.* Typhimurium. B) Amount of bioluminescence for the three representative serotypes at each time point.

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As a general trend observed for all serotypes, washing suppressed the reproduction of *Salmonella* on chicken skin, probably due to physical removal of bacteria (Figure 3.7). In non-washed skin samples, Salmonella numbers increased steadily over the two hour incubation period as indicated by bioluminescence quantification (Figure 3.6B), with final flux numbers showing an increase ranging from 143% (*S.* Schwarzengrund) to 459% (*S.* Newport) compared to initial measurement at time zero. Washed skin samples showed a lower increase in bioluminescence at the conclusion of the experiment compared to the non-washed skin samples, demonstrating the effectiveness of simple agitation in water for suppressing *Salmonella* growth on chicken skin.

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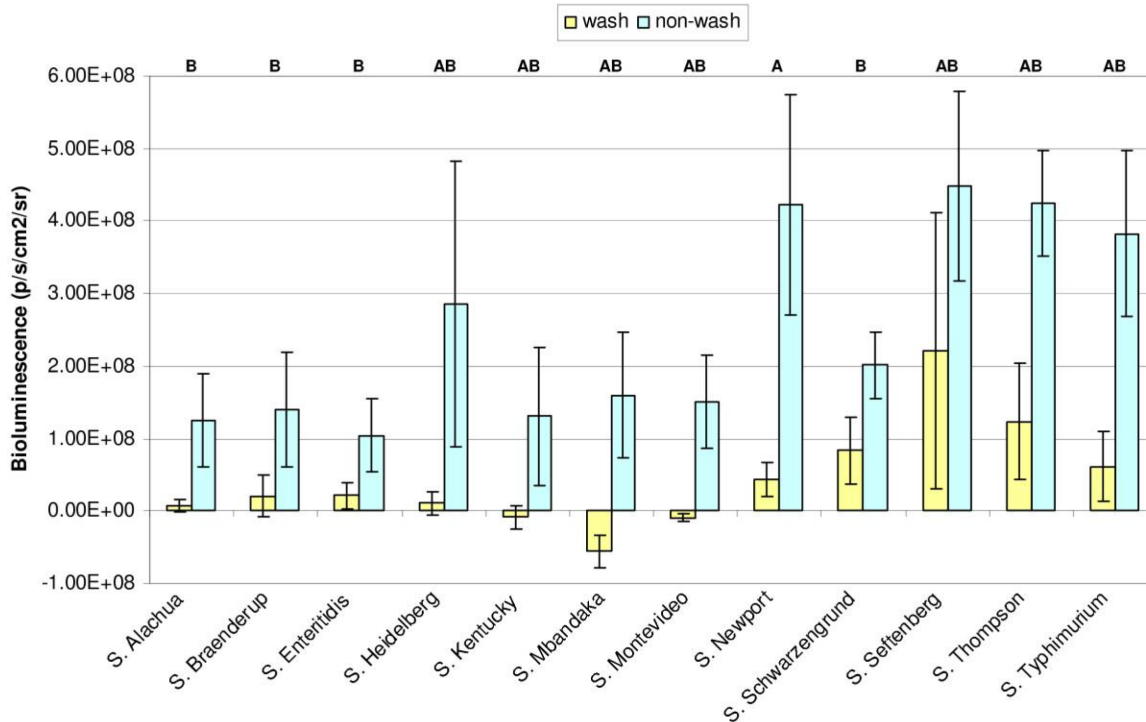

Figure 3.7 Comparison of the amount of *Salmonella* before and after 2 h incubation

The change in bioluminescence on chicken skin following 2 h incubation (relative to pre-wash bioluminescence) for washed and non-washed treatments is shown. Letters at the top of the graph indicate statistical groupings for the mean differences between washed and non-washed treatments within each serotype as determined by Tukey's test. Values with the same letter are not significantly different ($P \leq 0.05$).

For all twelve serotypes, the washed treatments had a significantly lower change in bioluminescence than the corresponding non-washed treatments after the 2 h wash period. However, there was serotype variation in the effectiveness of washing (Figure 3.7). For three serotypes (*S.* Kentucky, *S.* Mbandaka and *S.* Montevideo), washing reduced the number of *Salmonella* present on chicken skin at 120 m compared to 0 m (pre-wash). For the other serotypes, although washing did not decrease the number of *Salmonella* on chicken skin, as indicated by bioluminescence measurements, it was effective in significantly reducing the growth relative to non-washed samples.

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When the differences in bioluminescence between washed and non-washed treatments at 120 m were compared across serotypes, the decrease in bioluminescence for *S.* Newport caused by washing was significantly greater than the decrease in bioluminescence for *S.* Alachua, *S.* Braenderup, *S.* Enteritidis and *S.* Schwarzengrund (Figure 3.7). Thus, based on the difference in bioluminescence between washed and non-washed treatments, washing was observed to be most effective in removing *S.* Newport and lease effective for *S.* Alachua, *S.* Braenderup, *S.* Enteritidis and *S.* Schwarzengrund. However, it is interesting to note that the two serotypes that attached to chicken skin most effectively (*S.* Senftenberg and *S.* Thompson; [Figure 3.5]) were also able to increase their bioluminescence output in the wash treatment most effectively (3.7 and 2.7 fold increases, respectively, relative to pre-wash) over the 2 h incubation period.

Summary

In this chapter, the initial version of our luciferase reporter system was established in twelve serotypes of *Salmonella* isolated from the poultry processing continuum. The minimum detectable limit was estimated for each serotype and the plasmid to have an average half-life of 7 d without antibiotic pressure. Certain instances when antibiotic pressure cannot be applied, length of observation must be taken into consideration when designing experiments using plasmid pAK*lux*1. Bioluminescent *S. enterica* serotypes labeled with plasmid pAK*lux*1 should be suitable for short term experiments. *In vivo* experiments as well as studies with longer time courses will require a stable construct for the reporting system. In response to this limitation, the following chapter will introduce our research devoted to cloning the *lux*CDABE operon into a Tn-7 based transposon

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system that will insert the *lux* operon into the chromosome at a specific location and confer stability to the *lux* cassette.

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CHAPTER IV

DEVELOPMENT OF CHROMOSOME-BASED REPORTER SYSTEM FOR *SALMONELLA ENTERICA*

In this chapter, we aimed to develop a stable bioluminescence reporter system for several *S. enterica* serotypes. We used serotypes isolated from the broiler production continuum, including post-hatchery, prior to harvest, arrival at the plant, pre-chill tank, and post-chill tank. Furthermore, we used these serotypes to conduct a series of experiments to monitor cellular metabolic activity and pathogen attachment to chicken skin under simulated processing conditions by quantifying bioluminescence from the bacteria using a luminometer.

*The following chapter has been submitted for publication and is published in the open access journal BMC Microbiology.

Howe K, Karsi A, Germon P, Wills R, Lawrence ML, Bailey RN: **Development of stable reporter system cloning *luxCDABE* genes into chromosome of *Salmonella enterica* serotypes using Tn7 transposon**. *BMC Microbiol* 2010, **10**:197

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Methodology

Bacterial Serotypes and Growth Media

Eleven *S. enterica* serotypes from the poultry production continuum were selected from our catalogued library of isolates for this study (*S.* Alachua, *S.* Braenderup, *S.* Enteritidis, *S.* Heidelberg, *S.* Kentucky, *S.* Mbandaka, *S.* Montevideo, *S.* Newport, *S.* Schwarzengrund, *S.* Senftenberg, and *S.* Typhimurium). *S. enterica* serotypes were grown with Luria-Bertani (LB) media and agar plates at 37°C. Plasmid selection within *S. enterica* was conducted in media that contains ampicillin (Amp) at concentration 100 µg/mL. LB media was supplemented with 0.1% arabinose to induce transposition when appropriate.

Construction of Plasmid pBEN276

The *lux*CDABE operon was amplified from the genome of *Photorhabdus luminescens* with primers PG131 (gatgctacctcgaggtacaaccagtttgcaagatg) and PG132 (tacgctcaggatccgaattcactcccttgccatc) and cloned in plasmid pCR21 (Invitrogen) to yield plasmid pBEN139. The *Xho*I-*Bam*HI restriction fragment from plasmid pBEN139 that includes genes *lux*CDABE was subcloned in plasmid pBEN129, a derivative of plasmid pACYC184 [124], into *Xho*I and *Bam*HI sites to yield plasmid pBEN135. The *Xho*I-*Not*I fragment from plasmid pBEN135 carrying *lux*CDABE was subcloned into plasmid pGRG25 [125] to yield plasmid pBEN275. The promoter for housekeeping gene *frr* [126] was amplified from the *E. coli* K-12 MG1655 genome with primers PG209 (gtctgactcgaggaattcttcccgtgatggataaataag) and PG210 (catcactcgaggttacgaatccttgaaaacttg) and cloned into the *Xho*I site of plasmid pBEN275 to yield plasmid pBEN276.

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Bioluminescence Tagging of *Salmonella enterica* with Plasmid pBEN276

Chromosome based expression of luciferase was established in eleven *S. enterica* serotypes using plasmid pBEN276. *S. enterica* serotypes were grown to logarithmic phase (OD_{600} = 0.6-0.8) in preparation of washing with 15% v/v glycerol solution (4x) to create electrocompetent cells. Cells were stored at -80°C until transformation. Transformation of six *S. enterica* serovars (*S.* Alachua, *S.* Heidelberg, *S.* Kentucky, *S.* Mbandaka, *S.* Newport, and *S.* Senftenberg) with plasmid pBEN276 were conducted by electroporation using Gene Pulser II System with parameters 2.5 kV, 25 µF, and 400 Ω. Five *S. enterica* serovars (*S.* Braenderup, *S.* Enteritidis, *S.* Montevideo, *S.* Schwarzengrund, and *S.* Typhimurium) were transformed with parameters 1.8 kV, 25 µF, and 600 Ω. Cells recovered in SOC media at 37°C for 1 h. Following recovery, bacteria suspension was spread on LB + Amp agar to incubate at 37°C for approximately 16 h to select for successful transformation. Amp resistant colonies were cloned in LB media supplemented with arabinose at 30°C for approximately 16 h to initiate the transposition reaction. Cultures were streaked on LB agar to incubate at 42°C for approximately 16 h to cure plasmid pBEN276. Colonies were picked and cloned in LB media and LB + Amp media at 30°C for approximately 16 h. Inhibition of growth by Amp confirmed loss of plasmid pBEN276. The presence of the *lux* operon was detected using IVIS Imaging System 100 Series integrated with Living Image Software v2.50.

Characterizing Bioluminescence Properties of Chromosome-Based Luciferase Expression

Bioluminescent *S. enterica* serotypes were grown to stationary phase in LB media and confirmed with bacterial density values (OD_{600}) using Thermomax spectrometer.

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Serotypes were arranged as quadruplicate samples in black clear-bottom 96-well microtiter plate. A dilution series was prepared for each sample. For each dilution series, the first four columns consisted of 10-fold dilutions (1×10^{0} to 1×10^{-3}), and the remaining eight wells consisted of doubling dilutions (5.00×10^{-4}, 2.50×10^{-4}, 1.25×10^{-4}, 6.25×10^{-4}, 3.13×10^{-5}, 1.56×10^{-5}, 7.81×10^{-6}, 3.91×10^{-6}, 1.95×10^{-6}, 9.77×10^{-7}, 4.88×10^{-7}, 2.44×10^{-7}). Bioluminescence was measured with IVIS Imaging System 100 Series with 10 s exposure at 37°C and quantified using Living Image Software v2.60. Bioluminescence was normalized by dividing total flux by OD_{600} readings. Minimum detectable bacterial densities were determined from the last two dilutions using plate counts on LB agar.

Linear correlation formulas were calculated for each serotype using bioluminescence values and bacterial population numbers determined by duplicate plate counts. The minimum detectible number for each serotype was identified using the number of bacteria present in the last dilution that displayed detectable bioluminescence above background. Colony counts aided in calculation of the theoretical amount of bioluminescence produced per colony forming-unit (CFU) for each serotype.

Chromosome Stability of *lux* Operon in *Salmonella enterica*

Transgene stability was assessed in the chromosome of eleven *S. enterica* serotypes by subculturing in LB media approximately every 24 h for a period of 14 d. For each passage, each culture was inoculated at a 10-fold dilution into fresh 0.3mL cultures of LB media in black clear-bottom 96-well microtiter plate in quadruplicate. Bacterial density (OD_{600}) and bioluminescence was determined after approximately 12 h of growth every 3 d. Bioluminescence was measured using IVIS Imaging System with exposure

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time 15 s at 37°C and normalized by dividing total flux with OD_{600} readings. The average normalized bioluminescence for each serotype at each passage revealed the ability of each serotype to maintain the *lux* operon in the chromosome without selected pressure.

Evaluation of Bioluminescence Expression at Varying Temperatures

An experimental model was developed to investigate the sensitivity of our chromosome based luciferase reporter system as an indicator of metabolic activity at varying temperatures. Bioluminescence expression is a reliable barometer of metabolic activity due to its requirement of $FMNH_2$ for the luciferase reaction to occur. All bioluminescence and bacterial density measurements were conducted using LMax Luminometer (Molecular Devices) with 1 s exposure and SpectramaxPlus 384 spectrophotometer (Molecular Devices), respectively. Cultures of bioluminescent *S. enterica* serotypes were grown to stationary phase, approximately 16 h of incubation at 37°C. Cultures were diluted 10-fold with fresh LB media and 0.2 mL of the bacteria suspension was transferred to black clear-bottom 96-well microtiter plate to incubate at 37°C. Next, the samples remained at 25°C for 10 m and bioluminescence and bacterial density readings were taken at the conclusion of this time period. Finally, the plate was refrigerated at 4°C for 2 h, with periodic interruption every 30 m to measure bioluminescence and bacterial density.

Chicken Skin Assay for Real-Time Monitoring of Bioluminescent *Salmonella enterica*

Cultures of bioluminescent *S. enterica* serovars Mbandaka and Montevideo were prepared in quadruplicate for this assay. Each culture were grown to stationary phase and then diluted to approximately 1 x 10^6 CFU/mL in sterilized distilled water. One mL of

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bacteria suspension was added to 8 mm circular chicken skin sections in black clear-bottom 24-well tissue plate as outlined for the skin attachment model. Each sample plate incubated at room temperature (25°C) for 1 h to allow bacteria to attach to skin. Following incubation, the excess bacterial suspension was vacuum removed and each well gently washed with sterilized distilled water. This process was repeated twice to extract unattached bacteria.

S. Mbandaka and *S.* Montevideo were subjects for our chicken skin wash assay with the addition of temperature as a treatment. The treatment group received chilled water (4°C) and the control group received untreated water, or ambient temperature water (25°C). After removal of excess bacteria suspension, initial bioluminescence from bacteria attached to skin sections was quantified using IVIS imaging system with exposure time 15 s. One mL of 4°C sterilized distilled water was added to each experimental well for each serotype. The control group for each serotype received one mL of 25°C sterilized distilled water. Treatment samples were refrigerated at 4°C for a time period of approximately 2 h on a rotating stage at 200 rpm. The control plate remained at 25°C for a time period of approximately 2 h on a rotating stage at 200 rpm. At the conclusion of washing, water containing removed bacteria was vacuumed from each well to remove unattached bacteria and the samples were warmed to 37°C for 5 m. Bacteria attached to the skin was quantified by measuring bioluminescence from the chicken skin samples using IVIS Imaging System. Bioluminescence for each well was normalized by dividing the total flux of each well by the corresponding bacterial density value of the original bacterial suspension.

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The difference between the initial total flux of the samples before treatment (0 m) from the final total flux after treatment (120 m) was calculated to determine the effect cold washes had on microbial removal from the surface of chicken skin. The difference value was averaged among the quadruplicates to produce a mean difference value for each serotype. The mean difference for a serotype of the chilled wash group was compared to its counterpoint in the wash group by ANOVA using PROC GLM SAS 9.1 to evaluate the effectiveness of chilled wash to remove *S. enterica* from poultry skin. Variables in this model included replicate, treatment, and their interaction. Pairwise comparison of the means was also conducted using Tukey procedure. A significance level of $P \leq 0.05$ was applied in all statistical analyses.

Results and Discussion

Construction of Plasmid pBEN276

Plasmid pGRG25 features a site-specific recombination system based on the bacterial transposon Tn7 [125]. The Tn7 system inserts at the *att*Tn7 site and is oriented specifically such that the right end of Tn7 is adjacent to the 3' end of *glmS* gene [127], and it has been used for transgene insertion into the chromosome of *Escherichia coli, Salmonella* and *Shigella* [125]. Plasmid pGRG25 is a temperature-sensitive delivery plasmid that can be cured after transgene insertion at the *att*Tn7 site by culturing at 42°C. Plasmid pBEN276 contains the *lux*CDABE operon between the Tn7 transposon arms on plasmid pGRG25, and its expression is driven by the *E. coli frr* promoter (Figure 4.1), which controls expression of a house-keeping gene encoding ribosome recycling factor. Thus the *lux* operon will be expressed constitutively. The chromosomal insertion point is

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specific and insertion of *lux* operon does not disrupt the function of *glmS* gene, therefore it is highly unlikely that bacterial physiology will be affected adversely [125].

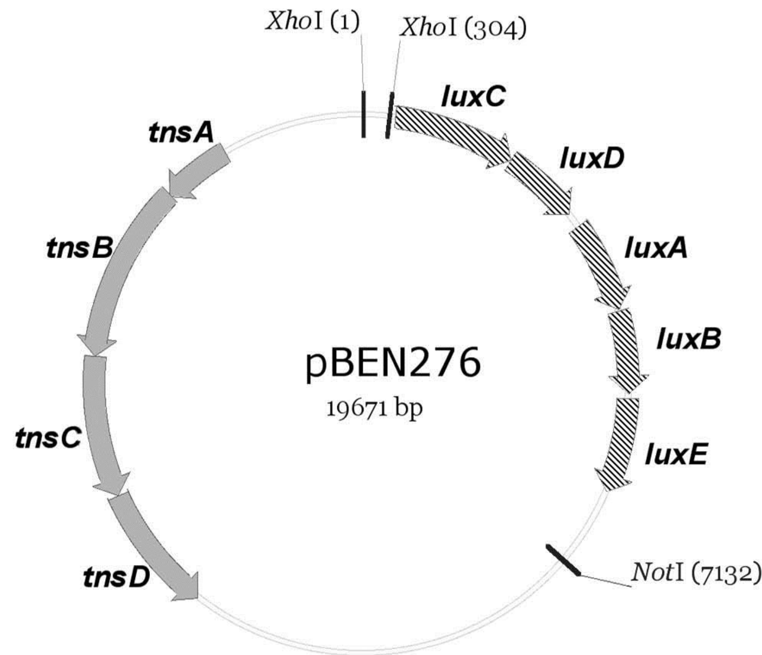

Figure 4.1 Plasmid pBEN276 vector

Genes TnsABCD encode the polypeptides required for transposition. The luciferase genes are encoded in the *lux* operon which is flanked by the Tn7 transposon arms (vertical bars at restriction sites *Xho*I and *Not*I). The expression of *lux* genes is driven by *E. coli frr* gene promoter which is located between the *Xho*I sites.

Characterizing Bioluminescence Properties of Chromosomal Luciferase Expression

Plasmid pBEN276 was utilized to insert the bacterial *lux* operon into the chromosomes of eleven *S. enterica* serotypes. Results indicated bioluminescence correlated well to bacterial population in all serotypes used, as exemplified in *S.* Montevideo ($P < 0.0001$, $R^2 = 0.994$) (Figure 4.2). The minimum detectable concentration of all eleven serotypes was, in decreasing order (CFU/mL): *S.* Kentucky –

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8.00 x 10^4; *S.* Mbandaka – 4.99 x 10^4; *S.* Enteritidis – 3.10 x 10^4; *S.* Schwarzengrund – 2.78 x 10^4; *S.* Montevideo – 1.74 x 10^4; *S.* Alachua – 1.07 x 10^4; *S.* Typhimurium – 6.72 x 10^3; *S.* Senftenberg – 6.40 x 10^3; *S.* Heidelberg – 5.28 x 10^3; *S.* Newport – 4.64 x 10^3; *S.* Braenderup– 4.16 x 10^3. Minimum detectable numbers of *S. enterica* expressing bioluminescence from the chromosome were higher compared to minimum detectable numbers of *S. enterica* expressing plasmid-based bioluminescence [128]. One possible explanation for this difference is a copy number effect where a single copy of the *lux* operon is inserted into the chromosome with the Tn7 system and only physiological levels of the luciferase protein will be expressed. In contrast, multiple copies of the gene are expressed in plasmid systems. For example, plasmid pAK*lux*1 is a pBBR1 derived plasmid which characteristically has a medium copy number (~30 copies/cell) [129]. Another possible explanation is due to promoter effect whereas the *frr* promoter drives expression of *lux*CDABE in the Tn7 system, and the *lacZ* promoter drives expression in the pAK*lux*1 plasmid system [128]. Our previous work with plasmid pAK*lux*1 showed that bioluminescent *S. enterica* carrying plasmid pAK*lux*1 emit, on average, 6.3351 p/s/cm^2/sr per CFU [128]; in comparison, *S. enterica* carrying the *lux* operon in the chromosome emit, on average, 0.0795 p/s/cm^2/sr per CFU. The intended purpose for this system is to use it as a screening tool for potential pathogen mitigation strategies, and this threshold of detection is sufficient for this purpose.

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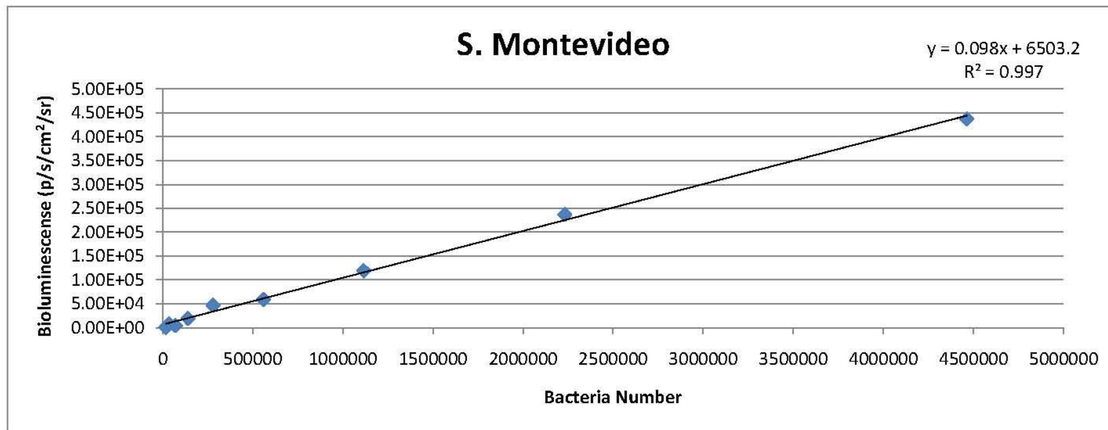

Figure 4.2 Correlation of bioluminescence against bacterial numbers

The correlation between bioluminescence signal and bacteria numbers for representative serovar *S.* Montevideo.

Two different sets of parameters were used to successfully transform all eleven *S. enterica* serotypes. These discrepancies may possibly be due to increased activity of restriction endonucleases in certain serotypes. The lysozyme lysis method for screening restriction endonucleases has been used to show there is increased restriction nuclease activity in some *Salmonella* serotypes [130]. Plasmid pGRG25 is a low copy plasmid and the large size of the plasmid (14-kb) plus the *lux* operon (7.1-kb) may make it vulnerable to restriction endonucleases. Limited compatibility between the bacteria and plasmid may be an issue and cause the range of application for this reporter system to be limited.

Chromosome Stability of *lux* Operon in *Salmonella enterica*

Our group evaluated the stability of the *lux* operon in the chromosome following transposition by subcloning bioluminescent *S. enterica* serotypes under non-selective conditions for 14 d at 37°C. Previous work from our group with plasmid-based bioluminescence expression showed the plasmid was unstable without antibiotic selection

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[110, 128]. The average half-life of plasmid pAK*lux*1, which contains the *lux*CDABE cassette, was approximately 7 d in *S. enterica* serotypes without antibiotic selection. This current study provides evidence for a 14 d period indicating stability of the *lux* operon in the chromosome of these eleven *S. enterica* serotypes with minimal bioluminescence flux (Figure 4.3). A notable observation was low initial expression of bioluminescence from *S.* Schwarzengrund (10^5 p/s/cm^2/sr). This serotype increased bioluminescence expression over the course of the experiment and reached similar levels to the other serotypes at approximately day 10 (10^7 p/s/cm^2/sr). The difference observed for *S.* Schwarzengrund is interesting. It is important to note that the Tn7 transposon system does not insert randomly in the *Salmonella* chromosome, but insertion occurs only at the *att*Tn7 site [41, 42]. Therefore, '*lux*CDABE mutants' occurring from undesirable insertions are not possible. Bacterial density values (OD_{600}) for *S.* Schwarzengrund were also similar to bacterial density values for the other serotypes. The contrast in bioluminescence expression may be attributed to a possible difference in host serotype background. Determination of the cause of this serotype-specific effect is beyond the scope of the current research. It is of interest that expression of bioluminescence in *S.* Schwarzengrund was also lowest in the plasmid *lux* system, pAK*lux*1, reported previously [128]. These results indicate plasmid pBEN276 can be utilized to construct a stable reporting system within the chromosome of *S. enterica* serotypes for use in extended *in-vitro* and *in-vivo* trials.

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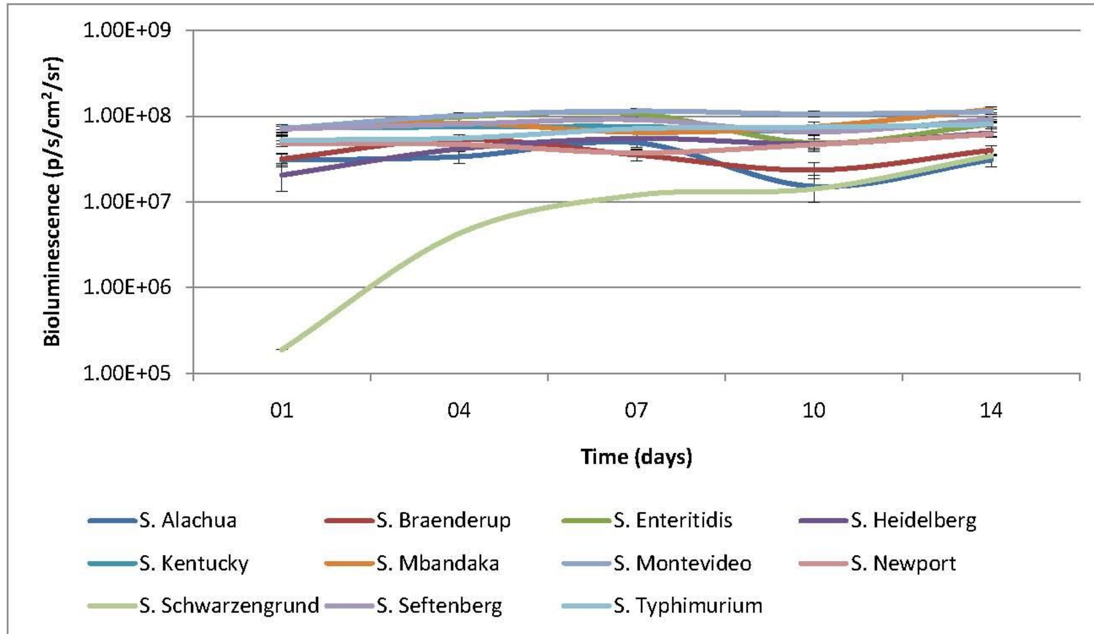

Figure 4.3 Stability of transgene in *Salmonella enterica* serotypes

S. enterica serotypes carrying transgene *lux*CDABE in their chromosome were subcloned under non-selective conditions for 14 d. At every third passage, bioluminescence was measured and normalized for cell density (OD_{600}).

Evaluation of Bioluminescence Expression at Varying Temperatures

*lux*CDABE encodes bacterial luciferase which catalyzes the oxidation of reduced flavin mononucleotide ($FMNH_2$) and a long chain aliphatic aldehyde in the presence of O_2 to produce flavin mononucleotide (FMN) and acid with light emission. Because $FMNH_2$ production is dependent on a functional electron transport chain, only metabolically active bacteria emit light [131]. Thus, bioluminescence imaging (BLI) provides a sensitive real-time measurement of the effects of various chemical, biological and physical stimuli on bacterial metabolism [132]. We utilized our bioluminescent *S. enterica* serotypes to validate our model under a temperature range similar to that which bacteria in food products are commonly exposed (host to ambient to refrigeration).

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Therefore we investigated the relationship between cellular metabolic activity, characterized by bacterial light production, and temperature variation. The temperatures selected were 37°C, 25°C, and 4°C.

Mesophiles, such as *Salmonella,* grow best in moderate temperatures (15-40°C) with normal enzymatic activity. In this experiment luciferase reaction within *S. enterica* was monitored. At 37°C and 25°C bioluminescence measurements were consistent within the replicates of the different serotypes. However, a change in temperature will have an impact on enzyme kinetics. Decreasing temperature, to 4°C, will slow molecular motion and inhibit the luciferase reaction. Decreasing temperature will also decrease the rate of metabolism, which translates to decreased concentration of substrate, $FMNH_2$, available for the luciferase reaction. At 4°C we observed an expected reduction in bioluminescence signal compared to readings at the two higher temperatures, 37°C and 25°C (data not shown). However, over the time required (approximately 1 min) to complete bioluminescence measurements at 25°C a rapid increase in the bioluminescence signal was observed between the first and the last wells read. Data showed that luciferase activity is restored shortly after removal from refrigeration temperature. Therefore, the temperature effect is minimalized after introduction to ambient temperatures (≥25°C). These results validate that our bioluminescence reporting system can monitor bacteria within a temperature range that food products are commonly exposed.

The stage on our luminometer has adjustable temperature with the lowest temperature setting being 25°C. Future work will include a refrigerated state for the reading stage to eliminate temperature variation.

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Chicken Skin Assay for Real-Time Monitoring of Bioluminescent *Salmonella enterica*

Salmonella represents a major issue for the poultry industry, and due to its persistence during the processing of chicken carcasses, the pathogen is difficult to eliminate the bacteria from chicken carcasses without proper cooking. The physiological mechanisms enabling the bacteria to persist throughout the processing continuum are not fully understood. Food safety necessitates attention to be focused on the comprehension of the mechanics of attachment of the bacteria in order to limit *Salmonella* as a food safety concern.

We have developed a chromosome based luciferase experimental model capable of real-time monitoring of bacteria viability and evaluating pathogenic mitigation strategies. Furthermore, the potential of this model may be of practical use in shedding light on how *Salmonella* is able to persist during the processing continuum. To demonstrate the potential value of this model as a screening tool, the performance of our bioluminescent *S. enterica* serotypes on chicken skin sections at two temperatures in an aqueous environment was evaluated. Serovars *S.* Mbandaka and *S.* Montevideo were selected for this skin attachment experiment based on the consistent bioluminescence expression observed within these serotypes (Figure 4.3). Individual aqueous solutions, each containing a *S. enterica* serotype, were prepared and introduced to chicken skin samples according to the skin attachment protocol (described previously). Separate black clear-bottom 24-well tissue plates, containing replicates of each serotype, were placed on a rotating stage at 4°C and 24°C for 2 h. Following this period, bioluminescence imaging was collected after a 5 m interval at 37°C for both serotypes, indicating bacteria numbers on chicken skin following cold and warm washes (Figure 4.4). Our previous work

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demonstrated washing with 25°C water suppressed the reproduction of *Salmonella* on chicken skin likely through the physical removal of bacteria [128]. Given that *Salmonella* is a mesophile, refrigeration temperatures further limit bacterial growth and the bacteria become metabolically static. Bioluminescence values at post-wash (4°C) were not shown to be significantly different compared to pre-wash values for both serotypes ($P \geq 0.25$). Bioluminescence values at post-wash (25°C) were greater compared to pre-wash values but the difference was not shown to be significantly different ($P \geq 0.125$). The increase in bioluminescence following the 25°C wash period is due to increased bacteria growth under favorable metabolic conditions (temperature) and nutrients provided by the chicken skin in solution. Our chromosome based luciferase model was able to quantify a change in bacteria number by monitoring bioluminescence following treatment.

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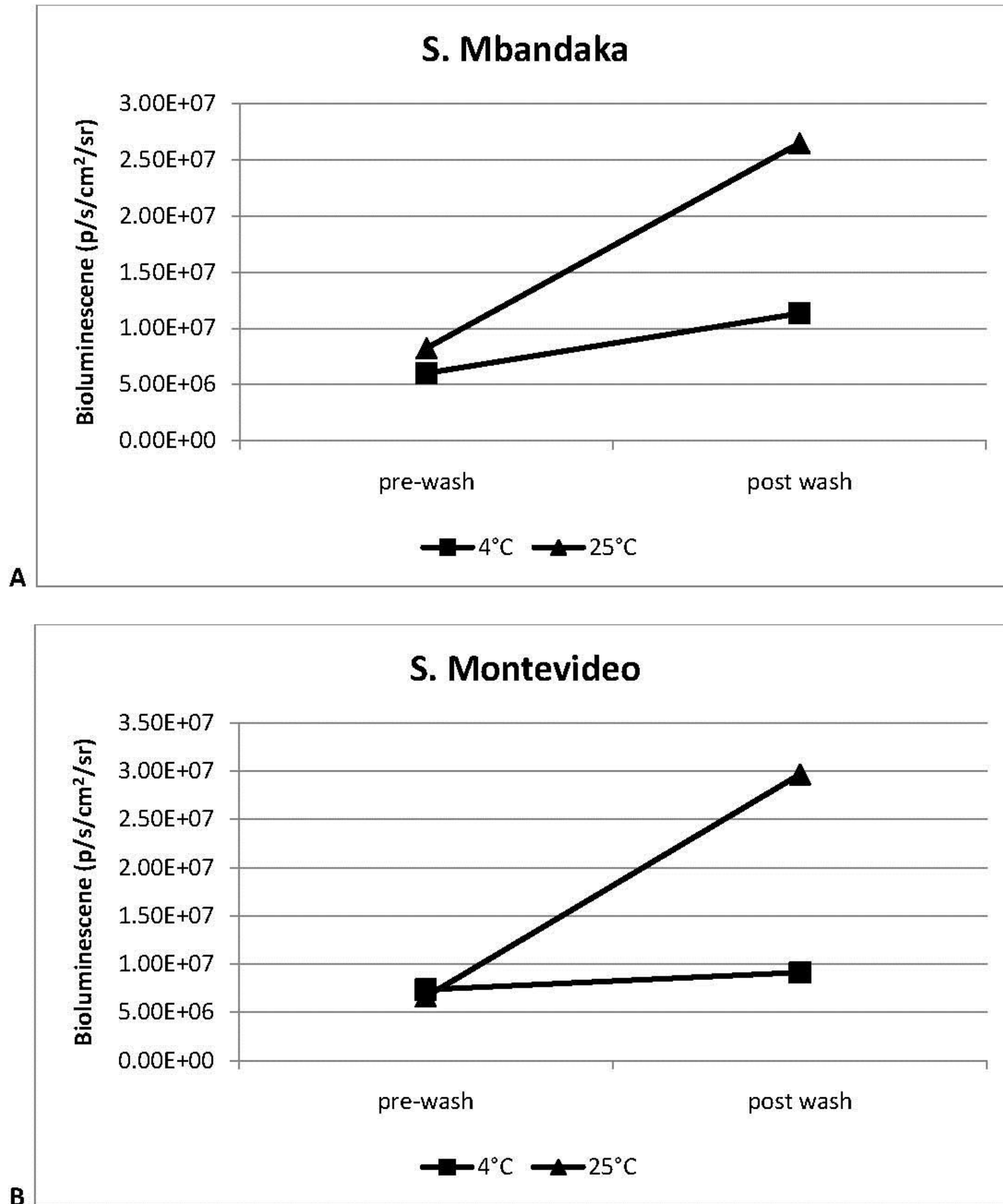

Figure 4.4 Comparison of the amount of *Salmonella* before and after 2 h incubation

The change in bioluminescence on chicken skin following 2 h incubation for 25°C and 4°C water washes. A) *S.* Mbandaka. B) *S.* Montevideo.

Results from our model provide the framework for further studies to evaluate the efficacy of pathogen mitigation strategies, i.e. antimicrobial compounds and processing

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parameters that may be applied in poultry processing to control *Salmonella.* Future work with our chromosome based luciferase reporter system in our chicken skin model will feature an extended time course to better reflect the duration of exposure to conditions bacteria might be subjected to in the poultry processing environment.

Summary

In this chapter, a novel, real-time reporting system was successfully introduced into *Salmonella enterica* serotypes. The *luxCDABE* operon was transposed from plasmid pBEN276 to the insertion site *attTn7* in the bacteria chromosome. The transposed gene was shown to be stable without external selection. This reporting system was shown to respond quickly following decreased metabolic activity due to refrigeration. Additional results with our chicken skin attachment model provide support for application of our model in trials simulating processing plant environmental conditions, with specific focus on immersion chilling. Extended research with our model will look at mechanisms of interaction of *Salmonella* with chicken skin using eukaryotic cell monolayers.

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CHAPTER V

DETERMINATION OF MOLECULAR COMPONENTS INVOLVED IN *SALMONELLA* INFECTION OF NONPHAGOCYTIC CELLS

This research is focused on the underlying bacteria-cell interactions of the invasion event initiated by *Salmonella* that occurs at the site of the epithelium lining of the gastrointestinal tract of its host. A standard gentamicin protection assay was conducted to study invasion in an adenocarcinoma colon epithelial cell line by *S. enterica* serovars Kentucky and Typhimurium. Deletion mutation strains were created for each serotype with λ Red recombination and PCR products to disrupt type III secretion system (T3SS) expression. Three comparison studies were conducted on the invasion results between strain and corresponding mutant, between serotypes, and between corresponding serotype mutant strains.

Methodology

Bacterial Strains and Plasmids

S. enterica serovars Kentucky and Typhimurium (hereafter referred to as Kentucky and Typhimurium, respectively) were isolated from the field as part of previous work to isolate and catalogue *Salmonella* specimens from the poultry processing continuum [109]. Plasmids pKD46, pKD3, and pCP20 were purchased from Coli Genetic Stock Center at Yale University (Hartford, CT).

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Preparation of Competent Cells and Electroporation

In order to prepare competent cells, a colony of *Salmonella* was inoculated in 5 mL LB media and incubated at 37°C with agitation at 200 rpm for approximately 16-20 h. The following day, 2 mL of the culture were transferred to freshly prepared 100 mL LB media and incubated at 37°C with agitation at 200 rpm. If appropriate, 10 mM L-arabinose was added to culture to prepare λ Red recombinase. Following 1 h incubation, OD_{600} was observed every hour while in lag phase and every 15-20 m once in log phase. Once OD_{600} reached 0.6-0.8, the culture was placed on ice for approximately 15 m to suspend growth. The culture was aliquoted equally into four 50 mL centrifuge bottles and kept on ice until ready to centrifuge. Each tube was centrifuged at 10,000 rpm for 10 m at 4°C. The supernatant was immediately poured off and 25 mL cold sterilized glycerol water (15% glycerol) was added to each bottle to resuspend the bacteria. The centrifuge process was repeated with fresh glycerol water. After the third spin, the supernatant was poured off and the bacteria pellets from the four tubes were combined into one tube with 25 mL glycerol water. The tube was centrifuged once more and the supernatant poured off and bacteria resuspended in the remaining liquid. Preparation of cells for electroporation was complete.

Standard electroporation was followed as 100 µL competent cells were combined with 2 µL DNA in 1.5 mL microcentrifuge tube. Solution was transferred to 1 mm gap cuvette and pulsed at 1.8 kV. Cells recovered in 500 µL S.O.C. media at 30°C for 1-5 h. Cultures were spread on LB agar with appropriate antibiotic and incubated at 30°C with agitation at 200 rpm for approximately 24 h.

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Construction of SPI Deletion Mutations

A series of deletion mutations were created within *Salmonella* Pathogenicity Island-1 (SPI1) in Kentucky and Typhimurium following a method developed by Datsenko and Wanner to delete structural genes critical to the proper function of T3SS [133]. This method has been successfully applied to inactivate genes within *E. coli* and *S. enterica* serovars Typhimurium and Enteritidis [12, 134, 135]. Briefly, phage λ Red recombinase, encoded on plasmid pKD46 replaced a chromosomal sequence with a PCR fragment that has homologous regions adjacent to the chromosomal sequence (Figure 5.1). The 40-nt homologous regions were designed from *S. enterica* Kentucky genome shotgun sequence (GenBank accession no. ABAK02000001.1) and *S. enterica* Typhimurium str. SL1344 (NCBI Reference Sequence: NC_016810.1). The 1.1-Kbp PCR fragment was generated using the 40-nt homologous extensions as primers (Table 5.1) from template pKD3 (Figure 5.2).

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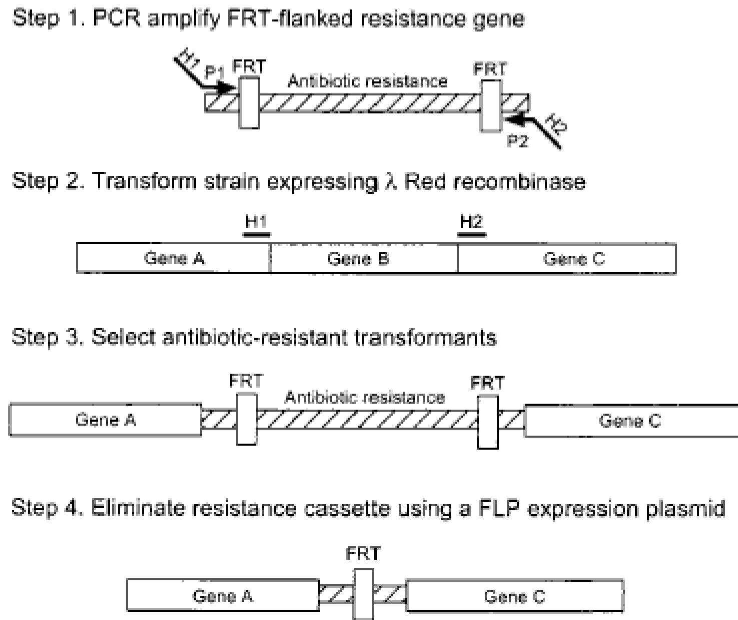

Figure 5.1 Gene disruption strategy using PCR products

To create the deletion mutation within our strain, (step 1) an antibiotic resistance gene was PCR amplified with primers which had 40-bp tails that were homologous to the flanking regions adjacent to the gene of interest. (step 2) λ Red recombination replaces the chromosomal sequence with the antibiotic resistance markers. (step 3) The antibiotic-resistant transformants were selected. (step 4) The resistance gene was removed by a helper plasmid. H1 and H2 refer to the homology regions. P1 and P2 refer to priming sites.

Table 5.1 Primer sequences

Primer	Sequence	Length
CVM29188.01F	TCATTTAATTGCCTCCTGACCTCTATCCAGATAAACACGAgtgtaggct ggagctgcttc	60
CVM29188.02F	TTATATTGTTTTTATAACATTCACTGACTTGCTATCTGCTgtgtaggctg gagctgcttc	60
CVM29188.03F	TCAATGCCGTACCTCGTTTTCTTGTGGCTGAATAACGTCTgtgtaggct ggagctgcttc	60
CVM29188.01R	GCGGAAATTATCAAATATTATTCAATTGGCAGACAAATGAcatatgaa tatcctccttag	60

The CVM29188.01F, CVM29188.02F, and CVM29188.03F primers harbor 40 nucleotides (in uppercase) that bind upstream of the *invG* gene, *invA* gene, and *spaS* gene, respectively, and 20 nucleotides (in lowercase) that correspond to P1. CVM29188.01R harbors 40 nucleotides (in uppercase) that bind downstream of *invG* gene, and 20 nucleotides (in lowercase) that correspond to P2.

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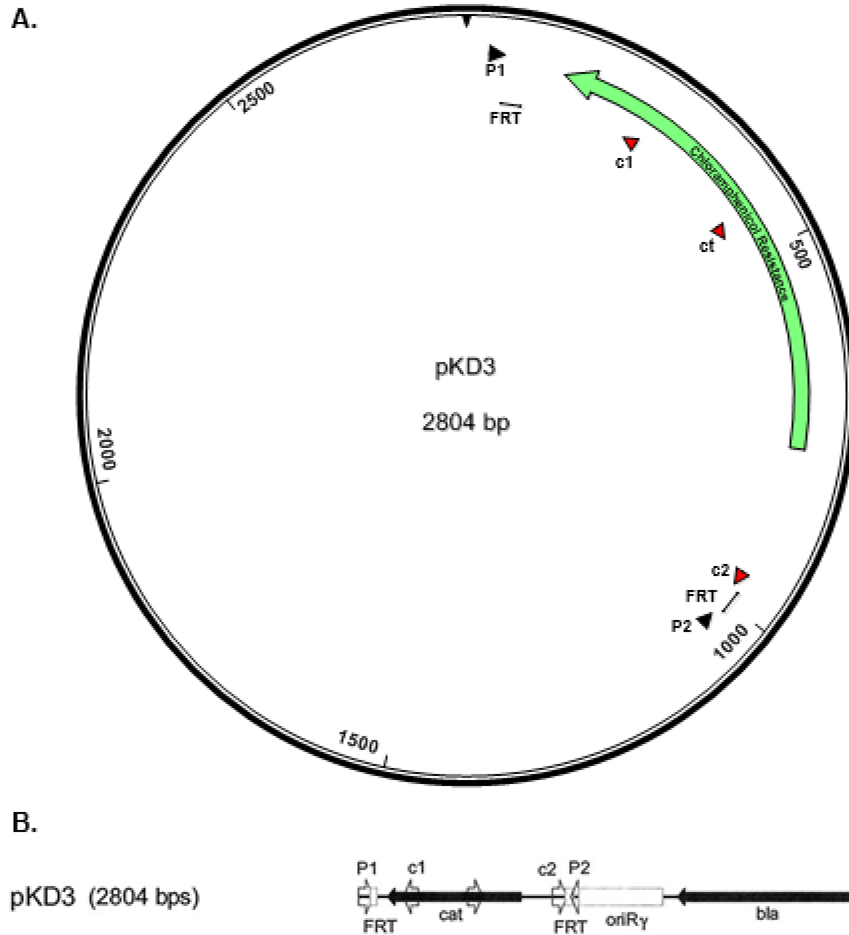

Figure 5.2 Template plasmid pKD3

(A) Arrowheads indicate location and orientation of priming sites. P1: priming site 1; P2: priming site 2. c1, c2, and ct: common test primers. FRT (FLP recognition target) sites flank chloramphenicol acetyl transferase gene at (51-84) and (981-1,022). Numerals identify nucleotide location in template plasmid pKD3 sequence (GenBank accession no. AY048742.1). (B) Linear representation of template plasmid pKD3.

The amplified PCR fragment from plasmid pKD3 encodes chloramphenicol acetyl transferase (CAT). Plasmid pKD46 transformants containing the PCR fragment after electroporation were selected for successful recombination events within the chromosome using chloramphenicol (Cm) antibiotic pressure, (10 μg/mL). Following selection, the resistance gene was eliminated using helper plasmid pCP20, which targets

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specific FRT (FLP recognition target) sites flanking the resistance gene for recombination [136]. Three deletion mutation events were scheduled for structural genes: *invG, invAEG,* and *spaSRQPO-invJICBAEG*.

Growth Kinetics

Growth analysis was conducted for wild-type Kentucky and Typhimurium and their corresponding strains with mutation Δ*spaSRQPO-invJICBAEG* to determine if bacteria replication was affected by the mutation. Separate cultures were prepared by inoculating fresh LB media with one colony of each serotype and strain and incubated for approximately 16 h at 37°C with agitation at 200 rpm. Following incubation, OD_{600} values for each culture were adjusted to 1.000 in fresh LB media. Triple technical replicates were prepared for each sample by diluting the culture 1/1000 in fresh LB media and incubated at 37°C with agitation at 200 rpm. At scheduled time points, starting at 0 h and occurring every hour to 14 h, OD_{600} was observed for each sample. The average OD_{600} values were determined from triple technical replicates for each serotype and strain. The averages were plotted against time (m) to create a growth curve depicting bacteria replication as a function of time.

Further growth analysis was conducted to establish viable bacteria number estimates in relation to OD_{600} values. Separate cultures were prepared by inoculating fresh LB media with one colony of each serotype and strain and incubated for approximately 16 h at 37°C with agitation at 200 rpm. Following incubation, OD_{600} values for each culture were adjusted to 1.000 in fresh LB media. Quadruple technical replicates were prepared for each sample by diluting the culture 1/1000 in fresh LB media and incubated at 37°C with agitation at 200 rpm. At time point 0 h, an aliquot from each

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culture was obtained and diluted 10^{-3} in 0.01 M phosphate buffered saline (PBS) (Sigma-Aldrich, St. Louis, MO) and 25 µL aliquots were spread on LB agar to enumerate colony forming unites (CFU). Following 6 h period, an aliquot from each sample was removed, diluted 10^{-5} and 10^{-6} in 0.01 M PBS and 25 µL aliquots were spread on LB agar to enumerate CFU. The previous step was repeated for a successive 6 h period and data collected as previously outlined. Data from the quadruple technical replicates at each time point was gathered to generate a XY scatter plot for each serotype and strain to predict bacteria number in relation to OD_{600}. The data was statistically analyzed using linear regression model (PROC REG) SAS for Windows v9.3. All statistical comparisons were two-sided using $P \leq 0.05$ as the significance level.

Cell Culture

Human colorectal adenocarcinoma epithelial Caco-2 cells (ATCC HTB-37; ATCC, Manassas, VA) were grown in Eagle's Minimum Essential Medium (EMEM) (ATCC, Manassas, VA), supplemented with 20% fetal bovine serum (FBS) (Access, Vista, CA) and 2 mM L-glutamine (EMEM-FBS), and maintained at 37°C in 5% CO_2 and 95% air, in 75 cm^2 flasks, unless stated otherwise. Trypsin-treated cells were seeded into 12-well tissue culture plates (Becton and Dickinson and Company, Franklin Lakes, NJ) at approximately 1 x 10^4 cells per well and grown at 37°C and 5% CO_2/95% air. Cells averaged 2-4 d to reach 95-100% confluent monolayers (approximately 5 x 10^5 cells/well). Cell media was exchanged every 2-3 d. Cell number was determined by hemocytometer counts.

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Bacterial Infection of Caco-2 Cell Monolayers

To determine rate of invasion of Caco-2 cells by Kentucky, Typhimurium, and their corresponding mutant strains, a gentamicin protection assay was performed as described previously [137, 138], with modifications. Briefly, fresh LB media was inoculated with one colony (Kentucky, Kentucky [Δ*spaSRQPO-invJICBAEG*], Typhimurium, Typhimurium [Δ*spaSRQPO-invJICBAEG*]) and incubated for approximately 16 h at 37°C with agitation at 200 rpm. Cultures were adjusted to OD_{600} = 0.800 and then diluted 1/1000 in pre-warmed complete cell media to achieve a predetermined bacteria number and transferred to Caco-2 monolayer cells at multiplicity of infection (MOI) 1:1. Tissue culture plates were briefly centrifuged at 400 rpm for approximately 1 m to bring bacteria into close proximity with the cell monolayer prior incubation for 1 h at 37°C. Removal of cell media with bacteria preceded 2x washes with 0.01 M PBS. Pre-warmed cell media supplemented with gentamicin (200 μg/mL; Gibco) was added to eliminate extracellular bacteria while plates incubated for 1 h at 37°C. Cell media solution was removed and saved to be spread (100 μL) on nonselective agar to confirm antibiotic action. Monolayers were washed twice with 0.01 M PBS, then 500 μL cold Triton X-100 (0.1% v/v 0.01 M PBS) was added to lyse cells. Cell suspension was collected, diluted 10^{-1} or 10^{-2}, and spread onto nonselective agar in 100 μL aliquots. Viable bacteria were enumerated and adjusted to estimate CFU/mL. Each strain consisted of eight replicates for each trial and the assay was repeated for three separate trials. The data was statistically analyzed using mixed linear model (PROC MIXED) SAS for Windows v9.3. Strain was the fixed effect and random effects were trial and trial(strain). The least square means were used to determine statistically significant differences

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between strains with respect to the mean CFU/mL. All statistical comparisons were two-sided using $P \leq 0.05$ as the significance level.

Annotation

For annotation, the whole genome shotgun sequence of *S. enterica* Kentucky str. CVM29188 (GenBank accession no. ABAK02000001.1) and whole genome sequence of *S. enterica* Typhimurium str. SL1344 (NCBI Reference Sequence: NC_016810.1) were uploaded to the RAST (Rapid Annotation using Subsystem Technology [ref]) server in FASTA format. The genomes were compared on function-base using RAST (http://rast.nmpdr.org/) to identify serotype-specific protein-encoding, rRNA, and tRNA genes and assign function to the genes.

Results And Discussion

Construction of SPI Deletion Mutations

Three sites within SPI1 were targeted for deletion to disrupt T3SS function in Kentucky and Typhimurium. PCR products were generated from pairs of 60-nt primers that included 40-nt homology extensions and 20-nt priming sequences complementary to plasmid pKD3 as template (Table 5.2). The PCR products were purified and then transformed into bacteria carrying the λ Red helper plasmid as described previously to create three separate deletion events within the structural genes of T3SS. The mutations were precisely targeted for *invG, invAEG*, and *spaSRQPO-invJICBAEG* (Figure 5.3).

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Table 5.2 Gene disruptions using λ red recombinase

Mutation	Homology Extensions	Priming Sites
ΔSPI1(*invG*)1705	40 nt; Ha: 2345733; H2: 2347418	P1; P2
ΔSPI1(*invA-invG*)4902	40 nt; Hb: 2342536; H2: 2347418	P1; P2
ΔSPI1(*spaS-invG*)11769	40 nt; Hc: 2335669; H2: 2347418	P1; P2

All disruptions were made in pKD46 transformants of BW25113. Extension lengths are given first. Numerals identify the 3' nucleotide of the extension in the Kentucky genome shotgun sequence (GenBank accession no. ABAK02000001.1). One primer had the Hχ (a, b, or c) extension and the 3' sequence for priming site 1 (P1). The other primer had the H2 extension and the 3' sequence for the complement of priming site 2 (P2). Priming sites P1 and P2 associated with plasmid pKD3 [133].

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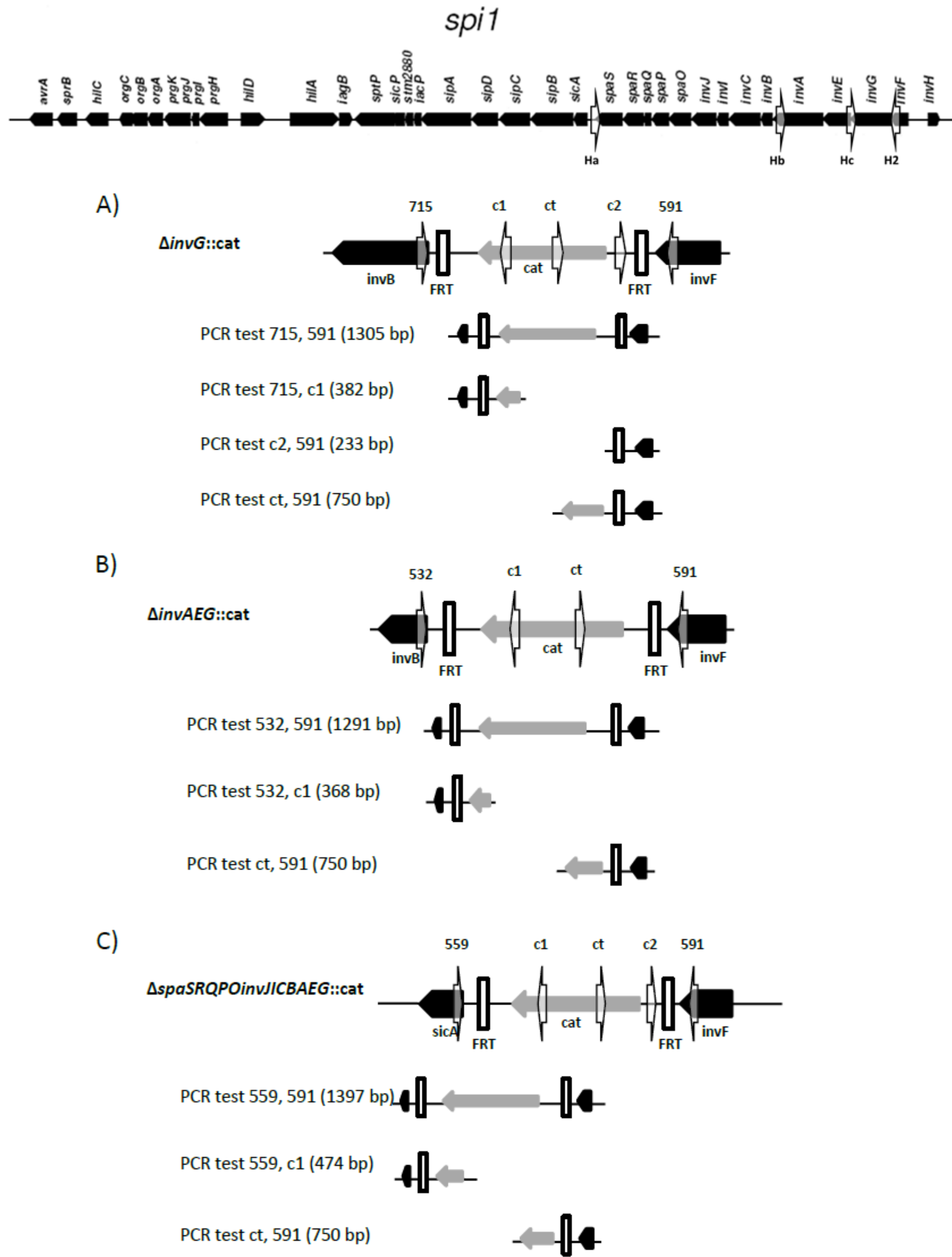

Figure 5.3 Structures of type III secretion system mutations

Top line shows *Salmonella* Pathogenicity Island-1. (A-C) Structures of the mutations generated using plasmid pKD3 as template. Numerals above the structures refer to locus-specific primers. The predicted PCR products are shown. C, complement; Hχ, homology χ (a, b, c, or 2).

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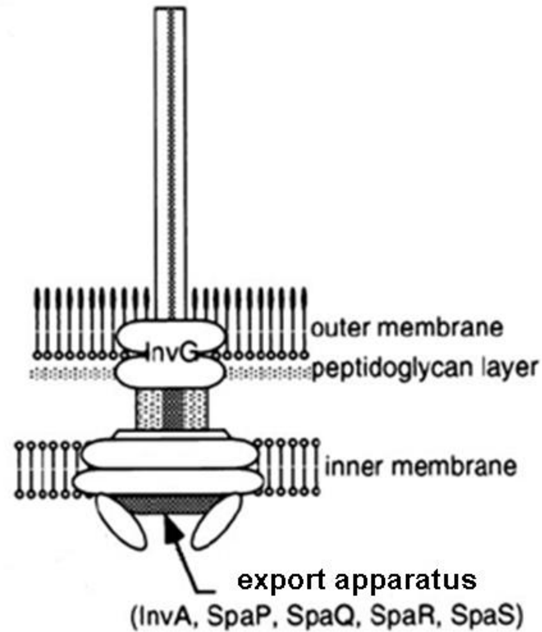

Figure 5.4 Type III secretion system basal structure

The basal structure spans the outer membrane, peptidoglycan layer, and the inner membrane. The outer membrane ring is formed by *inv*G. The export apparatus is associated with the inner membrane and consists of *inv*A, *spa*S, *spa*R, *spa*Q, and *spa*P.

Mutations of this nature interrupt the proper formation of the basal structure that anchors the needle to the bacterial membranes and disrupts T3SS function (Figure 5.4). The outer membrane ring of the basal structure is formed by the integral membrane protein *invG* [139]. The export apparatus complex serves as a platform for the assembly of the basal structure and is composed of highly conserved integral membrane proteins: *spaS*, *spaR*, *spaQ*, *spaP*, and *invA* [140]. Protein *spaS* has additional function to regulate secretion specificity of the virulence effectors through the base [141].

Cm resistance (10 µg/mL) phenotype indicated successful mutations. Antibiotic resistance gene was eliminated by FLP recombinase, expressed from helper plasmid pCP20, which targets the repeated FRT (FLP recognition target) sites flanking the

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resistance gene. Elimination of the antibiotic resistance gene leaves an 85-nt scar (Figure 5.5) in place of the deleted gene(s) that has stop codons in all six reading frames [133], thereby creating nonpolar gene deletions. Downstream gene expression can continue due to a ribosome binding site and start codon that is encoded in the scar sequence [133].

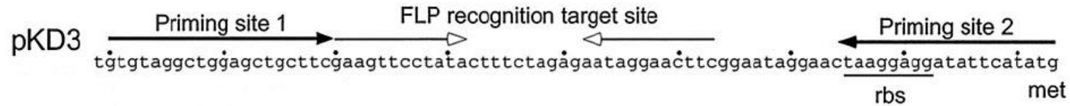

Figure 5.5 Sequence after FLP-mediated excision

Predicated ‘scar’ sequence remaining after FLP-mediated excision of the chloramphenicol resistance gene. Arrows with open arrowheads show the FRT site inverted repeats. The ribosome binding site (rbs) and methionine (met) start codon are marked.

All gene deletion mutations were verified by PCR tests using locus-specific primers and CAT-specific primers revealing that all strains had new junction and locus-specific fragments of the predicted sizes (Figure 5.6). Following elimination of the resistance gene, PCR test with locus-specific primers indicated the deletion mutants produced the expected junction fragments and verified mutations occurred in the designed location.

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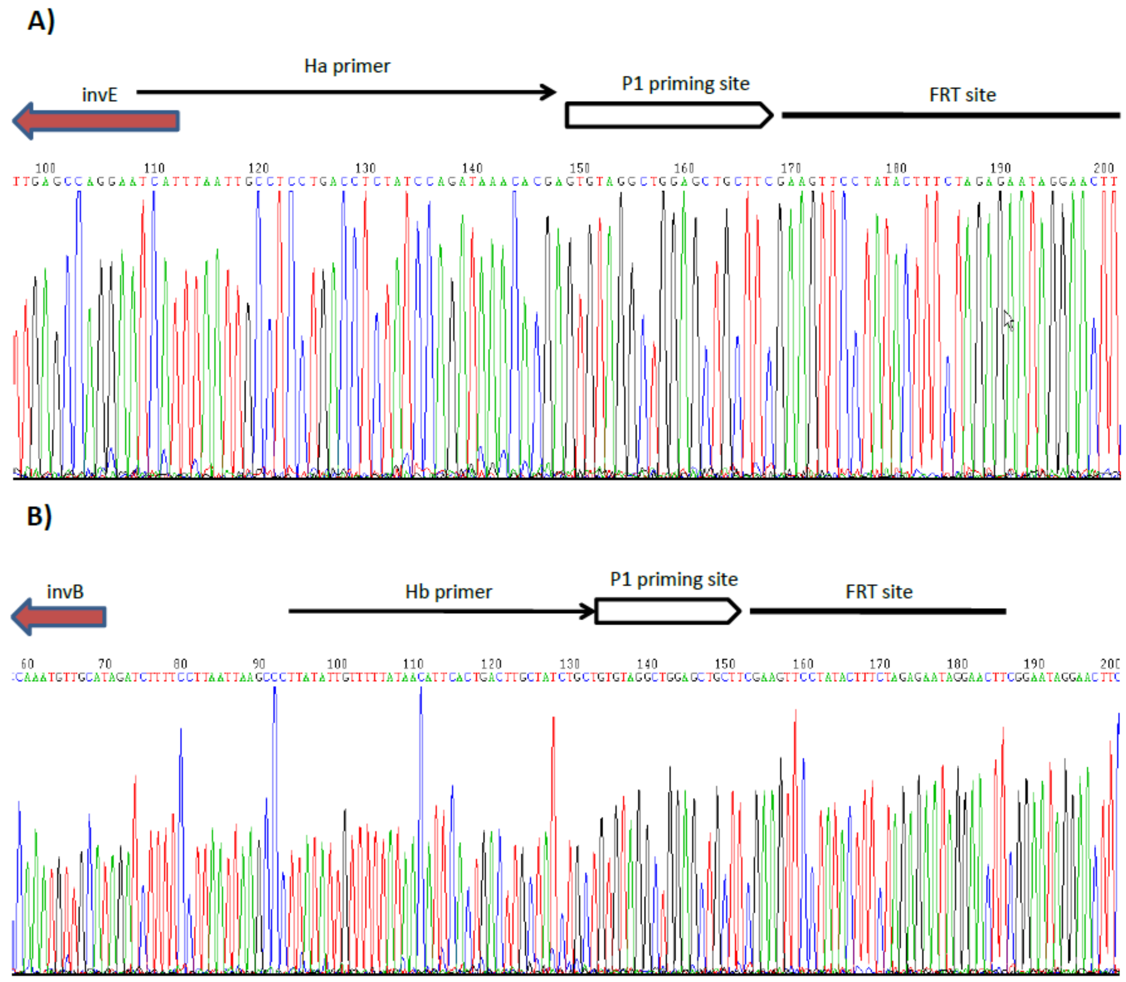

Figure 5.6 Sequence of type III secretion system mutation junctions

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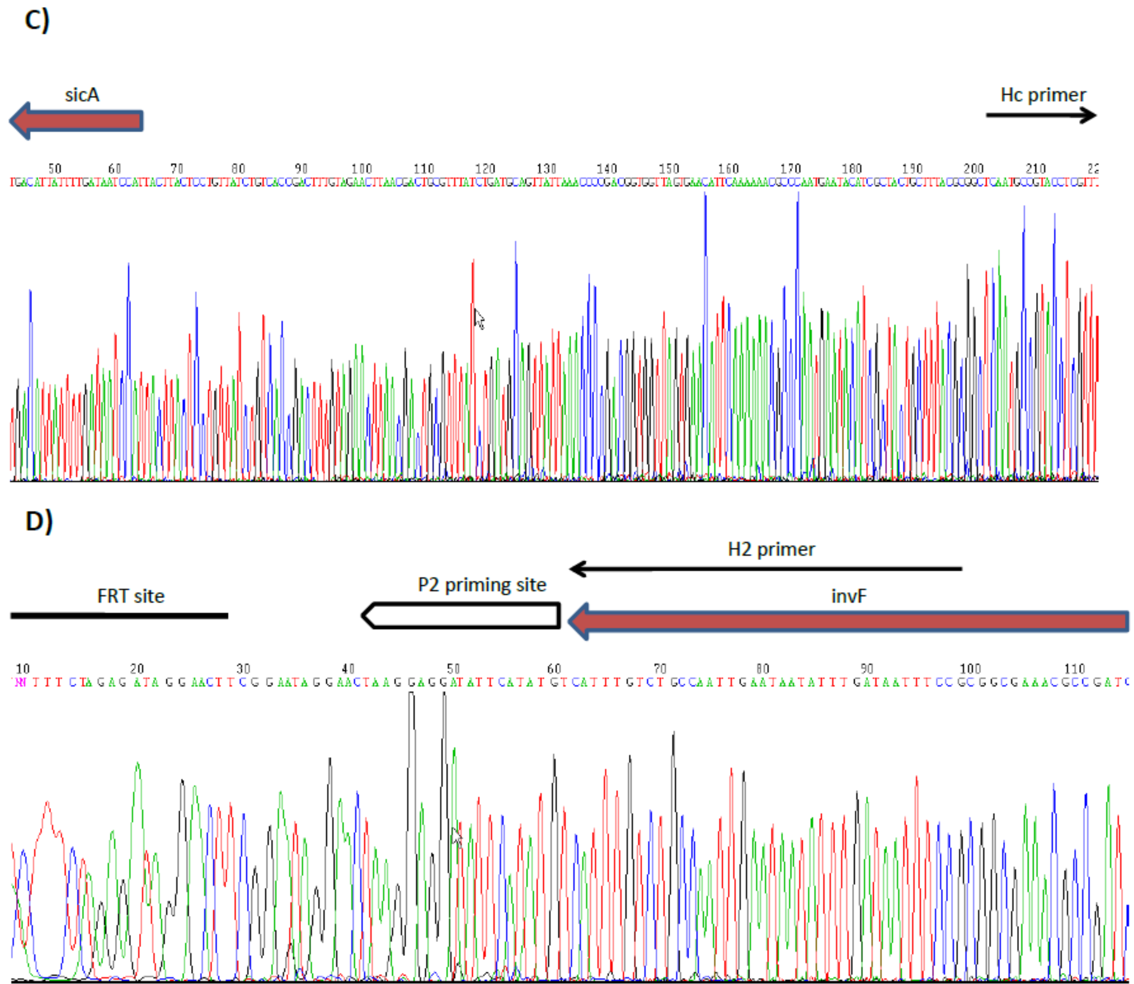

Figure 5.6 (Continued)

Chromatograms of the newly formed DNA sequences from Kentucky following recombination. A) The 5' sequence of the ΔSPI1(*invG*)1705 mutation. B) The 5' sequence of the ΔSPI1(*invA*-*invG*)4902 mutation. C) The 5' sequence of the ΔSPI1(*spaS*-*invG*)11769 mutation. D) The 3' sequence of the three mutations.

Growth Kinetics

Growth analysis was conducted for Kentucky, Typhimurium, and the corresponding mutation strains to determine if bacteria replication was affected by the deletion mutation. Bacterial cultures were adjusted to equal concentration, diluted 1/1000 in media and OD_{600} values were determined from 3 h to 14 h at every hour during incubation at 37°C at 200 rpm. The OD_{600} values were averaged from triple technical

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replicates and plotted as a function of time (m) for each serotype and strain (Figure 5.7). Deletion mutations created within SPI1 were not observed as affecting bacterial replication by Kentucky or Typhimurium in relation to their corresponding wild-type strains.

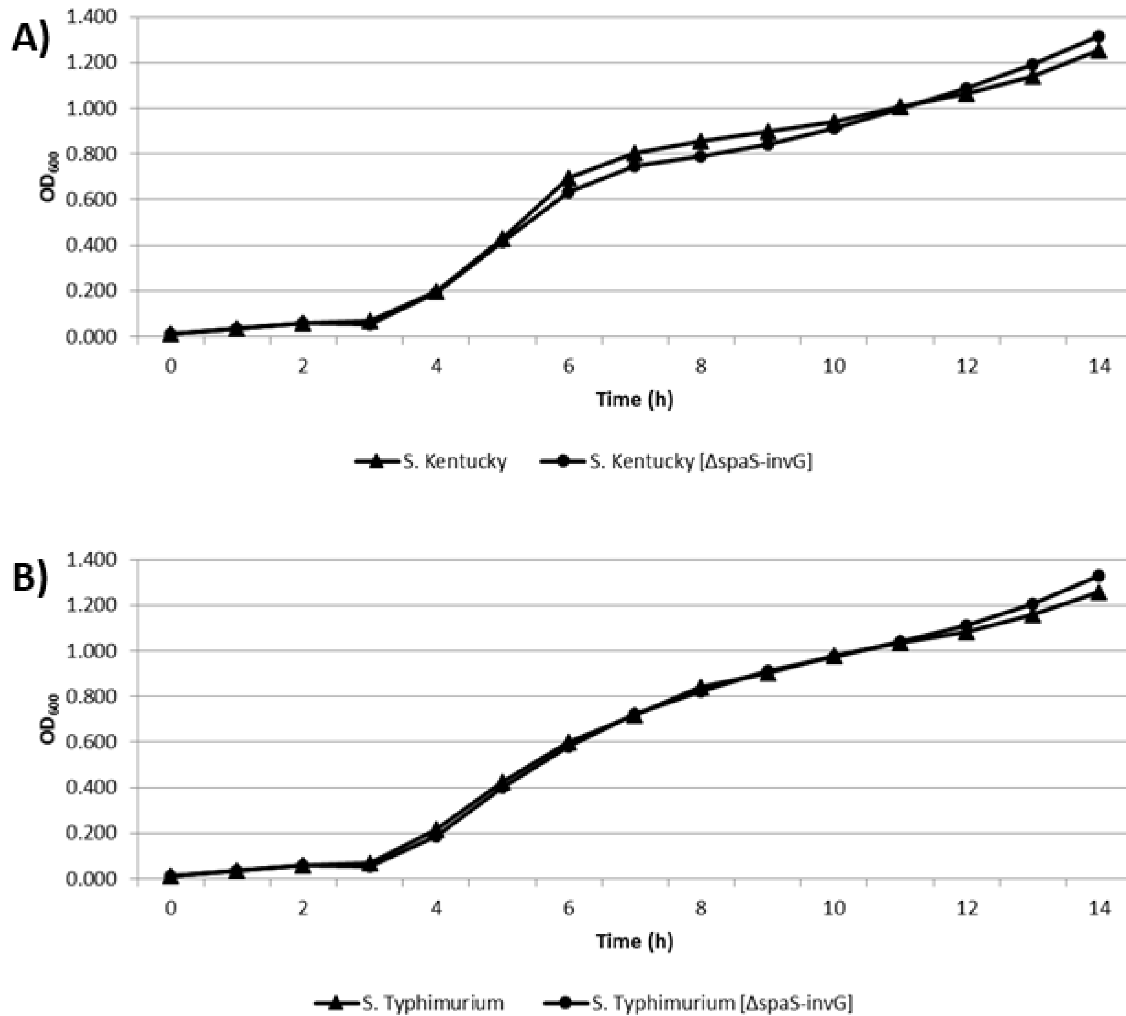

Figure 5.7 Bacterial concentrations as a function of time

Average of triple technical replicates for A) Kentucky, B) Typhimurium, and corresponding deletion mutant strain (Δ*spaS-invG*) plotted as OD_{600} values on the *y*-axis as a function of time (m) on the *x*-axis.

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Further growth analysis was conducted to confirm viable bacteria number relative to OD_{600} values for each serotype and strain. At time point 0 h, cultures were diluted 10^{-3} in 0.01 M PBS, and at time points 6 h and 12 h, cultures were diluted 10^{-5} and 10^{-6} in 0.01 M PBS and 25 µL were spread on LB agar. Colony forming units were counted and adjusted to CFU/mL from four technical replicates for each serotype and strain at 0 h, 6 h, and 12 h. For all serotypes and strains, the model used for linear regression analysis indicated a significant portion of the variation in CFU/mL was due to OD_{600}. The statistical analysis, including regression equation, R-squared value, *P*-value, for each for each serotype and strain follows (Figure 5.8).

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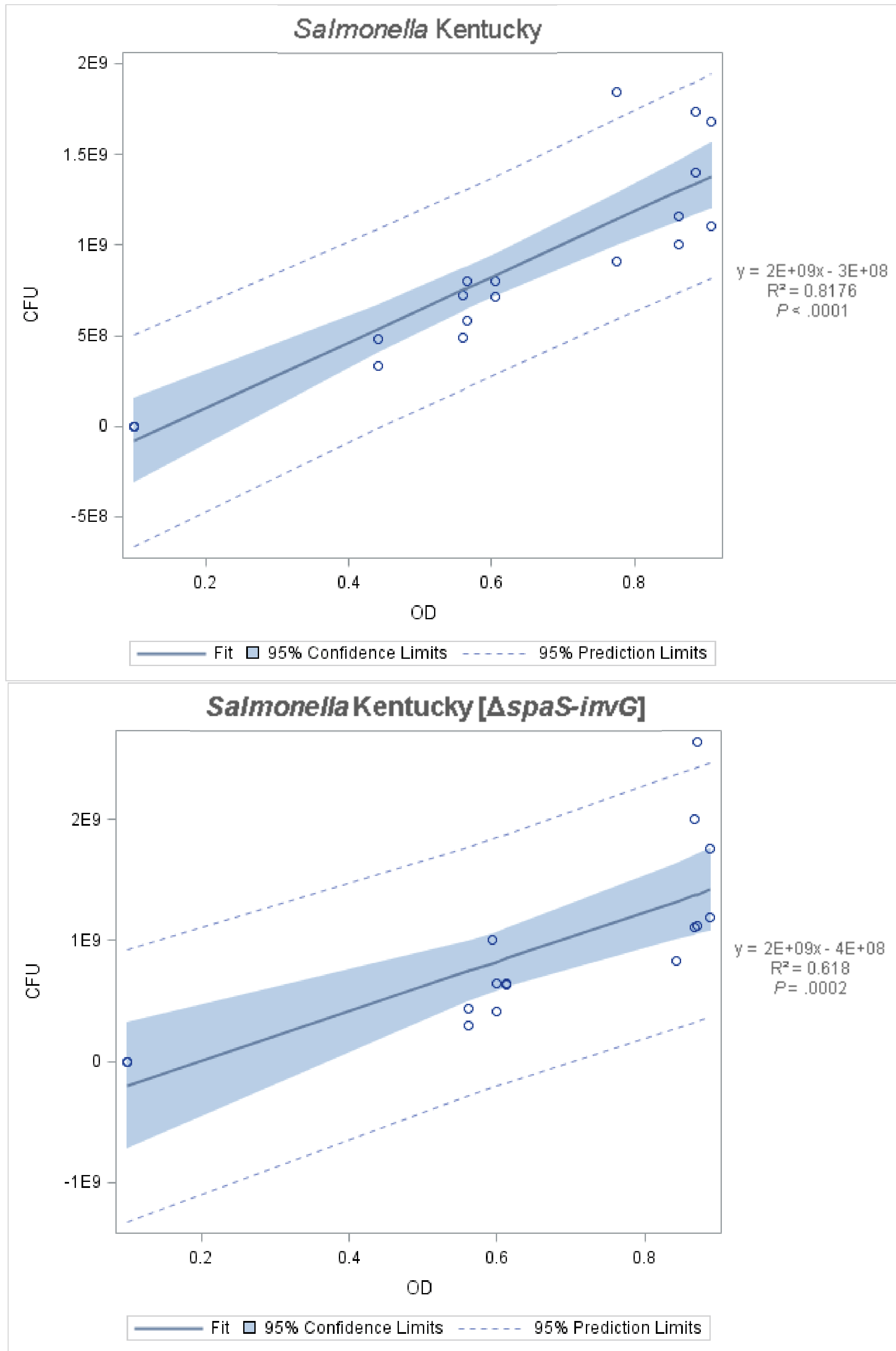

Figure 5.8 Fit Plot for Colony Forming Units (CFU) / mL

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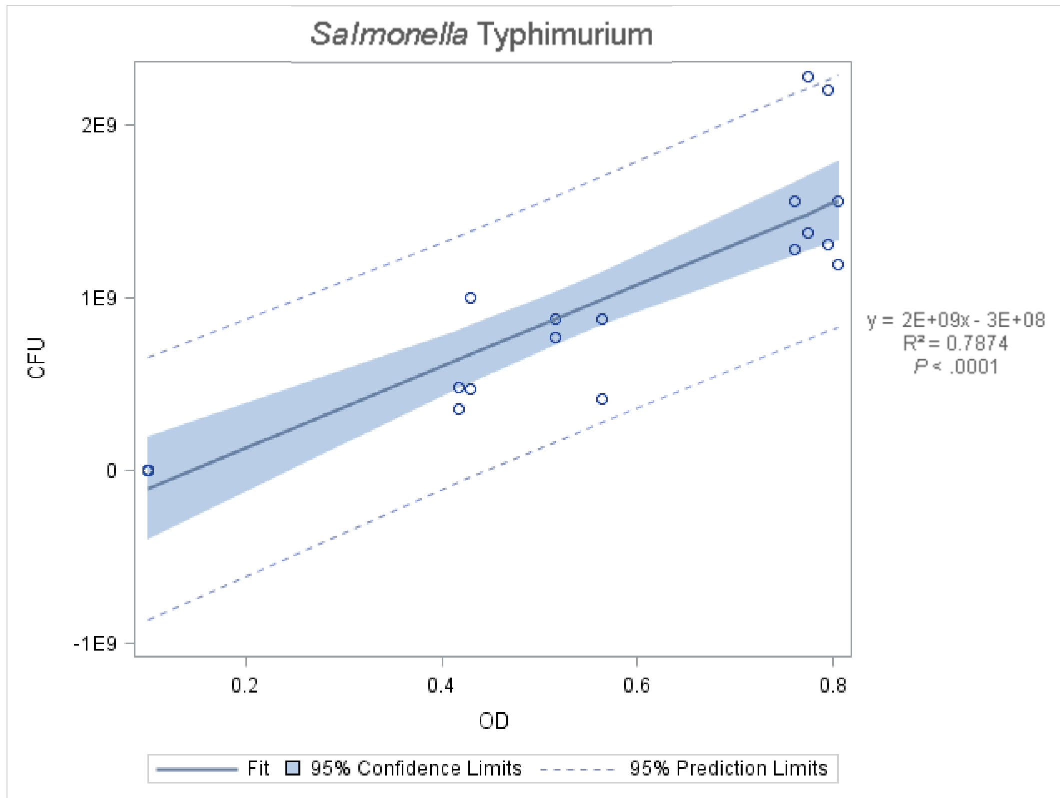

Figure 5.8 (Continued)

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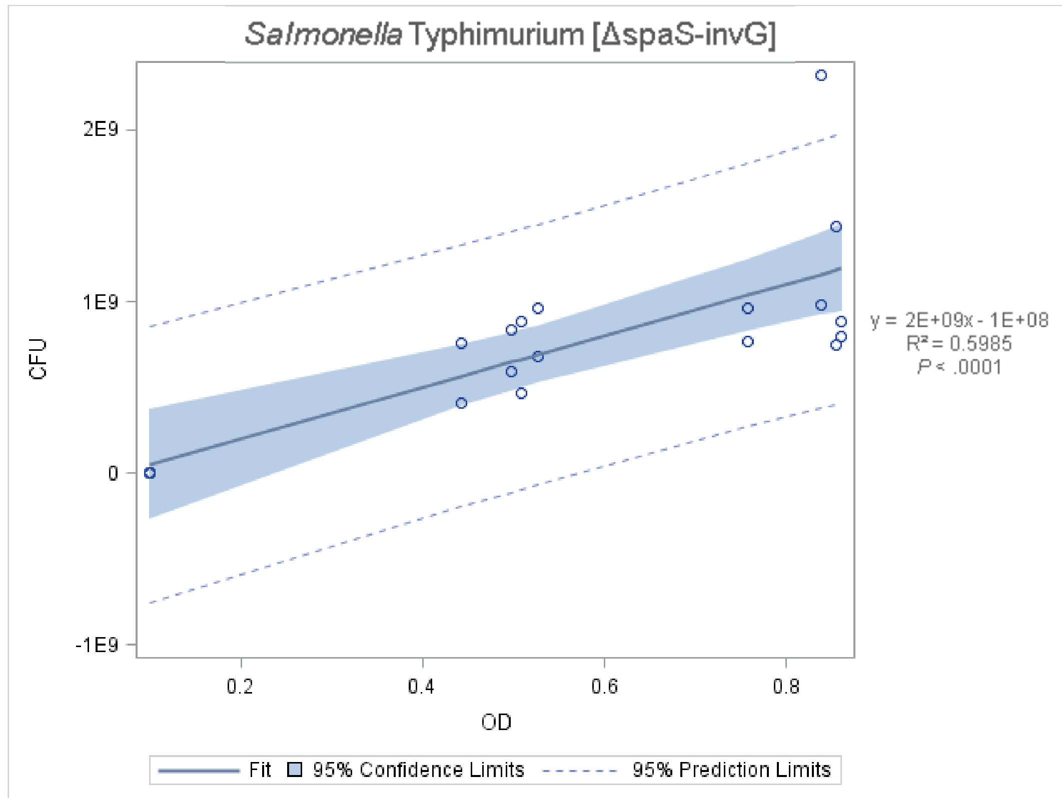

Figure 5.8 (Continued)

At each time point, cultures were diluted either 10^{-3} or 10^{-5} and 10^{-6} in 0.01 M PBS and 25 µL of bacteria suspension were spread on LB agar. Colony forming units (CFU) from each dilution were enumerated and adjusted to CFU/mL. CFU/mL along the *y*-axis was plotted as a function of OD_{600} on the *x*-axis in XY scatter plot to predict CFU relative to OD_{600}. The regression equations, R-squared value, and *P*-value were determined for each serotype and strain.

Bacterial Infection of Caco-2 Cell Monolayers

A standard gentamicin protection assay was modified for this study to determine the capacity of each strain to infect a monolayer of human colorectal adenocarcinoma cell line Caco-2. Following 1 h invasion time, extracellular bacteria were eliminated by supplementation of new cell media with gentamicin. Following subsequent 1 h incubation period, the cell media with gentamicin was removed and 100 µL was spread on nonselective media (LB, MacConkey, or Brilliant Green agar) to confirm the elimination

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of extracellular bacteria. The absence of CFU verified that gentamicin at concentration 200 µg/mL for 1 h was sufficient to eliminate extracellular bacteria (data not shown). Caco-2 cells were lysed with cold 0.1% v/v Triton X-100 diluted in 0.01 M PBS and the cell suspension collected. Wild-type Typhimurium was diluted 10^{-1} in 0.1 M PBS, the remaining three cell suspensions were not diluted, and 100 µL of the suspensions were spread on nonselective media. Viable bacteria numbers as a result of invasion were determined from plate counts and adjusted to estimate bacteria number per mL (Figure 5.9). Statistical significance of invasion rates for each strain was determined with SAS Windows v9.3 using mixed linear model and significance level $P \leq 0.05$.

Invasion Rates of Wild-type Kentucky and Typhimurium

As wild-type Typhimurium has adapted to humans as a host, a relatively high rate of invasion, approximately 185,625 CFU/mL, in comparison to Kentucky was expected (Figure 5.9). The invasion rate for wild-type Kentucky was significantly less in comparison to wild-type Typhimurium, approximately 3921 CFU/mL. Though Kentucky is currently the most frequently isolated serotype in commercial poultry, Kentucky is less commonly associated with human disease. Kentucky was not among the 20 most frequently reported serotypes listed for the United States in 2011 [142]. Based on a review of recent scientific literature, to our knowledge, this is the first study to demonstrate *in vitro* invasion of a human cell line by Kentucky.

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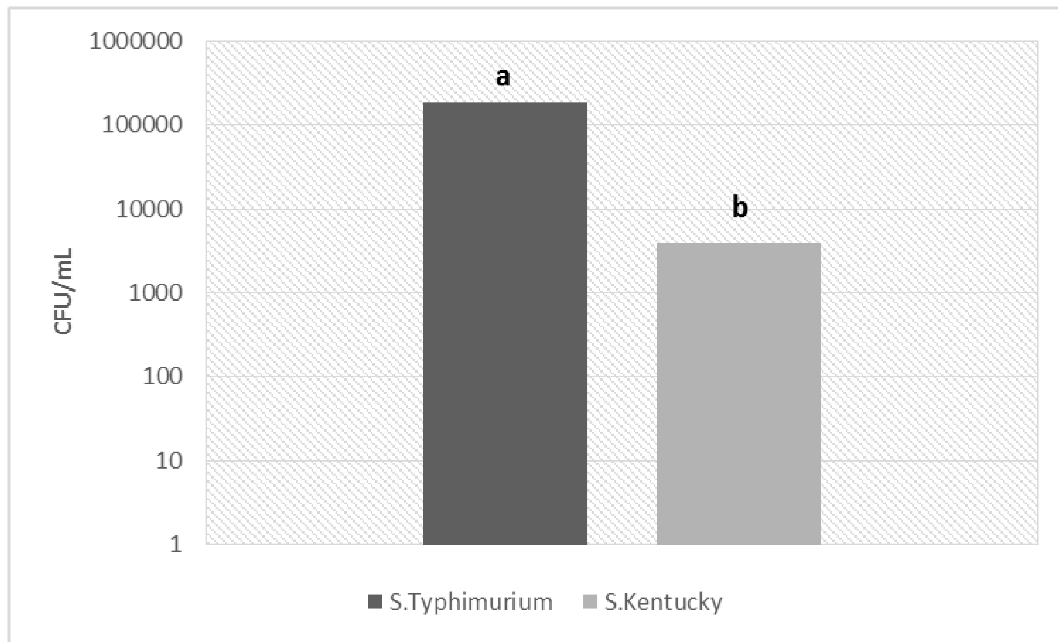

Figure 5.9 Estimated rate of invasion of Caco-2 cell monolayer by wild-type strains

Wild-type strains are expressed along the x-axis. Bacteria number are expressed along the y-axis and estimated per mL for 1 h invasion period. Strains with the same letter are not statistically different at $P \leq 0.05$.

The frequent isolation of Kentucky serotypes in commercial poultry, especially MDR strains, is significant from a public health standpoint. Angulo et al. suggests that resistance reservoirs in food animals can possibly promote the spread of resistance determinants among bacteria and lead to antimicrobial-resistant infections in humans [143]. Within the last decade, a MDR isolate of Kentucky displaying high-level resistance to ciprofloxacin was disseminated internationally with poultry suspected as the potential major vehicle for infection [105]. The strain identified ST198-X1 CIP^R infected nearly 500 patients in France, England and Wales, Denmark, and the United States during 2002-2008 with travel to northern Africa in common [105]. Kentucky isolates have been reported in Europe from travelers returning from northern Africa [106, 107] and more recently, Kentucky isolates with similar macrorestriction patterns to the isolates observed

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in Europe and Africa have been reported in Canada [108]. A majority of the *S*. Kentucky strains identified were ciprofloxacin-resistant [106-108], and many of the strains contained *Salmonella* genomic island 1 variants [108], resulting in treatment failure with traditional antimicrobials against nontyphoidal *Salmonella* infections due to high level of resistance.

Certain genetic factors have been hypothesized to be responsible for the emergence of Kentucky in poultry. In a comprehensive study of *S. enterica* serotypes, Kentucky was shown to have a slight growth advantage over other serotypes in mildly acidic environments (pH = 5.5), which may be beneficial in the mildly acidic environment of the cecum [144]. However, studies examining the colonization factors contributing to the emergence of Kentucky in poultry remain few. This serotype shares virulence genes with other *Salmonella* serotypes which are implicated in the colonization and infection of the gastrointestinal tract, however, mechanisms differ between serotypes, and even within an individual serotype, underscoring how gene expression can significantly affect these functions. The discrepancy of Caco-2 cell invasion rate between Typhimurium and Kentucky is possibly due to discrimination in target host by serotype related to genetic factors impacting host specificity including those linked with recognition and adhesion to intestinal surfaces and mechanisms of invasion of epithelial cells.

The factors initiating colonization for Typhimurium have been studied extensively for multiple hosts. Recognition and adhesion by *Salmonella* Typhimurium str. 798 to enterocytes in mouse and pig were related to phase variant expression of type I fimbriae [75]. Additional findings focused on *Salmonella* Typhimurium highlighted the

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important role fimbriae has to facilitate adherence and adaptation to a eukaryotic cell surface [145]. Genes associated with lipopolysaccharide biosynthesis have been determined to influence intestinal colonization of 3-week-old chicks by *Salmonella* Typhimurium F98 [146]. Less is known about the genes associated with recognition and adhesion for Kentucky and given the range colonization mechanisms can vary between hosts and within an individual serotype, the identification of factors influencing host colonization and adaptation unique to Kentucky would seem practical to comprehending its recent emergence in poultry.

The recent rise of Kentucky may correspond with its acquisition of a ColV virulence plasmid [103]. Enhancement of colonization induced by ColV plasmid has been observed for *E. coli* in animal models [147, 148] and ColV plasmid has been associate with conferring virulence factors such as resistance serum [149] and oxidative stress [150], promotion of iron acquisition [151], and metal transporters [150]. Of 293 isolates of Kentucky that were examined for the presence of ColV plasmid-associated genes, 213 (72.9%) contained all of the expected genes [152]. In the same study, a comparison between three sequenced Kentucky ColV plasmids, whose host strains differed in temporal and spatial isolation and source, revealed the plasmids were essentially identical. Considering the typical heterogeneous genetic nature of ColV plasmids [153] and the congruency of the ColV plasmids isolated from the Kentucky strains, a single horizontal transfer event may have occurred that resulted in clonal expansion of a prominent clonal type of Kentucky in poultry [152]. The presence of the ColV plasmid is a compounding factor that may enhance the persistence of Kentucky in the avian host and suggests that Kentucky has the ability to acquire and disseminate large plasmids encoding

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MDR and virulence [103]. As some plasmids are capable of autonomous restriction and modification, determinants for host specificity may be integrated into plasmid DNA molecules and then acquired by the pathogen. Given the prominence of Kentucky in commercial poultry and poultry products [98-101] and the latest cluster of MDR Kentucky cases isolated from humans [106-108, 154, 155] associated with ColV plasmid-associated genes [152], a potential opportunity exists for Kentucky to adapt, expand its host range and become a considerable zoonotic pathogen.

Invasion Rates of Wild-type and Mutation Strains

The deletion mutation (Δ*spaS-invG*) of the structural genes associated with T3SS decreased the rate of invasion for both serotypes in comparison to their wild-type strain. Human cells are typically not the specified host for Kentucky and so the invasion rate for the wild-type strain is limited (3921 CFU/mL). The loss of T3SS function reduced invasion 38.1% from the wild-type to 2425 CFU/mL (Figure 5.10) which was not significant ($P \leq 0.05$). Invasion by Typhimurium was significantly affected by the disruption of T3SS function, decreasing invasion 85.1% from the wild-type to 12445 CFU/mL. This reduction of invasion rate resulted from the inactivation of T3SS function, which signifies the prominent role this multi-protein complex system contributes to facilitating invasion, a finding that corresponds with previous scientific findings concerning the role of T3SS [80, 89, 156, 157]. Active invasion of epithelial cells by the mutation strains represents possible alternative mechanisms of invasion that may act independently and/or in a supplemental role to T3SS.

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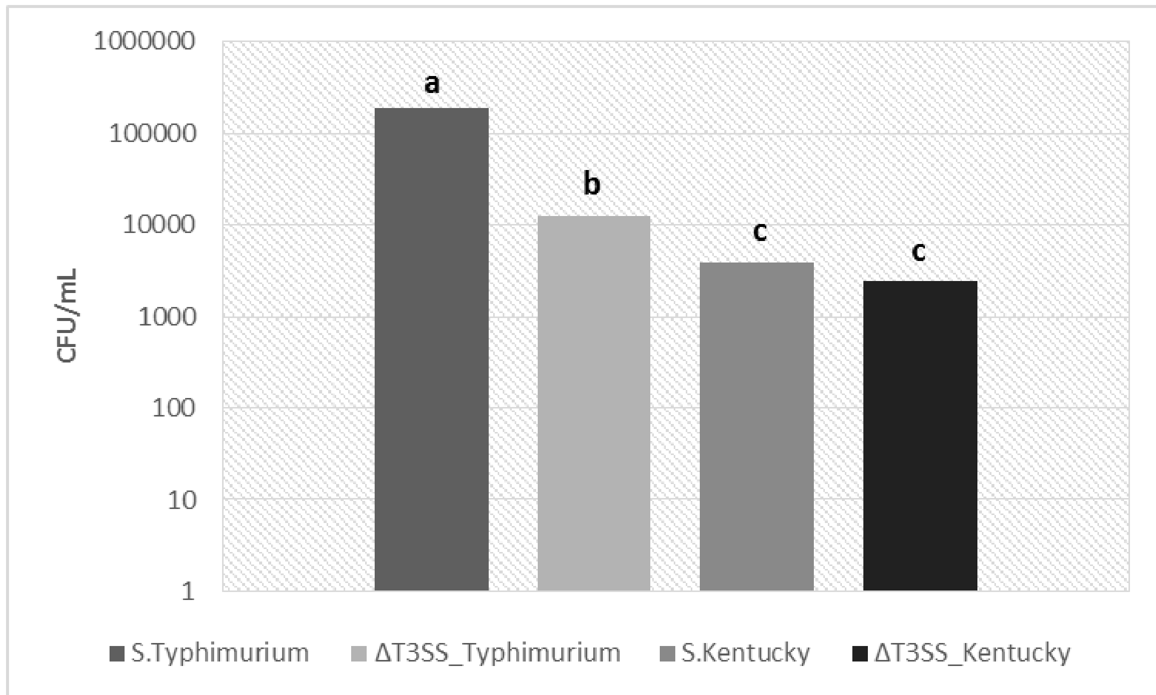

Figure 5.10 Comparison of rate of invasion of Caco-2 cell monolayer

Strains are denoted along the *x*-axis. Bacteria number are expressed along the *y*-axis and estimated per mL for 1 h invasion period. Strains with the same letter are not significantly different at $P \leq 0.05$.

Our results correspond with previous findings that suggest infection by *Salmonella* can occur in a manner independent of T3SS [10, 89, 90, 158]. The *Salmonella* species appearing in the aforementioned studies were serotypes with broad host ranges. To our knowledge, this is the first study to replicate invasion, suggestive of T3SS-independent mechanisms, by a *Salmonella* serotype in a host not traditionally within its range. Due to the nature of invasion by Kentucky in the case of human colorectal epithelial cells, this possible alternative mechanism may exist as a minor invasive function with an 'indiscriminative' host range. *Salmonella* is capable of infecting diverse hosts and cells types and is an implication of this research that an 'indiscriminative' mechanism may be evolutionarily beneficial to target a broad range of hosts.

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An outer membrane protein has shown promise as an entry mechanism able to induce cellular invasion independently of T3SS. Rck is a 19-kDA outer membrane protein encoded on the large virulence plasmid [91] whose expression was necessary and sufficient to enable non-invasive *E. coli* and Rck-coated beads to adhere to and invade epithelial, fibroblastic, and endothelial cells through modulation of actin-mediated internalization [92]. The role of Rck in *Salmonella* invasion has been demonstrated *in vitro* after growing *Salmonella* in swarming culture conditions [159], however, its precise role in *Salmonella* pathogenesis and its cellular receptor are poorly understood.

Additional factors have been postulated to influence surface cell rearrangements of enterocytes and affect invasion of eukaryotic cells. Outer membrane protein PagN interacts with haperain sulfate proteoglycans on the surface of eukaryotic cells and overexpression of PagN in a noninvasive *E. coli* strain induced cell invasion [160, 161]. However, replication of PagN activity has occurred under limited environmental conditions and although PagN mediates interaction between *Salmonella* and mammalian cells, its role as an invasin remains uncertain. Reports conflict over the role of type VI secretion system [162, 163], though deletion of *Salmonella* Pathogenicity Island-6 resulted in decreased ability of *Salmonella* Typhimurium to enter cultured HEp-2 tissue culture cells [162].

The internalization into nonphagocytic cells of the intestinal epithelium is central to the pathogenesis of *Salmonella*, especially in relation to the maturation of infection. Most certainly it is viable to expect multiple mechanisms that perform overlapping functions to preserve the invasion process. The majority of the invasion factors studied manipulate host signaling pathways to activate host actin nucleation to mediate the

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uptake of *Salmonella* into host cells [164, 165]. However, recently *Salmonella* Typhimurium was shown to manipulate additional signaling pathways leading to actomyosin-mediated contractility, suggesting a flexibility to target different cell types and/or subcellular regions [166, 167].

Summary

In this research, two strains of *Salmonella enterica* were investigated for their ability to induce internalization by human colorectal adenocarcinoma epithelial Caco-2 cells. Serovar Typhimurium is well adapted for invasion of human cells. Serovar Kentucky has been identified in commercial poultry, especially MDR strains, however this serotype is not typically a zoonotic pathogen. Disparities in the ability of each serovar to invade Caco-2 cell monolayers were shown. The Type III Secretion System (T3SS), a known mechanism for active cell invasion, was disrupted through genetic deletion of structural genes associated with function of the secretion system. The genetic mutation caused a decrease in the efficiency of invasion in comparison to the control, though complete cessation of invasion was not seen. Future research from this work includes a closer examination of the factors associated with serovar and host that affect host specificity for invasion, as well as, mechanisms that may play a supportive role to T3SS.

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CHAPTER VI

CONCLUSION

The research presented here is an extensive study on the specie of Gram-negative bacteria *Salmonella* and its implication as a major food borne pathogen in the present food processing continuum. Within this study, a bioluminescent reporting system for *Salmonella* was introduced as plasmid pAK*lux*1. This reporting system is a major step forward in laboratory detection and tracking of *Salmonella*. Whereas conventional culturing techniques followed a time frame of 48 to 72 hours, bioluminescence imaging provides real-time quantification and tracking of live bacteria within its host. Additionally, the luciferase reaction does not require any exogenous substrates. Application of this reporting system with a chicken skin attachment model developed for this research, yielded results that demonstrated the variation that *Salmonella* serovars show in their ability to attach to chicken skin. The ability of a serovar to attach to chicken skin may correlate with its ability to persist in poultry production. Further work with this reporting system and attachment model indicated that simple washing in an agitated water bath can remove contaminating *Salmonella* from chicken skin, however this method alone cannot eliminate the *Salmonella* completely due to strong bacterial attachment to skin. Therefore, additional methodologies should be used in poultry processing to eliminate *Salmonella* from the chicken skin to prevent foodborne disease outbreaks.

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A limitation of the pAK*lux*1 reporting system is that stability experiments showed the plasmid was not as stable in *Salmonella* as in other Gram-negative species, and this reporting system may not be useful for long-term experiments. To address this limitation, plasmid pBEN276 was constructed to transpose the *luxCDABE* operon into the chromosome of *Salmonella* at one specific site. Strains harboring the gene insertion were rapidly detected following metabolic stagnation due to refrigeration temperatures upon return to ambient temperature. This result was further confirmed in a chilled water experiment with our chicken skin attachment model. Our results showed that washing in chilled water further enhanced the effect the agitated water bath had on decreasing *Salmonella* on chicken skin by further suppressing replication due to metabolic stagnation. Bacteria quantification was done with bioluminescence imaging and demonstrated that this stable reporting system could be employed in experiments replicating immersion chiller settings. The immersion chiller tank is a step within commercial processing of broiler carcasses that has been indicated as the point of most significant cross-contamination. Further research is needed to investigate the mechanisms of interaction between *Salmonella* and chicken skin that lead to initial contamination of the skin and the factors that enable persistence of the bacteria in the host.

Salmonella enterica serovar Kentucky has close association with poultry and poultry products and is usually among the most commonly isolated serovars as listed by U.S. Department of Agriculture (USDA) and Food Service Inspection Service (FSIS). Traditionally, *S.* Kentucky is not a zoonotic pathogen though recent reports have shown cases of *S.* Kentucky isolated from humans. The factors limiting or enhancing host specificity remain unclear. In this research, *Salmonella enterica* serovar Typhimurium, a

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known human pathogen, and *S.* Kentucky were genetically mutated to disrupt the function of the Type III Secretion System (T3SS). The T3SS is a unique protein delivery system that is involved with the induction of internalization of the bacteria by previously nonphagocytic cells. Using human colorectal adenocarcinoma epithelial Caco-2 cells as the matrix in a gentamicin protection assay, an experiment was designed to determine the rate of invasion by the two *Salmonella* serovars and their corresponding mutant strains. Results showed that *S.* Kentucky was able to actively invade the eukaryotic monolayer, though at a rate that was significantly lower than *S.* Typhimurium. Additionally, the mutated strains were able to initiate invasion of the eukaryotic monolayer, though not as effectively as the wild-type strains. Serovar discrepancies for invasion were seen with the mutated strains as well. The results from the invasion assay provided the basis for additional research to the factors delineating host specificity. These factors may act as potential targets as vaccination candidates. Extended research may also be aimed at the mechanisms of invasion that supplement the role the T3SS plays in host invasion.

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CHAPTER VII

FUTURE RESEARCH

The body of work accumulated for my dissertation has provided a strong basis upon which future research may stem from. One direct extension of my research may possibly involve further study of the Type III Secretion System and possible alternative purposes of the protein transport system. A hypothetical scenario may include recombination of the effector genes with therapeutic genes and manipulating the secretion system as a drug delivery system. Other possible scenarios may include directing expression of the secretion system and/or the target cell to expand the range of the needle complex to transport proteins of interest. Research aimed at harnessing the precision and seamless ease with which nature carries out biological procedures is akin to summiting Mount Everest.

One aspect of my dissertation research that I was not able to address was the inter-species interactions of *Salmonella* while in the gastrointestinal tract. With anywhere between 300 and 1000 different species of bacteria comprising the flora of the large intestine of the human, the interactions and communication occurring within this environment seem extraordinarily vast. How a species is able to navigate and process signals from its immediate environment remain hidden within its genetic code. For example, SdiA is a quorum sensing regulator. *Salmonella* can activate this regulator by detecting acyl homoserine lactones (AHL), however, *Salmonella* cannot synthesize AHL

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itself but detects AHL molecules from other microbes. Complex, sophisticated mechanisms of sensing and interacting with the environment, such as this one, compose an intricate web of communication that allows the proper function of the organ. A disruption to this web can unbalance the environment and lead to malfunction, tissue injury, even disease if equilibration is not restored. Quorum sensing is a possible area of research that shows promise for inter- and intra-species communication. Broader areas of research that are attractive include sociomicrobiology, systems biology, and metabolic modeling.

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