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ABSTRACT

Most *Salmonella enterica* serovars are believed to have a cyclical lifestyle involving both host-associated and environment-associated, persistent phases. Their ability to persist in the environment increases the probability that they will be transmitted. Our hypothesis is that the genetic factors required for cellular aggregation and biofilm formation are important for host-to-host transmission. A link between biofilm formation, environmental persistence and transmissibility has not been observed, due to the lack of an appropriate model.

We developed a murine model of *Salmonella* transmission allowing us to study the genetic factors involved in the transmission process. To test the role of aggregation and biofilm formation we used the ΔcsgD mutant, which is deficient in both processes. We also engineered luciferase reporter strains of *Salmonella enterica* serovar Typhimurium (*Salmonella* Typhimurium) to track infection within a mouse population before the onset of clinical signs using bioluminescent imaging.

We determined that mice shed high levels of *Salmonella* Typhimurium in their feces when pre-treated with streptomycin. To observe the transmission efficiency of *Salmonella*, we tracked their spread from infected mice to naive mice, and determined that *Salmonella* could be transmitted only after pre-treatment with streptomycin. We compared the shedding potential and colonization levels of mice challenged with either wild-type *Salmonella* Typhimurium or the ΔcsgD mutant and determined them to be statistically similar when challenged separately. We found that wild-type *Salmonella* Typhimurium persisted in fecal pellets at higher levels than the ΔcsgD mutant. We compared both the short- and long- transmission potential of the ΔcsgD mutant to wild type *Salmonella* Typhimurium, and found that the mutant did not have a defect in either process.

Though not observed in our model, we believe that environmental persistence and biofilm formation are important for the transmission of *Salmonella* due to its cyclical lifestyle. The model we generated remains useful to test the role of other genes in transmission. It can be further refined to more accurately mimic environmental transmission of *Salmonella*. Further understanding of the transition of *Salmonella* from infected hosts to the environment and back into new hosts will aid in reducing its environmental persistence and transmission.
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5.1 Discussion

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LIST OF ABBREVIATIONS

Amp – ampicillin
BCC – Burkholderia cepacia complex
c-di-GMP – bis-(3’-5’)-cyclic dimeric guanosine monophosphate
Cam – chloramphenicol
CDC – Centers for Disease Control, United States of America
CFU – colony forming unit
CPS – counts per second
FMNH$_2$ – riboflavin phosphate
GALT – gut associated lymphoid tissues
i.v. – intravenously
Kan – kanamycin
LoD – limit of detection
LPS – lipopolysaccharide
MCS – multiple cloning site
MLNs – mesenteric lymph nodes
PCR – polymerase chain reaction
PMLs – polymorphonuclear leukocytes
rdar – red, dry and rough
SPI1 – Salmonella pathogenicity island 1
SPI2 – Salmonella pathogenicity island 2
tafi – thin, aggregative fimbriae
Tet – tetracycline
T3SS – type III secretion system
WT – wild-type
1.0 INTRODUCTION

1.1 Introduction to Salmonella

1.1.1 Classification of Salmonella

The genus Salmonella consists of two species: Salmonella enterica and Salmonella bongori. S. enterica can be further divided into six different subspecies: S. enterica subsp. enterica, S. enterica subsp. arizonae, S. enterica subsp. diarizonae, S. enterica subsp. salamae, S. enterica subsp. houtenae and S. enterica subsp. indica. Due to previous naming conventions, these subspecies are sometimes referred to as S. enterica subspecies I (enterica), II (salamae), IIIa (arizonae), IIIb (diarizonae), IV (houtenae) and VI (indica). Prior to its designation as a distinct species of Salmonella, S. bongori was referred to as S. enterica subspecies V. These subspecies further contain many different serovars or serotypes of Salmonella, such as the S. enterica subsp. enterica serovars Typhimurium, Enteritidis, Typhi and Choleraesuis. Almost 2600 serovars of Salmonella have currently been described. Of these, 60% belong to S. enterica subsp. enterica, as do 99% of all Salmonella isolates taken from humans and domestic mammals. Salmonella can also be loosely divided into two groups based on the diseases they typically cause in humans: typhoidal and nontyphoidal Salmonella. Typhoidal Salmonella, such as the S. enterica subsp. enterica serovars Typhi and Paratyphi, cause a systemic disease known as typhoid fever characterized by fever, abdominal pain and the presence of a rash, while nontyphoidal Salmonella, such as Salmonella enterica subsp. enterica serovar Typhimurium (referred to as Salmonella Typhimurium), typically cause a self-limiting gastroenteritis.

1.1.2 Disease, pathogenesis and epidemiology of Salmonella infections

Human infections with nontyphoidal species of Salmonella typically present as self-limiting gastroenteritis with symptoms of nausea, abdominal pain, diarrhea, vomiting and headache. The incubation period before the onset of clinical signs varies depending on the individual and the inoculation dose but typically ranges from 6 – 72 hours. Systemic infection can occur in rare cases leading to complications such as bacteremia, meningitis, encephalitis, osteomyelitis and endocarditis. Risk factors for severe disease following infection include immunosuppression, recent antibiotic use, HIV infection and both extremes of age. As most nontyphoidal Salmonella (NTS) infections are typically self-limiting, antibiotic therapy is not
recommended except in severe cases, and in some cases may even prolong the duration of disease and/or shedding of the bacteria. Due to the increasing presence of antibiotic-resistant isolates of various *Salmonella* serovars (such as *Salmonella Typhimurium*) in foodborne infections, the avoidance of antibiotic use will likely become even more pronounced in the future.

Upon encountering a susceptible host, nontyphoidal *Salmonella* species, like *Salmonella Typhimurium*, begin by colonizing the terminal ileum and colon, causing gastroenteritis. Using a type III secretion system (T3SS) encoded by the *Salmonella* pathogenicity island 1 (SPI1) bacteria are able to penetrate and invade intestinal epithelial cells using various effector proteins secreted by the SPI1 T3SS, preferentially targeting M cells. Once inside epithelial cells *Salmonella* are able to invade tissue mononuclear cells (i.e. macrophages and dendritic cells) and using a second T3SS – encoded by the *Salmonella* pathogenicity island 2 (SPI2) – they sculpt the intracellular environment to their benefit. A local inflammatory immune response is induced, resulting in large infiltration by polymorphonuclear leukocytes (PMLs) into the intestinal lumen resulting in the characteristic intestinal inflammation and diarrhea. Infection of a healthy individual with nontyphoidal *Salmonella* typically results in pathology that is limited to the intestine, but immunocompromised individuals may experience a systemic disease aided by the presence of *Salmonella* in immune cells and their dissemination throughout the body.

Nontyphoidal *Salmonella* are a major contributor to foodborne bacterial infections, causing an estimated 94 million infections worldwide each year leading to 155,000 deaths. In the United States, nontyphoidal *Salmonella* are the leading cause of both hospitalization and death by foodborne illness, and they are estimated to cost the Canadian economy $846 million each year. Foodborne outbreaks of *Salmonella* are commonly seen and typical sources of infection include food products such as beef, chicken, pork, eggs, dairy products and fresh produce. According to a study from the Centers for Disease Control (CDC), the most commonly isolated *Salmonella* serovars in the U.S. in 2009 were *Salmonella Enteritidis*, *Salmonella Typhimurium*, *Salmonella Newport*, *Salmonella Javiana* and *Salmonella Heidelberg*. Novel sources of *Salmonella* infection continue to emerge, likely due to the adaptability of *Salmonella*, their ability to persist for prolonged periods of time in numerous food products, as well as new trends in how the population is consuming food. The emergence of a global marketplace sourcing products from many countries with differing standards of microbial...
surveillance as well as an increasing population of aging individuals suggests that foodborne illnesses will likely remain a significant problem in the future\(^{111}\).

1.1.3 *Salmonella* life style

Most *S. enterica* subsp. *enterica* serovars are believed to have a cyclical lifestyle involving both a host-associated, infectious phase and an environment-associated, persistent phase\(^{155}\). The host range for many *Salmonella* serovars encompasses a large spectrum of animals including, but not limited to humans, primates, mice, chickens, pigeons, cows, pigs and lizards\(^{21,45}\). Differences in the specificity of host restriction as well as in disease severity are thought to be due to the existence of both a core set of genes shared by most serovars of *Salmonella* (genes related to metabolism, DNA replication, etc.) as well as variable areas of the genome related to virulence and the colonization of specific hosts\(^{21,40,45,116}\). This suggests that an individual serovar may be well adapted for the infection of their typical host yet poorly adapted for the infection of a sufficiently different host species. Different *Salmonella* serovars can also be classified as host-generalist, host-adapted or host-restricted. Host-adapted and host-restricted serovars of *Salmonella* typically cause invasive diseases and spend the majority of their life inside of infected hosts where they have developed specific strategies to evade immune detection\(^{147}\). Host-generalist serovars typically cause gastroenteritis in a broad range of hosts and the environment plays a larger role in their life style and transmission\(^{11}\). This difference in life style is thought to be largely due to the loss of genes in host-restricted and –adapted serovars of *Salmonella*, resulting in their increased dependency on their host\(^{11}\).

Infected hosts act as reservoirs of *Salmonella* who then seed the environment with bacteria where they persist until they encounter another susceptible host\(^{106}\). The ability of *Salmonella* cells to persist in the environment for prolonged periods of time increases the chances that they will successfully transmit to a new host and continue the infectious cycle\(^{155}\). Studies have shown that an individual strain of *Salmonella* can be isolated in an area from both infected animals themselves as well as from environmental sources nearby (i.e. soil, slurry, cages, etc.) even after disinfection, which act as a source of further infection and propagation\(^{5,41}\). Certain *Salmonella* serovars are also able to persist for periods of several weeks in water sources such as ponds, surface water and sewage and these can act as environmental sources of contamination as well\(^{18,96}\). These observations support the idea that the environment and
environmental reservoirs play a significant role in the persistent life style of *Salmonella*. The role of biofilms in this persistent phenotype of *Salmonella* will be explored in a subsequent section.

1.1.4 *Salmonella* mouse models

The majority of information regarding *Salmonella* pathogenesis and infection comes from the use of mouse models. In these models, it has been observed that *Salmonella* invade gut epithelial cells, some with a preference for M cells, in the small intestine before entering the gut associated lymphoid tissues (GALT) that lie underneath the epithelium. From the GALT, *Salmonella* spread systemically throughout the body and enter the spleen and liver, where they reside in phagocytic cells, as well as the mesenteric lymph nodes. In these models, the murine gut has much less inflammation than what is observed in human gastroenteritis. Resistance to murine *Salmonella* infection is primarily controlled by the mouse solute carrier family 11α member 1 locus (Slc11α1, formerly known as Nramp-1) that aids in acidifying phagosomal compartments in macrophages. Susceptible Slc11α1 mice, such as C57BL/6 and Balb/c mice, are used as an acute infection model of *Salmonella* infection as they typically succumb to infection due to their inability to control *Salmonella* replication and spread. In a chronic model of *Salmonella* infection, resistant Slc11α1+ mice are used as they are able to control *Salmonella* replication in the spleen and liver, resulting in a sub-acute, persistent and chronic infection that can last for a period of months.

To more closely mimic human gastroenteritis, the streptomycin-treatment mouse model of *Salmonella* infection was developed. First published in 2003, this model proved to display many of the hallmarks of human gastroenteritis in mice: epithelial ulceration, edema, induction of intercellular adhesion molecule 1 and a large proliferation of PML/CD18+ cells were all observed using this model. The streptomycin model involves treating mice with a single dose of the antibiotic streptomycin prior to challenge with *Salmonella*, which results in a transient disruption of the microbiota. This leads to high levels of gut colonization in the colon and cecum and high levels of intestinal inflammation within the first 24 hours. *Salmonella* blooms in the murine gut within 4 – 6 hours post-infection, resulting in high bacterial densities in the large intestine and high levels of bacterial shedding in the feces. Compared to bovine and primate models – the typical models used to study gastroenteritis – there are identical virulence
factor requirements in the streptomycin mouse model, suggesting it is a more economical model to effectively study the effects of *Salmonella*-induced gastroenteritis.\(^8\)

### 1.2 *Salmonella* biofilms

#### 1.2.1 Composition of *Salmonella* biofilms

A bacterial biofilm is defined as a structured community of bacteria encased in a self-produced extracellular matrix, typically attached to a biotic or abiotic surface\(^{137}\). Biofilms represent a major and common form of bacterial life in the various natural environments\(^{128}\). Biofilm development is thought to be a sequential process beginning with unaggregated planktonic cells which reversibly bind to a surface and form microcolonies of aggregated cells followed by the formation of larger, hardier macrocolonies. Mature biofilms are thought to be dynamic, multilayered structures where cells are free to disperse back into the environment as both planktonic cells or clusters\(^{108,128,159}\). *Salmonella* biofilms consist of both protein and polysaccharide components linked together in an extracellular matrix, and this matrix mediates interactions with the environment surrounding the bacteria\(^{55}\). The protein components of *Salmonella* biofilms consist largely of curli fimbriae (previously referred to as thin, aggregative fimbriae – Tafi)\(^{33,121}\) and secreted BapA\(^{92}\), while the polysaccharide components are composed mainly of cellulose and an O-antigen capsule\(^{55}\), with small levels of other polysaccharides such as LPS\(^{137}\). The rdar morphotype (red, dry and rough) has been characterized as one of the major biofilm types of *Salmonella*, and is characterized by its unique colony morphology\(^{151}\). rdar colonies take on a red color when grown in the presence of the dye Congo red due to staining of various components of the biofilm extracellular matrix, and deficiencies in the production of these components can be observed due to abnormal staining\(^{162}\).

The structural components of curli fimbriae are encoded by the *csgBAC* operon, where CsgA is the major structural subunit (*csg* for curli synthesis genes. *agf*, for aggregative fimbriae, was used previously but *csg* will be used going forward in this thesis). Research has shown that curli fimbriae are involved in the initial attachment phase of biofilm formation where bacteria adhere to a surface\(^{6,97}\), as well as in further interactions with both host and bacterial cells, linking cells together in a matrix\(^{154}\). They have been implicated in the attachment of cells to abiotic surfaces and are typically produced only at temperatures below 30° C\(^{123}\), suggesting they play a significant role outside of hosts. BapA is a large protein that is secreted from cells and associates
at the cell surface along with other biofilm components\textsuperscript{92}. It is thought to play a role in the physical connections of cells in a biofilm, strengthening and reinforcing the bonds made by the other biofilm components\textsuperscript{137}. It was found that BapA-deficient mutants had reduced invasion of epithelial cells as well as reduced colonization of host organs, indicating that it may play a role in host cell colonization and invasion, providing a potential link between biofilm formation and virulence\textsuperscript{92}.

Cellulose biosynthesis is controlled by the \textit{bcsABZD} and \textit{bcsEFG} operons and requires indirect activation of the trans-membrane protein AdrA (described in Section 1.2.2 below). Cellulose is composed of repeating (1→4)-\(\beta\)-linked D-glucose chains; the chains align to form a hydrophobic, inert matrix that, along with curli fimbriae, traps and holds cells together in the biofilm\textsuperscript{162}. This matrix is highly resistant to both strongly acidic and strongly alkaline solutions, in agreement with its proposed role in resistance\textsuperscript{162}. Cellulose is also thought to play a critical role in adhesion to external surfaces due to its sticky texture and its ability to facilitate long-range cell-to-cell interactions\textsuperscript{137}. The \textit{Salmonella} O-antigen capsule is structurally very similar to the O-antigen component of LPS, with a few substitutions to the side-chains of the 4-sugar repeating unit\textsuperscript{55}. The \textit{yihU-yihO} and \textit{yihVW} operons, which code for the production of the O-antigen capsule, are conserved throughout the \textit{Salmonella} genus, suggesting they likely play an important role in the life cycle of these bacteria\textsuperscript{55}. The O-antigen capsule is composed of >2300 repeat units which is ~100 times greater than that found in the LPS chains of most typical \textit{Salmonella}\textsuperscript{55}. It is hypothesized to play a role in protecting the biofilm-encased cells against desiccation and therefore likely also involved in environmental survival\textsuperscript{55}.

\textbf{1.2.2 Regulation of \textit{Salmonella} biofilm formation}

\textit{Salmonella} biofilm formation is regulated by a highly complex regulatory network involving many different interconnecting systems\textsuperscript{137,150}. CsgD is the major transcriptional response regulator that regulates the expression of the different structural components of the biofilm matrix\textsuperscript{54}. It has been implicated in the regulation of the \textit{csgBAC} operon which encodes curli fimbriae\textsuperscript{160}, the \textit{bapABCD} operon which encodes BapA\textsuperscript{92} and the \textit{yihU-yihO/yihVW} operons which encode the \textit{Salmonella} O-antigen capsule\textsuperscript{55}. Biosynthesis of cellulose is likewise indirectly regulated by the binding of CsgD to \textit{adrA}; this results in the production of bis-(3′–5′)-cyclic dimeric guanosine monophosphate (c-di-GMP), an important secondary messenger, which
allosterically activates the enzymes BcsA and BcsB. Recently, a CsgD-independent pathway of cellulose production was identified, suggesting that other regulatory methods exist separate from CsgD. Mutants in csgD lack multicellular behavior and do not produce the major biofilm components.

The regulation of CsgD itself is also complex. One method of its regulation is through the activity of 6 different diguanylate cyclases and phosphodiesterases which regulate the intracellular pool of c-di-GMP. There are also many different environmental signals that regulate its transcription: temperature, osmolarity, nutrient levels, iron concentration, pH and oxygen levels have all been shown to influence the transcription of csgD. Typically, stationary growth in conditions of high cell densities and low nutrients activate the expression of csgD. Many different trans-acting regulators have been implicated as well: H-NS, OmpR, IHF, CpxR and MlrA have all been shown to affect the expression of csgD. The variety of inputs that regulate the expression of csgD allow its expression to be fine-tuned in response to varying environmental conditions, increasing the sensitivity of biofilm formation as a physiological response. A study found that csgD expression was bimodal in a population of cells; cells either had high expression of csgD or had its expression turned off, with little intermediate expression observed. High expression of csgD was associated with the population of aggregated cells while low expression of csgD was associated with planktonic, single cells. It has been hypothesized that the bistable expression of csgD exists in order to maximize the survival potential of a population of cells under changing environmental conditions, ensuring that a subset of the population survives regardless of the conditions encountered.

RpoS (σS), encoded by rpoS, is another major regulator of biofilm formation. As a sigma factor, it regulates the transcription of many genes that are involved in the stress response and environmental survival, both situations where biofilm formation could occur. It has been shown that over 25% of the RpoS regulon was upregulated in biofilm-associated cells, as well as that wild-type Salmonella had 3 times the transcription levels of RpoS as a csgD mutant, which was unable to form biofilms. RpoS has also been implicated in the regulation of csgD, adrA and the csgBAC operon, suggesting it plays a significant role in overall biofilm regulation. The PhoPQ two-component regulatory system has also been shown to regulate biofilm formation by repressing their formation, possibly through indirect regulation of RpoS levels in a cell. PhoP has been shown to both stabilize RpoS through the activity of IraP, as well as to enhance its
degradation through the activation of RstA\textsuperscript{25,144}. This suggests that PhoPQ is involved in the tight regulation of RpoS levels in a cell, which impact the ability of \emph{Salmonella} to form biofilms. Overall the high level of regulation of biofilm formation in \emph{Salmonella} underlines both its importance as well as the high energy costs of producing a biofilm; the process of producing the components of the biofilm matrix is a costly endeavor, and would not be started until a cell received the input from many different systems.

\subsection*{1.2.3 Survival advantages of biofilm formation}

Aggregation of \emph{Salmonella} cells via the rdar morphotype was shown to provide a virulence disadvantage against wild-type cells\textsuperscript{151}, suggesting that aggregation, and therefore biofilm formation, has a function separate from \emph{Salmonella} virulence. The rdar morphotype itself is generally associated with curli fimbriae production, cellular aggregation and biofilm formation\textsuperscript{32,153}. Supporting its role in non-host survival and environmental persistence, it was found that the genes involved in curli synthesis (an important indicator of rdar morphology) remain inactive inside of a mouse and are expressed only after being shed into the external environment\textsuperscript{151}. The rdar morphotype was also found to be highly conserved throughout \emph{Salmonella}\textsuperscript{153}, supporting the idea that it plays a role in a significant portion of the \emph{Salmonella} life style.

It has been shown that rdar-positive cells, when compared to mutants in \textit{csgD} (biofilm transcriptional regulator), \textit{csgA} (curli fimbriae) and \textit{bcsA} (cellulose production), had increased resistance to prolonged periods of desiccation in the absence of nutrients\textsuperscript{152}. The majority of cells in rdar colonies grown on agar remained alive after a period of nearly 2 months and colonies that had been peeled off of agar and desiccated for up to 9 months in plastic wells continued to harbor viable cells. The mutant strains in question all had reduced survival, indicating that curli fimbriae and cellulose both played a role in the long-term survival of these cells. Cellulose was also observed to play a role in protecting rdar colonies against sodium hypochlorite (bleach), a common disinfectant, confirming earlier reports of the vital role of cellulose in resistance to sodium hypochlorite\textsuperscript{133}. A study looking at the survival benefits of the O-antigen capsule in similar conditions found that it was highly involved in resistance to desiccation\textsuperscript{55}. O-antigen capsule mutants (\textit{\textDelta}yihO, \textit{\textDelta}yihQ and \textit{\textDelta}Pyih) had significant reductions in their survival after desiccation to levels similar to that of the \textit{\textDelta}csgD mutant, which had the
lowest survival observed in other studies\textsuperscript{152}. In a later study it was found that rdar cells had upregulated many genes involved in osmoprotection, suggesting a mechanism through which rdar cells could resist desiccation\textsuperscript{150}.

Various other studies have shown that \textit{Salmonella} biofilms impart resistance to many disinfectants, sanitizers and antibiotics. A study comparing the survival of biofilm-associated and planktonic cells of \textit{Salmonella} Weltevreven found that biofilm cells resisted both chlorine and iodine at much higher concentrations than planktonic cells\textsuperscript{80}. Biofilm cells required both increased contact time as well as higher concentrations than planktonic cells to be completely eradicated. A similar study found that 2-day old biofilms had increased resistance to three different disinfectants when compared to planktonic cells\textsuperscript{109}. Biofilms of the \textit{S. enterica} serovars Senftenberg and Agona were found to be resistant to disinfectants based on cationic tensides, glutaraldehyde and hypochlorite (bleach), while ethanol-based disinfectants appeared to be quite effective. They also found that surface-dried \textit{Salmonella} retained many resistance properties against these disinfectants, suggesting that desiccation-resistant cells are resistant to these disinfectants as well. Various antibiotics have also been tested to demonstrate the resistance of biofilm-associated cells compared to planktonic cells. In one study, planktonic cells were susceptible to most antibiotics tested (5 of 7) while biofilm-associated cells were resistant to all antibiotics tested except enrofloxacin and ampicillin\textsuperscript{112}. In another study, biofilm-associated \textit{Salmonella} were found to have almost 2000-times the resistance to ciprofloxacin than planktonic cells\textsuperscript{138}. This is especially of interest because ciprofloxacin and other third-generation cephalosporins are regularly used to treat nontyphoidal \textit{Salmonella} infections\textsuperscript{137}; if biofilm-associated cells are involved in human infections, this could reduce the efficacy of treatment in these patients.

\textbf{1.3 Bacterial transmission}

\textbf{1.3.1 Role of transmission in the bacterial life cycle}

Transmission plays a large role in the lifestyle and fitness of bacterial species and is both a vital and required component in ensuring their maintenance in a host population\textsuperscript{15,94}. The cyclical nature of transmission suggests that it encompasses distinct stages of host infection and colonization, the exit from a host into the environment as well as movement from the environment into a subsequent host\textsuperscript{94,106}. While both host infection and colonization are
important in the overall cycle of transmission, they remain well-represented areas of *Salmonella* research; the factors involved in inducing the exit from a host into the environment as well as in facilitating the movement into a new host remain poorly understood, and are the focus of this thesis. As mentioned previously, many *Salmonella* species have a lifestyle that involves a significant environmental stage in between host colonization events that can last for prolonged period of time. This is not uncommon for pathogens: the transmission of many pathogens from host-to-host involves the environment as a tool to gain access to subsequent hosts. The ability of bacteria like *Salmonella* to replicate outside of their primary host – such as in the external environment – increases their odds of transmission by allowing them to remain in the environment for extended periods of time until a susceptible host can be found. The ability to transmit from host to host also increases the overall fitness of bacteria, especially when conditions become unsuitable for continued persistence in the current host.

The process of transmission must also exist as a balance between the ability to transmit to subsequent hosts and the appropriate level of virulence in the current host. If the primary transmission event occurs after a large amount of damage has been done to the infected host it will be unlikely to support subsequent transmission events, reducing the total transmission potential of each host. This would not be to the benefit of the bacteria as each host would have a limited number of opportunities where transmission could occur due to the significant damage each event caused. A general trade-off model has previously been proposed, suggesting that any pathogen must make trade-offs between transmission and host survival. Low levels of colonization typically result in reduced virulence and prolonged host survival but low pathogen transmission. High levels of reproduction in the host result in increased virulence and shorter periods of host survival but bring the benefit of high levels of transmission potential. Ideally, a balance would be found with optimal survival for both the host and pathogen. Alternatively, it could be said that the high extra-host stability of bacteria like *Salmonella* decreases the fitness costs of virulence in this situation; it allows the bacteria an alternate strategy of continued persistence regardless of whether their hosts survives. Regardless, this suggests that bacterial persistence and transmission have evolved through a balance of selection for both bacterial and host interests, resulting in overall homeostasis between the propagation of bacteria and their maintenance in a host.
Bacterial transmission is a poorly understood topic at present. Many host and bacterial determinants involved in the process that facilitate host-to-host transmission remain unknown while the topic continues to be poorly represented in microbial research. It is estimated that most *Salmonella enterica* subsp. *enterica* serovars have significant portions of the genes in their genomes (20 – 30%) with unknown functions. It is possible – even probable – that a large portion of these genes have functions in environmental persistence and transmission and are not involved in virulence. The time between host colonization events is a key period where interventions could be directed in order to reduce the transmission of pathogens like *Salmonella* within the human population, and in turn reduce the adverse impact that these bacteria have on the healthcare system. This is especially important for pathogens where pharmacological interventions have limited use, and these types of targeted interventions have been theorized to result in substantial reductions in the rates of transmission and infection seen in a heterogenetic population. In order to effectively develop these kinds of interventions against *Salmonella*, its interactions with both the external and host environments must be well-characterized and key transmission routes must be identified. Interventions must also be directed against all aspects of a pathogen’s transmission; for example, poor sanitation procedures result in the contamination of water supplies, which may then contaminate food sources and these food sources enter society as contaminated food products that negatively impact the healthcare system. Interventions would need to touch on proper sanitation procedures, the monitoring and handling of livestock and the testing of food before it enters the human environment among all other potential aspects. As both population movement and growth increase in the near future the horizontal transmission of pathogens is likely to become a greater problem, and such interventions will likely become necessary to prevent the mass dissemination of disease.

**1.3.2 Epidemiological studies examining *Salmonella* outbreaks and transmission**

A large majority of the research directed at *Salmonella* transmission exists as epidemiological studies examining the sources of outbreaks. These studies are largely retrospective but remain useful in identifying the potential sources of *Salmonella* infection and the reasons that these outbreaks occurred. In general, these epidemiological studies examine the potential sources of *Salmonella* infection or the risk factors that predispose individuals to infection with *Salmonella*. While useful at determining such potential sources of *Salmonella*...
infection, the length of time they take to produce actionable results often results in a delay of immediate aid (i.e. product recalls). By the time the source has been identified it is possible that many contaminated products have already been consumed, resulting in lowered efficacy of potential aid.

Contaminated water can be an important source of *Salmonella* outbreaks; *Salmonella* species were the causative agents of 19% of all drinking water-associated outbreaks in the United States from 1971 – 2006. The source of a 2008 outbreak of *Salmonella* Typhimurium in the town of Alamosa, Colorado was traced to the local water supply. During the outbreak 434 cases of *Salmonella* infection were reported, 124 of which were laboratory-confirmed, 20 which resulted in hospitalization and 1 which resulted in death. The majority of cases were thought to be unreported with an estimated total of 1,300 infections (15 – 20% of the town’s population). The source of contamination was determined to be animal fecal contamination of a supply tank that was part of the water supply; due to the town’s water being unchlorinated, this contamination spread to the population. A study looking at a small 2008 outbreak of *Salmonella* serotype I 4,[5], 12:i:- (a serotype similar to Typhimurium) in a rural town in Texas had similar findings. Contamination at a water source was hypothesized to be the cause of infection, likely from animals or a nearby septic tank. These and similar reports highlight the importance of water as a source of mass *Salmonella* transmission as well as the potential for animal shedding as a contaminant, suggesting that interventions directed at these sources would be largely beneficial.

Contaminated water sources have also been implicated in *Salmonella* outbreaks associated with the consumption of contaminated fruit and vegetables. A 2008 study examining a *Salmonella* Newport outbreak in 2005 found that the source was tomatoes from Virginia that were contaminated from pond water used to irrigate the tomato fields. This strain caused 72 laboratory-confirmed cases of *Salmonella* infection across 16 states with an estimated 2,500 total cases. *Salmonella* was detected in a nearby pond that was, in some cases, sprayed onto tomato plants. Generally, tomato-related *Salmonella* outbreaks are highly geographically dispersed, which suggests that the initial point of contamination is generally the farm or distribution facility rather than the point of sale to a consumer. This implies that the most impactful location for direct interventions would be at the farm or distribution level. Similar to the previous findings, a 2009 study looking at an outbreak of *Salmonella* Saintpaul in Australia found that cantaloupe were the most likely source of infection. Although they were unable to determine the exact
source, a few farms examined during the trace-back investigation had positive environmental contamination for *Salmonella* species. A previous study had shown that *Salmonella* could be recovered from cantaloupe for up to 21 days if stored at 4° C and up to 14 days if stored at 20° C, suggesting that these bacteria were able to persist for prolonged periods of time on the surface of the fruit \(^{119}\). Cases such as these suggest that the contamination of fruits and vegetables with *Salmonella* commonly happens due to environmental contamination at the farm or distribution level.

A 2009 study examined the risk factors in children involved with bacterial reportable enteric infections (REI-B), specifically those with *Salmonella*, *Campylobacter*, *Shigella* and *E. coli* O157 infections \(^{39}\). Risk factors associated with *Salmonella* infection included the consumption of raw pork and shrimp, raw sprouts and bagged lettuce/spinach, the presence and use of a private well or septic system at home and the use of natural water sources for recreation use. Another study examining risk factors for developing *Salmonella* bacteremia in children determined that, along with previous cases of gastroenteritis and antibiotic exposure, patients were more likely to develop bacteremia during the summer as compared to the colder seasons \(^{66}\). A Canadian study examining the risk factors of *Salmonella* Enteritidis infection from 2007 – 2009 found that being between the ages of 0 – 4, travelling internationally as well as the spring months (March – May) were associated with higher risk of *Salmonella* infection \(^{146}\). Along with the typical risk factors of consuming contaminated food sources, these findings suggest that non-food sources also play an important role in the transmission of *Salmonella*.

### 1.3.3 Environmental reservoirs of *Salmonella*

Environmental reservoirs play a large role in the survival and persistence of *Salmonella* species, as well as aiding in their perpetuation. As discussed above, *Salmonella* are frequently detected in environmental water samples. *Salmonella* species have been isolated from diverse water sources all over the world: from rivers in Canada \(^79\) and the U.S. \(^{103}\), surface and potable water in South Asia \(^81\), rural irrigation water in Alberta \(^52\), drinking water in New Zealand \(^{141}\) and many other sources. The ubiquity and persistence of these bacteria underlines the number of potential sources of contamination and entrance into the human population, through other vectors as well as food products. As mentioned previously, contaminated water sources are thought to be
a significant contributor to the contamination of fresh produce with *Salmonella* and other bacteria\(^\text{10}\), highlighting another area where the indirect impact of water can be seen.

Rodents are also believed to be a natural vector of *Salmonella* that play a role in their transmission and persistence. Although food production animals are the main source of direct human infection, rodents are thought to act as vectors that help maintain *Salmonella* in an agricultural environment\(^\text{74}\). This is especially of concern in areas housing food production animals, such as farms, due to their direct link into human consumption. Studies have shown that the risk of *Salmonella* persisting on a farm after decontamination increased with the presence of rodents, suggesting they may act as a reservoir for subsequent bacterial seeding\(^\text{48,122}\). It has also been suggested that rodents act to amplify the presence of *Salmonella* on farms. In one study of poultry farms rodents were found to have three times the infection levels with *Salmonella* Enteritidis than the surrounding environment, suggesting the rodents act to amplify the levels of *Salmonella* present in a given environment\(^\text{73}\). Due to the high density of rodents on some farms and their tendency to cluster together, this allows for rapid horizontal transmission of *Salmonella* throughout the population, reinforcing its maintenance. It also allows rodents to act as a persistent source of *Salmonella*, where they constantly reintroduce bacteria into the environment and subsequently re-infect other food-producing animals\(^\text{102,131}\).

Livestock are another reservoir of *Salmonella* that frequently enter the human environment. Chickens, pigs and cows are all known to be potential carriers of *Salmonella* and represent a major route of human infection\(^\text{57}\). In chickens, colonization with *Salmonella* can remain subclinical in many animals, making it difficult to detect in the food production chain prior to its consumption\(^\text{8}\). One study found that the overall prevalence of *Salmonella* on conventional broiler poultry farms was approximately 28%\(^\text{2}\), and the consumption of chicken remains a risk factor for salmonellosis\(^\text{100}\). *Salmonella* Choleraesuis has been shown to spread throughout pig populations rapidly and remains infective even after 2 – 4 months in desiccated feces\(^\text{61}\). Similarly to poultry, *Salmonella* infections in pigs often remain asymptomatic and result in high levels of shedding in a significant proportion of the population\(^\text{88}\). A study looking at *Salmonella* rates on swine farms in the U.S. found that over 50% of farms tested positive for the presence of *Salmonella*\(^\text{69}\). Numerous *Salmonella* serovars are frequently isolated from cattle farms, with some serovars such as *Salmonella* Kentucky having a prevalence of up to 97% on *Salmonella*-positive farms\(^\text{68}\). Other serovars, such as *Salmonella* Cerro, were shown to persist
subclinically in cattle herds for periods of up to 3 years. The high shedding and persistence levels as well as the ability of *Salmonella* to colonize production animals at subclinical levels increases the presence of *Salmonella* on livestock farms, resulting in its frequent transmission to humans.

1.3.4 Existing models to study bacterial transmission

The current transmission models for *Salmonella* typically employ mouse models of chronic infections (i.e. Slc11α1+ mice). The most prominent model, published in 2008, demonstrated transmission using a natural model of *Salmonella* persistent (i.e. chronic) infection in 129X1/SvJ resistant mice. Mice infected with *Salmonella* in this model become chronically infected for 30 – 40 days post-infection and persistently shed bacteria in their feces. To study transmission, four mice were challenged with *Salmonella* Typhimurium and then mingled with one uninfected mouse 5 days post-infection for a period of 28 days. The uninfected mice began shedding *Salmonella* in their feces 1 – 2 days post-mingling and the shedding levels, fecal anti-*Salmonella* IgA levels, serum anti-*Salmonella* IgG levels and colonization levels were all indistinguishable from the initially challenged mice at the conclusion of the experiment, suggesting that infected mice were rapidly able to transmit infection to naive mice. The authors noted that transmission was highly efficient and that most transmission occurred from a small subset of the population (<30%) termed supershedders that were shedding ≥10^8 CFU/g feces of *Salmonella* and were able to transmit infection to 100% of their cage mates. The low and moderate shedders shed *Salmonella* at levels of <10^8 CFU/g feces and had no observable transmission even after co-housing for 28 days. Of note, after treatment with streptomycin all mice were converted to supershedders, underlining the importance of the resident microbiota in preventing the supershedder phenotype and high levels of transmission. While useful, this model had no reliable method of determining which mice became infected until the conclusion of the experiment due to the sub-acute nature of the chronically infected mice (i.e. there were no visible symptoms); animals had to be euthanized to determine if transmission had occurred. The large majority of the population (>75%) were poor reservoirs of infection; a small subset of mice were responsible for the majority of the transmission observed, meaning that large groups of mice would be required for any large-scale transmission experiments in order to ensure observable
transmission. There remains a niche for alternate transmission models to decipher further aspects of *Salmonella* transmission.

There have been other preliminary studies examining the transmission of *Salmonella* in other species, though not in completely characterized models. A 1994 study in swine examined the rate of transmission from infected pigs to *Salmonella*-free pigs when co-mingled in the same enclosure\(^4^9\). Eight infected pigs were co-mingled with nine uninfected pigs for 21 days; prior to co-mingling the two groups were housed in separate facilities. None of the uninfected pigs became symptomatic during the course of the experiment and only one of these pigs tested positive in both rectal and tonsil swabs for *Salmonella*. Some uninfected pigs did have detectable *Salmonella* in their cecum (6), ileum (4) or ileocolic lymph node (8), suggesting that although no pigs had overt signs of disease, sub-acute transmission had occurred. A 2006 study examined the transmission of antibiotic-resistant or -susceptible isolates of *Salmonella* Typhimurium in chickens\(^8\). Each cage contained two seeder chickens that were challenged with *Salmonella* and 10 uninfected chickens. Half of the cages were challenged with an antibiotic-resistant strain of *Salmonella* and half with an antibiotic-susceptible strain. Half of the cages also had their water supplies supplemented with chlortetracycline. In cages challenged with the resistant strain and supplemented with chlortetracycline there was a 90% transmission rate from seeder chickens to naive chickens. In the same groups without chlortetracycline the transmission rate dropped to 60%, suggesting the antibiotic treatment increased the transmission efficiency of the resistant strain. The chickens treated with chlortetracycline also had higher levels of bacteria in the organs sampled which may have been responsible for the increased transmission. In cages challenged with the susceptible strain and either supplemented with chlortetracycline or not, the transmission rates were 95% and 90%, respectively; there was no significant difference between these two groups. Currently, there are no transmission models that facilitate the study of specific genetic factors in the transmission process of *Salmonella* and the role they play in transmission.

Transmission studies also exist for bacterial species other than *Salmonella*. A 2012 study examined the role of *spo0A* in *Clostridium difficile* in persistence and transmission using a mouse model\(^3^8\). This work was done after the construction of a generalized model to study the spore-mediated transmission of *C. difficile* in mice\(^9^3\). Donor C57BL/6 mice were challenged with *C. difficile* and then mingled with uninfected mice treated with clindamycin under different conditions. Donor mice challenged with wild-type *C. difficile* and mingled for 1 hour resulted in
100% transmission, while donor mice challenged with the ∆spo0A and then mingled resulted in 20 – 40% transmission, depending on the strain. When the same experiments were done with a porous wall separating the donor and naive mice – to prevent coprophagy but allow contact – wild-type C. difficile still transmitted at 100% efficiency while the mutant strains did not have detectable transmission. In the same experiment done with a double porous wall – to prevent both coprophagy and contact but allow airborne transmission – wild-type C. difficile transmitted with 60% efficiency while the mutants again failed to transmit. Finally, to mimic environmental transmission donor mice were placed in cages for 1 hour and then both mice and feces were removed. The cages were left in a sterile environment for 16 hours and then naive mice were placed inside. Wild-type C. difficile transmitted at 100% efficiency while the mutants again failed to transmit. These results indicated that spo0A was required for efficient host-to-host transmission, and this model allowed for the effective assignment of function to the gene spo0A in the transmission and persistence process in C. difficile.

A 2015 study examining the transmission of Staphylococcus aureus following bacteremia determined the impact that a selection of genetic regulatory systems had on transmission. Infected mice were injected intravenously (i.v.) with S. aureus and then cohoused with naive mice for the duration of the experiment. They observed that naive mice became infected with S. aureus after co-housing, as demonstrated by the fecal shedding levels in naive mice matching those of the i.v.-challenged mice. This suggested that S. aureus could be transmitted to naive mice through the fecal-oral route after a period of bacteremia in the original host. ∆sae, ∆agr and ∆sae∆agr mutants were compared to the wild-type, and it was observed that naive mice caged with the double mutant had significantly lower amounts of bacteria in their feces, presumably due to reduced transmission. This suggested that the sae and agr regulatory systems, involved in the regulation of many virulence factors, also played a role in host-to-host transmission in S. aureus. Finally, they determined that antibiotic-resistant strains of S. aureus were preferentially transmitted to naive hosts. This model, while able to qualitatively compare different strains in their transmission, relied on the detection of S. aureus in the feces of naive mice to measure transmission. An ideal model would allow for a more accurate and quantitative measurements of transmission to naive mice.

Other transmission experiments exist as smaller aspects of larger studies. A study examining the gastrointestinal colonization of Pseudomonas aeruginosa in mice housed 1
infected mouse with 3 uninfected, antibiotic-treated mice and found that there was efficient transmission of disease without a contaminated water source. This suggested a role of the fecal-oral route in the transmission of *P. aeruginosa*\(^8\). In a study examining lipid modifications of ArnT in *Bordetella bronchiseptica* the transmission efficiency of a \(\Delta arnT\) mutant was compared to the wild-type\(^1\). A mouse challenged with wild-type *B. bronchiseptica* and then housed with 2 – 3 naive mice for 21 days transmitted with >80% efficiency to secondary mice. The \(\Delta arnT\) mutant did not transmit to any secondary mice, suggesting that ArnT plays a role in host-to-host transmission. Since these studies touched on small aspects of transmission, more comprehensive studies would be required to determine the overall requirements and determinants of transmission in bacteria.

### 1.4 Genetic manipulation of *S. enterica* chromosomal DNA

**1.4.1 Tn7 transposition**

Transposons are transposable genetic elements\(^1\) that are able to mobilize throughout the genome using specialized recombinases – known as transposases – that are typically self-encoded.\(^12\) Generally, transposons exist as mobile elements that, while typically bringing no benefit to the host, could potentially cause lethal mutations. That they remain so common in nature organisms suggests that they are particularly well suited for rapid dissemination throughout the genomes of many different organisms. The bacterial Tn7 transposon is a particularly sophisticated example and has many unique and beneficial features. The Tn7 system has two main mechanisms for transposition. One method results in highly specific, directed transposition into a specific site in the bacterial chromosome – the \(att\)Tn7 site – while the other preferentially targets conjugative plasmids\(^1\). Of particular interest is the chromosomally targeted method, as it ensures that transposition can occur without having negative fitness impacts on the host bacteria due to its site-specific nature.

The Tn7 transposon itself is flanked by the Tn7L (150 bp) and Tn7R (90 bp) regions which contain transposase-binding sequences, allowing for precise binding of the transposase and subsequent transposition\(^1\). DNA contained between these two sequences is excised and integrated into the transposition site using the specialized recombinase that is self-encoded by the transposition operon. Insertion of the transposon is orientation specific due to the asymmetric sequences of both the Tn7L and Tn7R ends, as well as the function of the Tn7 system itself,
The transposon contains all the necessary components for transposition: namely, the *tnsABCD* genes. Transposition with Tn7 minimally requires the *tnsABCD* operon (only one site selector is required) and the two Tn7 flanking sequences, Tn7L and Tn7R. The Tn7 transposon system also appears to have a broad host range and is able to function effectively in many different bacterial species. Using these beneficial elements different Tn7 systems have been developed that take advantage of the different aspects of the bacterial Tn7 transposition system.

TnsA and TnsB together form the heteromeric Tn7 transposase utilized by the system. The transposase introduces double-strand breaks at either end of the transposon, then excises and integrates the DNA at the *att* site. TnsA mediates 5’ strand breaking while TnsB mediates both 3’ strand breaking and joining. TnsD and E are alternative site selectors that direct the transposase to a particular insertion site, depending on which is active, by binding DNA. TnsD is a sequence-specific DNA binding protein that directs transposition to the *att* site – a chromosomal region found downstream of the *glmS* gene in many bacteria. The amino acid sequence of the recognition site in *glmS* was almost 100% conserved in 6 representative organisms that were examined (E. coli, Rhizobium leguminosarum, Bacillus subtilus, Candida albicans, Caenorhabditus elegans and Homo sapiens) making it an ideal candidate for such strict recognition. While the recognition site is in the 3’ end of the *glmS* gene, insertion occurs downstream of the gene itself; insertion of genetic material at this site has not been found to result in any fitness defects to the bacteria itself. TnsE preferentially directs transposition into conjugative plasmids, though the particular insertion regions have no recognizable DNA similarity. It is hypothesized that TnsE directs the transposase to regions of DNA structure, such as the β clamp of replicating DNA. TnsC is an ATP-dependent DNA binding protein that acts as a regulator of Tn7 transposition by bringing together the transposase (TnsAB) and the site selector (TnsD or TnsE); it activates the transposase when in contact with both the transposase itself (through TnsB) as well as the site selector.

1.4.2 Tn7-based transposition and cloning systems

Due to the ease-of-use, specificity, efficiency and broad host range of Tn7 transposition many different cloning systems have been established to take advantage of transposition. These systems all utilize the Tn7 system as a mechanism to introduce foreign DNA into the
chromosome in a very efficient and site-specific manner. Chromosomal insertion has numerous advantages over typical plasmid-based systems. Chromosomal DNA is inherently more stable than extra-genomic DNA\textsuperscript{101}; this heightened stability, along with the systems that monitor the integrity of chromosomal DNA\textsuperscript{161}, ensures that the inserted DNA persists and is not lost during subsequent replication and division, as plasmids can be\textsuperscript{101}. Using plasmid-based reporter constructs typically results in a significant proportion of cells in the population experiencing plasmid loss, removing them from the pool of reporters\textsuperscript{151}. Chromosomal insertion also results in expression levels more closely resembling physiological levels, allowing for a better approximation of function\textsuperscript{101}. Chromosomally integrated constructs also remove the need for antibiotic selection, which would typically be required for the maintenance of plasmids but is not always desired (i.e. introducing bacteria into the environment or into human patients)\textsuperscript{101}. It has also been shown that the presence of cloning vectors in a cell can alter the virulence of certain bacteria, such as Salmonella\textsuperscript{89}. Finally, the site-specific nature of Tn7-based insertion results in repeatable and easily comparable chromosomal insertion. This allows the same construct to be inserted in different mutants, allowing for effective comparisons of expression dynamics. These are some of the reasons that chromosomal insertion systems are desired for cloning systems, and in particular why Tn7-based systems have become popular in recent years.

In 2005, a system was published that utilized the power of Tn7 transposition to develop a broad-based cloning and expression system\textsuperscript{26}. It used both pUC18T-based and pUC18R6K-based plasmids to design suicide delivery vectors that would allow the delivery of a construct into a cell that could then be targeted for insertion into the chromosome by the Tn7 transposition machinery. Main features of the plasmid were either ColEI (pUC18T) or R6K (pUC18R6K) origins of replication to allow the plasmids to function as suicide vectors in any non-Enterobacteriaceae (pUC18T) or in any strain not containing the pir gene required for replication (pUC18R6K). They also contained the bla gene encoding β-lactamase to allow for antibiotic selection. Each plasmid then contained a multiple cloning site (MCS) flanked by the Tn7L and Tn7R sites; this allowed the desired construct to be easily cloned into the MCS and be targeted for chromosomal insertion. A second, helper plasmid – pTNS1/2 – containing the genes encoding the transposition machinery (\textit{tnsABCD}) was used to mobilize the construct contained between the two Tn7 sites into the chromosome of the desired strain. Using this system, the authors demonstrated efficient transposition in \textit{P. aeruginosa} as well as demonstrating that
transposition occurred downstream of *glmS* at the *attTn7* site 100% of the time in *Pseudomonas putida*, a species that was not known to have a unique transposition site, when using the pTNS1 helper plasmid. To demonstrate the universal utility of the system they also demonstrated it working in *Yersinia pestis*, a species with no known site-specific integration cloning systems, as well as in *Burkholderia thailandensis*, a species whose complete genome was unknown at the time. Finally, they constructed *P. aeruginosa* strains containing chromosomally located expression systems to demonstrate the uses of the Tn7-based cloning system.

This mini-Tn7 system has been modified in different ways since its publication. Recently, a group published a modified system by adding a P*BAD* promoter and araC gene between the Tn7L and Tn7R sites to control the expression of the construct once inserted into the chromosome. This new plasmid – called pTJ1 – contains the trimethoprim-resistance encoding *dhfRII* gene as well. Using this new delivery vector, as well as the updated helper plasmid pTNS3, the authors demonstrated the function of the system by efficiently cloning their gene of interest into both *P. aeruginosa* and *Burkholderia cepacia* complex (BCC) species. The same group later published a separate modification of the mini-Tn7 system containing the *luxCDABE* operon contained between the Tn7L and Tn7R sites, whose expression was driven by the constitutive P1 integron promoter. The promoter region also allowed for the easy removal and insertion of any promoter of choice to facilitate promoter-expression studies. The authors demonstrated the utility of the promoter-luciferase constructs, both *in vitro* and *in vivo* using mice monitored on a bioluminescent imager.

A modified Tn7 system was published in 2006 which combined the helper and delivery vectors into a single plasmid – pGRG25. This plasmid contained a MCS flanked by the Tn7L and Tn7R sites to allow for simple ligation of the desired construct. The *tnsABCD* operon was contained on the same plasmid outside of the Tn7L and Tn7R ends to ensure it wasn’t excised along with the desired construct. Expression of the *tnsABCD* operon was driven by a P*BAD* promoter to allow for controlled expression of the transposition machinery; the araC gene was also contained on the plasmid, enabling arabinose-inducible expression of the *tns* operon. The vector had a temperature-sensitive pSC101 origin of replication enabling it to be cured from the recipient cells after transposition, as well as the β-lactamase-encoding *bla* gene for antibiotic selection. Using this system the authors confirmed that all cells contained the transgene at the *attTn7* site and that transposition occurred in 79% of cells that were tested. They tested the
system in various *E. coli* isolates and indicated that the system would work in any bacterium that supports the replication of pSC101-origin plasmids (*E. coli, Salmonella* and *Shigella* species); they also hypothesized that altering the origin of replication should result in effective function outside of the *Enterobacteriaceae*. This system had the benefit of requiring only a single plasmid, reducing the necessary work, as well as being claimed to be highly efficient. Due to this efficiency the authors claimed that drug selection was not necessary to obtain successful transposition.

The pGRG25 system was later modified by a subsequent group to contain the bacterial luciferase operon – *luxCDABE* – in the MCS, resulting in the plasmid pBEN276. The expression of the *lux* operon was driven by the *E. coli* frr promoter, which is a housekeeping gene encoding a ribosome recycling factor; this resulted in constitutive expression of the *lux* operon in subsequent reporter strains generated using this system. The indicated use for this system was to easily generate luciferase reporters in any strain of interest. They characterized the luciferase expression of reporters they generated and determined that the luciferase construct was stable in the chromosome for the tested period of time. Luciferase expression was also tested and found to be consistent over a range of temperatures including 25° C and 37° C, but not 4° C. Overall, this system demonstrated a method to efficiently generate luciferase reporters using a modified single-plasmid system.

### 1.5 *In vivo* bioluminescent imaging technologies

#### 1.5.1 Overview of *Photorhabdus luminescens* bacterial luciferase

Bacterial luciferases are the most widely distributed bioluminescence systems in nature and have long been of interest to researchers. Bacteria that produce bioluminescence are largely found in three genera: *Vibrio*, *Photobacterium* and *Photorhabdus*. The genes involved in bacterial luminescence are encoded by the *luxCDABE* operon. The luciferase reaction is catalyzed through the actions of both LuxA and LuxB, which form the 78 kD luciferase enzyme and together oxidize a long-chain fatty aldehyde in the presence of both oxygen and riboflavin phosphate (FMNH₂) to generate light. The remaining members of the *lux* operon – LuxC, LuxD and LuxE – are involved in the regeneration of the long-chain fatty aldehyde required for the production of light. If supplied with oxygen, FMNH₂ and a long-chain fatty aldehyde the luciferase enzymes will autonomously generate light mainly at 490 nm, along with a secondary
peak at 590 nm\(^{140}\). Bioluminescence can be detected using a specialized apparatus, such as a luminometer. In the mid-1980’s, the full luciferase gene cassette – the \textit{luxCDABE} operon – was successfully cloned and expressed in \textit{E. coli}\(^9\). Due to the current ease of use (i.e. not requiring the addition of exogenous substrates) bacterial luciferase is an increasingly popular system for many scientific applications\(^{29,63}\).

1.5.2 Applications of the bacterial luciferase reporter genes

Over the past few decades bacterial luciferase has been utilized in many different fields as a tool to visualize diverse biological processes\(^{63}\). LuxAB makes a notable reporter for biological systems due to the quick response time of its expression to variable conditions, allowing it to function as a real-time measurement of activity. Simple, unobtrusive detection methods also allow biological systems to be examined without disrupting their function, allowing for the continued observation of the same sample over a time-course\(^{29}\). These benefits have lead to its use in many different applications. A study in 1985 first demonstrated the use of the \textit{lux} operon as a way to measure gene expression\(^{47}\). The \textit{lux} operon was inserted into a promoterless mini-Mu transposon and \textit{E. coli} cells were mutagenized using this mini-Mu\textit{lux} transposon. Target gene interruption resulted in the expression of the \textit{lux} genes as a measure of target gene activity, with simple and sensitive detection of light as an indicator. A few years later, in 1989, \textit{E. coli} cells expressing a LuxAB fusion were shown to function as a visible reporter in the eukaryote \textit{Nicotiana tabacum}, underlining the use of bacterial luciferase as a cellular imaging technology in eukaryotic cells\(^{113}\).

The applications of bacterial luciferase are not limited to studying bacteria themselves; many studies have utilized bacterial luciferase in eukaryotic systems as well. A 1992 study used tetracycline-controlled transactivator-responsive luciferase constructs to monitor the highly sensitive luciferase expression as a measure of gene expression in HeLa cells\(^{58}\). Expression of luciferase in this system was tightly controlled by the addition of tetracycline in a highly responsive manner that allowed the generation of an “on/off” control for mammalian genes. Due to the short half-life of luciferase the authors were able to observe a rapid shutdown of transcription upon the addition of tetracycline. The \textit{lux} operon was first expressed natively in \textit{Saccharomyces cerevisiae} in 2003, with slight modifications required to the system itself, paving the way for numerous other studies using bioluminescent eukaryotic cells\(^{67}\). These studies
eventually lead to the generation of a human cell line autonomously expressing the bacterial *lux* operon. Using these cells, bioluminescent imaging can be performed using mammalian cell lines expressing the *lux* genes as probes. These cells have numerous advantages over traditional bioluminescent probes in that they do not require the addition of a substrate, have a low background level when used in a bioluminescent imager and have a long-lasting signal that can be easily detected over long time-courses. Bacterial luciferase systems do, however, require the use of greater numbers of reporter cells than firefly luciferase reporters in order to be detectable. In the future, eukaryotic-optimized *lux* systems will likely be further refined and optimized, reducing the potential downsides to their use and emphasizing the unique properties of the system.

Another common use of bacterial luciferase as a reporter involves the imaging of live animals using non-invasive bioluminescent imaging and luciferase reporter strains. The non-invasive nature of these types of imaging experiments come with the advantage of being able to track the same population of animals throughout an entire experiment; imaging the animals in a bioluminescent imager does not require their euthanization. The prolonged nature of bioluminescence also allows the study of reporters over a long period of time. Because whole animals are imaged at the same time, these studies allow the entire animal to be observed; you are not limited to looking at only the organs you already know are involved in a biological process. A 2011 study examining relapsing typhoid fever after antibiotic withdrawal used bioluminescent *Salmonella* to track their spread in mice. Using bioluminescent imaging they were able to determine that the mesenteric lymph nodes were a major reservoir for bacteria upon relapse. They observed that low levels of *Salmonella* remained in the MLNs even after antibiotic treatment and that the levels observed using imaging correlated well with bacterial counts when organs were homogenized and plated. Another study in 2009 examining murine intestinal colonization with *E. coli* using plasmid-based bacterial luciferase reporters found that bacterial counts correlated very closely with bioluminescent signals observed using a bioluminescent imager (BLI). The authors concluded that intestinal colonization could be inferred directly from the bioluminescence observed in an animal. These and other studies all demonstrate the powerful *in vivo* applications of bacterial luciferase in its many forms.
2.0 HYPOTHESIS AND OBJECTIVES

2.1 Hypothesis

Due to the cyclical lifestyle of many *Salmonella* serovars they are believed to spend significant periods of time in the environment \(^{155}\). The ability to replicate and persist outside of a host, such as in an environmental reservoir, has been hypothesized to greatly enhance the transmission potential of a pathogen \(^3\). It follows that factors involved in environmental persistence may also play a significant role in the transmission of *Salmonella* between hosts. Cellular aggregation and the formation of biofilms through the production of an extracellular matrix has been demonstrated to aid in the survival of *Salmonella* species in non-host environments and under varied stress-related conditions *in vitro*, such as prolonged desiccation \(^{152}\), treatment with disinfectants \(^{152,157}\), and exposure to antibiotics \(^{112}\). These processes do not have a clear role in virulence \(^{132,133}\), suggesting they may be involved in another, distinct part of the *Salmonella* lifestyle \(^{151}\).

I hypothesize that the genetic factors involved in cellular aggregation and biofilm formation are vital to host-to-host transmission as well. A link between biofilm formation, environmental persistence and transmissibility has not yet been demonstrated, which is likely due to the lack of an appropriate model to study transmission. It is possible to generate mutant strains of *Salmonella* Typhimurium, a nontyphoidal serovar of *Salmonella*, which lack the transcriptional regulation necessary for producing biofilm by knocking out the gene *csgD*. CsgD is a transcriptional regulator that controls the expression of many biofilm-related extracellular compounds \(^{54}\); \(\Delta csgD\) mutants do not aggregate and form biofilm under conditions that induce biofilm formation in wild-type *Salmonella* \(^{162}\). I hypothesize that *Salmonella* Typhimurium \(\Delta csgD\) mutants will have reduced transmission rates when compared to the wild-type in a murine model due to their inability to aggregate and form biofilms.

To test the transmission potential of many *Salmonella* Typhimurium strains I developed a murine model of *Salmonella* transmission during the course of my project. The purpose of this model is to monitor the spread of *Salmonella* Typhimurium from infected seeder mice to naive, uninfected mice in an experimental mouse population. By comparing the rates of transmission of both wild-type and mutant strains, I will gain insight into how these specific mutations alter the transmission potential of bacteria in our model and elucidate the role of these genes in the transmission process. Luciferase-producing *Salmonella* Typhimurium reporter strains I have
generated and bioluminescent imaging will be used to monitor the spread of *Salmonella* Typhimurium throughout a mouse population. Based on the findings of previous studies I hypothesize that the main route of infection in our model will be through the fecal-oral route, where uninfected mice become infected through exposure to fecal pellets contaminated with *Salmonella*<sup>91</sup>. The goal is to be able to observe the onset and spread of infection before the presence of clinical signs in the mice, giving me a more precise and accurate quantification of the transmission dynamics in the model.

### 2.2 Objectives

The specific objectives of my Master’s project were as follows:

1) Develop a working model of murine *Salmonella* Typhimurium transmission that allows for the study of genetic factors involved in the transmission process.

2) Using this model, test specific genetic factors predicted to be involved in the transmission of *Salmonella* Typhimurium.

3) Observe the temporal and spatial dynamics of murine *Salmonella* Typhimurium infection using a bioluminescent imager and *Salmonella* Typhimurium luciferase reporter strains.
3.0 MATERIALS AND METHODS

3.1 Bacterial strains and culture conditions

The *Salmonella enterica* subspecies *enterica* serovar Typhimurium (*Salmonella Typhimurium*) strains (both the wild-type and ΔcsgD mutant) used in all experiments were derived from the ATCC 14028 type strain. Various *Salmonella Typhimurium* WT/ΔcsgD Kan^R^/Cam^R^ luciferase reporter strains containing a chromosomal insertion of the *Photorhabdus luminescens luxCDABE* operon were used in all animal trials, imaging experiments and luciferase assays, and their generation is described below (referred to as *Salmonella Typhimurium* reporters). *E. coli* DH10B was used for general cloning and *E. coli* CC118 was used for replication of the pUC18R6KT-miniTn7T vector and its derivatives.

To generate the bacterial challenges used for murine infections, strains were streaked onto LB agar (supplemented with 50 µg/mL kanamycin [Kan] or 10 µg/mL chloramphenicol [Cam]) from frozen stocks. Isolated colonies were selected and grown for 16 hours in LB broth at 37°C, shaking at 200 rpm. These cultures were then used to generate the bacterial challenges, described below. For luciferase assays, the strains assayed were grown for 16 hours in 5 mL LB broth cultures supplemented with the appropriate concentration of antibiotic depending on the assay (50 or 100 µg/mL Kan, 5, 7, or 10 µg/mL Cam, 5, 7, 9, 100 µg/mL ampicillin [Amp], or 10 µg/mL tetracycline [Tet]). These cultures were diluted 1:600 into 150 µL assay media (i.e. LB broth, 1% Tryptone or LB broth without NaCl, supplemented with antibiotics), in a 96-well clear-bottomed black plate (Product #3631, Corning Life Sciences, Tewksbury, MA, U.S.A.) and overlaid with 50 µL of mineral oil. Luminescence and optical density (600 nm) measurements were performed every 30 minutes using a Victor X³ multi-label plate reader (Perkin-Elmer Life Sciences, Waltham, MA, U.S.A.).

3.2 Modifications to the pUC18R6KT-miniTn7T delivery vector

To facilitate cloning into the pUC18R6KT-miniTn7T plasmid²⁶ a poly-linker containing a *PacI* restriction site was inserted between the *SacI* and *KpnI* restriction sites contained in the multiple cloning site (MCS). The oligonucleotides Sac_Pac_Kpn1 and Sac_Pac_Kpn2 (see Appendix A) were annealed to generate the *PacI* poly-linker. pUC18R6KT-miniTn7T was digested with *SacI* and *KpnI* and the poly-linker was ligated into the MCS. The pHSG415-tnsABCD and pUC18R6KT-miniTn7T-*PacI* vectors were used for all subsequent experiments.
3.3 Generation of the pCS26 sig70-16 Cam\textsuperscript{R} and Tet\textsuperscript{R} vectors

To generate a chloramphenicol-resistant pCS26 sig70-16 vector\textsuperscript{13} a promoterless version of this plasmid containing a Cam\textsuperscript{R} gene was used (pCS26cam), prepared by B.D. Jones, M.G. Surette and R. DeVinney. The sig70-16 promoter region of pCS26 was polymerase chain reaction (PCR) amplified using the pZE05 and pZE06 primers (see Appendix A), purified, and digested with BamHI and XhoI. This was then ligated into BamHI and XhoI-digested pCS26cam and electroporated into \textit{E. coli} DH10B cells, which were incubated at 37° C for 1 hour in SOC media and plated on LB agar supplemented with 10 \(\mu\)g/mL Cam. Successful clones were screened by PCR using the same pZE05 and pZE06 primers as above.

To generate a tetracycline-resistant pCS26 sig70-16 vector, the pACYC184 plasmid was PCR amplified using the primers TetExtractFor2 (containing an EcoRI site) and TetExtractRev2 (containing a PstI site) (see Appendix A) to amplify the Tet\textsuperscript{R} gene and then digested with PstI and EcoRI. pCS26 sig70-16 Cam\textsuperscript{R} was then digested with PstI and EcoRI, ligated with the Tet\textsuperscript{R} gene and electroporated into \textit{E. coli} DH10B cells. Potential transformants were incubated at 37° C for 1 hour in SOC and plated on LB agar supplemented with 7 \(\mu\)g/mL Tet. Potential clones were grown overnight in LB agar supplemented with 7 \(\mu\)g/mL Tet, and the resulting plasmids were purified and screened by PstI and EcoRI digestion.

3.4 Cloning the luciferase construct into the \textit{Salmonella Typhimurium} chromosome

The pCS26\textsuperscript{13} vectors (pCS26 sig70-16 Kan\textsuperscript{R}/Cam\textsuperscript{R}) were PCR amplified using the primers pCS26\_Pac\_FOR and pCS26\_Pac\_REV (see Appendix A) to amplify the region containing the bacterial luciferase operon (\textit{luxCDABE}), the promoter (sig70-16), and the antibiotic resistance marker (Kan\textsuperscript{R} or Cam\textsuperscript{R}). The amplified PCR products (\textit{luxCDABE} sig70-16 Kan\textsuperscript{R}/Cam\textsuperscript{R}) were digested with \textit{PacI} and ligated into the \textit{PacI}-digested pUC18R6KT-miniTn7T-Pacl delivery vector to generate a Tn7 delivery vector containing the \textit{lux} construct (\textit{luxCDABE} sig70-16 Kan\textsuperscript{R}/Cam\textsuperscript{R}). The ligation mixture was electroporated into \textit{E. coli} CC118 cells followed by incubation in 1 mL SOC at 37° C for 1 hour to allow the pUC18R6KT-miniTn7T-Pacl- \textit{luxCDABE} sig70-16 Kan\textsuperscript{R}/Cam\textsuperscript{R} construct to replicate. Successful clones were grown overnight in LB broth supplemented with 50 \(\mu\)g/mL Kan or 10 \(\mu\)g/mL Cam, as well as 100 \(\mu\)g/mL Amp, and plasmid purification was performed. To screen the insert orientation of the \textit{luxCDABE} insert into the Tn7 delivery vector, plasmids were digested with \textit{NotI}, \textit{XbaI} and
EcoRV and ran on a 1% agarose gel for 1 hour at 100 V. Differences in band sizes indicated whether the
lux construct was ligated into the delivery vector in the “forward” or “reverse” orientation.

The final delivery vectors were then electroporated into electrocompetent Salmonella Typhimurium ΔcsgD cells containing the helper plasmid pHSG415-tnsABCD. Cells were incubated at 28° C in 1 mL SOC for 1 hour, and potential transformants were selected on LB agar supplemented with 50 µg/mL Kan or 10 µg/mL Cam and grown at 37° C overnight. Successful chromosomal insertion of the lux construct in any resulting transformants was confirmed using the primers glmSdetectFOR and glmSdetectREV (see Appendix A). These clones were used as donor strains to generate P22 phage lysates, as described below.

3.5 P22 phage transduction

Donor strains were streaked from frozen stocks onto LB agar supplemented with antibiotic (50 µg/mL Kan or 10 µg/mL Cam) and grown overnight at 37° C. Single colonies were selected from plates and grown in 5 mL LB broth for 8 – 16 hours, shaking at 200 rpm. P22 phage lysates were prepared by mixing 1 mL overnight culture with 4 mL P22 phage broth, at an MOI of approximately 0.01 – 0.1 PFU/cell, and incubated at 37° C for 10 – 16 hours with agitation at 200 rpm. Cell debris was removed by centrifugation (13,500×g, 2 minutes), the supernatant was transferred to a glass screw-top vial and 500 µL of chloroform (100 µL per mL of supernatant) was added as a preservative. P22 phage lysates were stored at 4° C.

Recipient strains were grown in 5 mL LB broth for 18 h at 37° C, shaking at 200 rpm. 1:1,000 and 1:10,000 dilutions of the donor P22 phage lysate were prepared in 0.85% NaCl and were mixed with any recipient strain cultures at ratios of 0.2:1, 1:1, and 5:1. The P22 phage and strain mixture was incubated at 37° C for one hour, without shaking, to allow for phage adsorption and transduction. Cells were pelleted by centrifugation (13,500×g, 1 minute), resuspended in 1 mL LB broth, plated on LB agar supplemented with 10 mM EGTA and 50 µg/mL Kan or 10 µg/mL Cam and incubated overnight at 37° C. To ensure that potential transductants did not contain lysogenic phage, colonies were selected and streaked out on EBU agar. White colonies were selected and the chromosomal region of insertion was PCR amplified using the primers pZE05/06 and glmSdetectFOR/REV (see Appendix A) and sequenced. Reporters whose sequences were confirmed were used for all subsequent experiments.
3.6 Luciferase assays

Strains grown overnight at 37° C in 5 mL LB supplemented with antibiotic (50 µg/mL Kan, 10 µg/mL Cam, 100 µg/mL Amp or 10 µg/mL Tet) were diluted to a final dilution of 1:600 in 96-well clear-bottomed black plates (Product #3631, Corning Life Sciences, Tewksbury, MA, U.S.A.) in the media being tested (LB broth, 1% Tryptone or LB broth without NaCl) supplemented with antibiotics (50 or 100 µg/mL Kan, 5, 7, or 10 µg/mL Cam, 5, 7, 9, 100 µg/mL Amp, or 10 µg/mL Tet). Before starting the assay 50 µL of mineral oil was overlaid on the wells to prevent drying out of the cultures. All luciferase assays were performed using a Victor multi-label plate reader (either a Victor³V 1420 or Victor X³; Perkin-Elmer Life Sciences, Waltham, MA, U.S.A.). Absorbance (OD at 590 nm) and luciferase (counts per second [CPS]) measurements were taken every 30 minutes, with periodic agitation (1 minute, approximately 10 minutes apart) for a total of 24 or 48 hours, as indicated.

3.7 Drop dilutions for cell enumeration

Dilution plates were prepared by adding 90 µL of PBS to each well in a 96-well clear plastic plate. A volume of 10 µL of the sample being enumerated was added to row A for a dilution of 1:10; 10 µL of sample from each row was then serially diluted through rows B – H, resulting in a series of dilutions from 10⁻¹ to 10⁻⁸. A specified volume from each well (4 µL for bacterial cultures, 10 µL for organ/fecal samples) was plated on LB agar supplemented with antibiotics, with each dilution well growing as a small cluster of colonies forming units (CFU). Colony numbers at each dilution spot were enumerated by sight, with the ideal count ranging from 3 – 30 colonies. Final sample concentration was determined by the following formula:

\[ \text{CFU/mL} = \text{colony count [CFU]} \times \left( \frac{1}{\text{drop volume [mL]}} \right) \times \left( \frac{1}{\text{dilution factor}} \right) \]

3.8 Plating efficiency testing

Strains being tested (WT Salmonella Typhimurium KanR and CamR sig70-16 reporters) were grown overnight in 5 mL LB broth cultures supplemented with either 50 µg/mL Kan or 10 µg/mL Cam. Flasks of LB broth (50 mL) were inoculated with 500 µL of either strain and grown at 37° C. From these flasks, 0.7 OD stocks were created (0.7 OD equals approximately 7x10⁸ CFU) of each strain individually, as well as a combined stock containing equal numbers of both strains (7x10⁸ CFU total). Each stock was serially diluted to 10⁻⁷ and plated in duplicate on LB
agar containing 50 or 100 µg/mL Kan, or 7, 10, or 15 µg/mL Cam to determine the plating efficiency of each strain on each media.

3.9 Streptomycin MIC testing

The WT *Salmonella* Typhimurium Kan\(^R\) reporter was grown at 37\(^\circ\) C overnight in LB broth, with agitation at 200 rpm. LB broth supplemented with streptomycin was serially diluted, with 8 replicates, in a 96-well clear-bottomed black plate (Product #3631, Corning Life Sciences), resulting in the following concentrations of streptomycin: 512, 256, 128, 64, 32, 16, 8, 4, 2, 1 and 0.5 mg/mL; a control column containing no streptomycin was included as well. A 5 mL, 1x10\(^7\) CFU/mL stock of *Salmonella* Typhimurium was prepared in LB broth, and 100 µL of this stock was pipetted into each well of the 96-well plate (each well had a final cell count of approximately 1x10\(^6\) CFU). The 96-well plate was then left overnight at 37\(^\circ\) C. OD\(_{590}\) readings were taken from each well using the Victor X\(^3\) multi-label plate reader (Perkin-Elmer Life Sciences) to determine the MIC of streptomycin with this strain.

3.10 Design and cloning of sig70c10 and sig70c35 promoters

The oligonucleotides sig70-16-10c2F and sig70-16-10c2R, and sig70-16-35c2F and sig70-16-10c2R (see Appendix A) were annealed to generate the phosphorylated Psig70c10 and Psig70c35 promoters. These promoters were ligated into BamHI and XhoI-digested and antarctic phosphatase-treated pCS26 sig70-16 Kan\(^R\)/Cam\(^R\) vectors, electroporated into *E. coli* DH10B cells, incubated in 1 mL SOC at 37\(^\circ\) C and plated on LB agar supplemented with 50 µg/mL Kan or 10 µg/mL Cam. These vectors were then cloned into the *Salmonella* Typhimurium chromosome and donor P22 phage lysates were generated, as detailed above.

3.11 Bioluminescent imaging

All bioluminescent imaging was performed using an IVIS Lumina II bioluminescent imager (Caliper Life Sciences, Hopkinton, MA, U.S.A.). Mice were anesthetized using 2.5% isofluorane, with a chamber flow rate of 1.5 L/min O\(_2\) and IVIS flow rate of 0.3 L/min O\(_2\). Photographs and luminescence measurements were taken with an f/stop of 1.2, with variable binning and time values to obtain representative images. All final measurements were converted to radiance values (photons/s/cm\(^2\)/sr) from counts per second (CPS).
3.12 Animal challenges and infections

Female C57BL/6 mice (6 – 8 weeks old) were purchased from Jackson Laboratory (Bar Harbor, ME, U.S.A.) and were assigned to cage groups either manually or randomized through the use of a randomization table generated using Microsoft Excel. Mice had their ears notched to allow individual mice to be differentiated during the course of an experiment. Bacterial challenges were prepared in 100 mM HEPES (pH 8) at $1 \times 10^4 – 1 \times 10^7$ CFU per 100 µL, depending on the trial. Cultures grown in LB broth at 37° C for 16 hours, with agitation, were diluted to the appropriate concentration in 100 mM HEPES. Challenges were delivered to mice by oral gavage in 100 µL volumes performed by VIDO Animal Care technicians. Competitive infections were performed using the same procedure, ensuring that both strains were present in the challenge at an approximately 1:1 ratio (i.e. in a $1 \times 10^7$ CFU challenge, both strains would be present at $\sim 5 \times 10^6$ CFU). The two strains were differentially marked using either a KanR or CamR resistance marker, allowing for selection of both strains separately after the experiment. Each challenge was serially diluted and drop dilutions were plated to quantify the number of bacteria of each strain present in each challenge.

3.13 Organ collection and processing from infected mice

Mice were monitored over the course of each experiment for weight loss and the appearance of clinical signs of infection. Mice that had lost >20% of their starting weights (weight score = 3), typically 4 – 7 days post-infection, were humanely euthanized, either with isofluorane or by cervical dislocation. The spleen, liver, cecum and mesenteric lymph nodes (MLNs) of each mouse were harvested by a VIDO Animal Care technician and collected in a 2 mL Eppendorf Safe-Lock Tube (Product #022363352, Eppendorf, Hamburg, Germany) containing 1 mL of PBS and a 5 mm stainless steel bead (Product #69989, Qiagen, Venlo, Netherlands). Organs were homogenized using a high-speed mixer mill (MM400; Retsch, Haan, Germany) for 4 minutes at 30 Hz. Homogenized organs were serially diluted and drop dilutions were plated to quantify the number of bacteria of each strain present in each organ.

3.14 Streptomycin pre-treatment and challenge of C57BL/6 mice

Mice were grouped into cages and had their ears notched according to the experimental challenge protocol (see above). Streptomycin pre-treatment was adapted from a previously
established protocol. Four hours prior to treatment with streptomycin, food and water were removed from the cages. 20 mg of streptomycin in 100 µL (from a stock solution of 200 mg/mL streptomycin sulfate) was delivered to each mouse by oral gavage. Food and water was immediately returned to each cage after treatment. Streptomycin treatment was always performed 24 hours before bacterial challenge. Food and water were removed from the cages 4 hours before challenge. Mice were then challenged with $1 \times 10^4$, $1 \times 10^5$, $1 \times 10^6$ or $1 \times 10^7$ CFU of *Salmonella Typhimurium*, the exact dosage and strain depending on the particular trial. Water was returned to the cages immediately, and food was returned 2 hours after challenge.

### 3.15 Fecal sample collection and processing

To collect individualized fecal pellets, mice were isolated and housed in plastic isolation containers each day until 3 – 4 fecal pellets were shed and collected (typically <30 minutes). Fecal pellets were then transferred to 1.5 mL Eppendorf tubes. Individual fecal pellets were collected using sterile forceps and either transferred to 2 mL Eppendorf Safe-Lock Tubes (Product #022363352, Eppendorf, Hamburg, Germany) containing 1 mL of PBSA and a 5 mm stainless steel bead (Product #69989, Qiagen, Venlo, Netherlands) for immediate processing or transferred into 48- or 96-well plates for long-term storage. Fecal pellets were homogenized using a high-speed mixer mill (MM400; Retsch, Haan, Germany) for 4 minutes at 30 Hz. The number of *Salmonella Typhimurium* present in each fecal sample was determined by serially diluting and plating each sample by drop dilution.

### 3.16 Collection and long-term storage of fecal pellets

Fecal pellets selected for long-term storage were collected using two different methods. Fresh fecal pellets were collected as above and stored in 48- or 96-well plates. Other fecal pellets were collected in a manner to maximize the number of pellets collected by collecting all fecal pellets in a cage. For this, mice were transferred to clean cages and the entire contents of the old cage (i.e. bedding, fecal pellets, etc.) were collected. 3 pellets were sampled immediately, following the above procedure, to determine the number of bacteria being shed. Remaining pellets were taken and stored in either 48- or 96-well plates (Product #3548 and #3596, Corning Life Sciences, Tewksbury, MA, U.S.A.). Plates were covered with lids and stored on a bench top at room temperature for a period of up to 6 weeks. Each week 6 – 10 pellets were randomly
selected and sampled as above to determine the number of *Salmonella* Typhimurium present in each fecal sample after long-term desiccation and storage at room temperature.

### 3.17 Experimental design of initial transmission trial

Mice were randomly sorted into cages and their ears were notched based on randomized assortment determined in Microsoft Excel. Eight seeder mice (two per cage) that had been pre-treated with streptomycin (as above) were challenged with $1 \times 10^5$ CFU of the WT *Salmonella* Typhimurium sig70-16 Cam$^R$ reporter. The three groups of naive mice (five per cage) had different initial treatments: group 1 – untreated; group 2 – pre-treated with streptomycin as described above (the day before co-housing); and group 3 – fasted for 20 hours (16 hours before co-housing and 4 hours after co-housing with seeder mice). Two seeder mice were randomly selected and placed into cages containing the naive mice; the two remaining seeder mice were left to observe.

For the duration of the experiment, shedding of the seeder mice was monitored by collecting and processing 3 fecal pellets from each mouse daily, and all mice were monitored for clinical signs of infection and weight loss, as described above. Mice were euthanized when they scored a weight score of 3 and their organs were collected and processed as described above to quantify the bacteria present in their organs.

### 3.18 Experimental design of short- and long-term transmission trial

A total of 30 naive mice (five per cage) and 12 seeder mice (three per cage) were randomly sorted into cages and their ears were notched based on randomized assortment determined in Microsoft Excel. The seeder mice were challenged with $1 \times 10^5$ CFU of one of the following sig70-16 reporter strains of *Salmonella* Typhimurium (one strain randomly assigned per cage): WT *Salmonella* Typhimurium Kan$^R$, *Salmonella* Typhimurium ΔcsgD Kan$^R$, WT *Salmonella* Typhimurium Cam$^R$ or *Salmonella* Typhimurium ΔcsgD Cam$^R$. The challenges were delivered double-blinded, with both researchers and Animal Care staff blinded to which group received which challenge. These seeder mice were randomly co-housed with a group of streptomycin pre-treated naive mice for the duration of the experiment (groups 1 – 4). The two cages of naive mice that were not co-housed with seeder mice (groups 5 and 6) had their cages seeded with 200 desiccated fecal pellets; 100 fecal pellets shed from mice challenged with the
WT *Salmonella* Typhimurium reporter and 100 fecal pellets shed from mice challenged with the *Salmonella* Typhimurium ΔcsgD reporter. Reciprocal groups of fecal pellets containing Kan$^R$ and Cam$^R$ *Salmonella* were used.

For the duration of the experiment, shedding of the seeder mice was monitored by collecting and processing 3 fecal pellets from each mouse daily, and all mice were monitored for clinical signs of infection and weight loss, as described above. Mice were euthanized when they scored a weight score of 3 and their organs were collected and processed as described above to quantify the bacteria present in their organs.

### 3.19 Ethics statement

All animal experiments in these studies were conducted in accordance with the Guidelines of the Canadian Council on Animal Care and the Regulations of the University of Saskatchewan Committee on Animal Care and Supply. All animal experiments were performed under Animal Use Protocol #20110057, which was approved by the University of Saskatchewan’s Animal Research Ethics Board.
4.0 RESULTS

4.1 Construction and characterization of *Salmonella* Typhimurium reporter strains

4.1.1 Generation of *Salmonella* Typhimurium reporters

To facilitate both *in vitro* and *in vivo* studies using *Salmonella* Typhimurium reporter strains and bioluminescent imaging (BLI), we generated luciferase reporters using a modified Tn7 transposition system. The overall objective of such a system was to chromosomally insert a luciferase reporter construct (*lux* construct) into the *Salmonella* chromosome, resulting in various reporter strains for use in subsequent imaging studies. The *lux* constructs themselves were sourced from the plasmid pCS26 (Figure 1A). The complete luciferase construct contained the *Photorhabdus luminescens luxCDABE* luciferase operon as a reporter, with expression controlled by a synthetic σ\(^{70}\)-dependent promoter – sig70-16 – as well as an antibiotic resistance marker (Figure 1B). The sig70-16 promoter sequence was based on the consensus from many different σ\(^{70}\)-controlled promoters in *E. coli* (described in detail in Section 4.1.7). Expression from this promoter was constitutive, due to the ubiquity of the σ\(^{70}\) sigma factor, resulting in reporters that constitutively expressed luciferase. Isolating and extracting the *lux* construct was achieved through digestion of pCS26 with PacI, removing the replication origin region of the plasmid.

pCS26 was designed in a way that allowed this system to be modular: the reporter genes and the desired promoter could be easily excised and exchanged with other modules in order to design an appropriate reporter construct for any experiment. The reporter construct could be changed by digesting pCS26 with NotI, removing the *luxCDABE* operon and ligating in a new reporter. The promoter region could be changed by digesting pCS26 with XhoI and BamHI and ligating in a new promoter. Finally, the antibiotic marker was changed from kanamycin-resistant (Kan\(^{R}\)) to chloramphenicol-resistant (Cam\(^{R}\)) through a series of cloning steps (see Section 3.3); the Cam\(^{R}\) marker could be removed from the pCS26cam plasmid using EcoRI and PstI restriction sites. Each of these features allowed for the customization of the reporter construct prior to Tn7 transposition, resulting in a variety of potential reporter strains. In the following studies we utilized luciferase reporters, carrying both Kan\(^{R}\) and Cam\(^{R}\) resistance markers.

Transposition through a Tn7-based system required two main elements: a delivery vector as a vehicle for the reporter construct and a helper plasmid encoding the transposition genes.
Figure 1: pCS26 and the luciferase reporter construct. A) The plasmid pCS26 was used to generate luciferase constructs. The promoter site (green) is flanked by Xhol and BamHI sites. Expression of the reporter genes (yellow; luxCDABE operon derived from Photorhabdus luminescens) is driven by the promoter at this site. Reporter genes are flanked by NotI sites. An antibiotic resistance marker (red) allows for antibiotic selection; both kanamycin-resistant and chloramphenicol-resistant versions of the plasmid were used. PacI sites flank the full luciferase construct. B) The luciferase construct consists of the reporter genes (lux operon), a promoter (sig70-16) and an antibiotic resistance marker. This construct is isolated through digestion with PacI or PCR amplification and inserted into the bacterial chromosome using Tn7 transposition.
necessary for transposition. The delivery vector selected was a modified version of pUC18R6KT-miniTn7T-\textsuperscript{26} – pUC18R6KT-miniTn7T-\textit{PacI} (Figure 2A). We modified the pUC18R6KT-miniTn7T vector by introducing a \textit{PacI} site into its multiple cloning site (MCS). This was achieved by introducing a poly-linker containing a \textit{PacI} site between the \textit{SacI} and \textit{KpnI} sites contained in the original MCS. After digesting pUC18R6KT-miniTn7T with both \textit{SacI} and \textit{KpnI}, we ligated the poly-linker into the delivery vector for efficient cloning of our various reporter constructs; \textit{PacI} was used to extract the reporter construct from pCS26. The MCS in pUC18R6KT-miniTn7T-\textit{PacI} was located between its Tn7R and Tn7L sites, and ligation of the reporter construct between these sites resulted in its integration into the bacterial chromosome through Tn7 transposition. The helper plasmid – pHSG415-\textit{tnsABCD} – was designed by PCR amplifying the \textit{tnsACBCD} operon from the plasmid pTNS2\textsuperscript{26} and ligating it into pHSG415 under the control of a chloramphenicol promoter (Figure 2B); this was done to increase the longevity of the \textit{tnsABCD} genes in the cell. Our initial transposition attempts using pTNS2 were unsuccessful (MacKenzie, Waldner and White; unpublished data); since pTNS2 was a suicide vector (i.e. it does not replicate in \textit{Salmonella}), we reasoned that it might have been cured from the cells before adequate amounts of the TnsABCD proteins could be produced. pHSG415-\textit{tnsABCD} contains a temperature-sensitive origin of replication that allowed for curing of cells in a more controllable manner, and as a consequence, better \textit{tnsABCD} expression.

Ligation of the \textit{PacI}-digested reporter construct into \textit{PacI}-digested pUC18R6KT-miniTn7T-\textit{PacI} resulted in two possible insert orientations due to the use of single restriction enzyme in the cloning. Delivery vectors containing the reporter construct in the orientation shown in Figure 1B were referred to as the forward orientation, while delivery vectors containing the reporter construct in the opposite orientation were referred to as the reverse orientation. As Tn7 transposition is orientation-specific, subsequent chromosomal insertion would occur in the same orientation as in the delivery vector. To screen the orientation of the miniTn7T-reporter constructs, we digested these vectors with \textit{XbaI}, \textit{EcoRV} and \textit{NotI} and ran them on a 1% agarose gel (Figure 3). Delivery vectors containing the reporter construct in the forward orientation resulted in visible bands at approximately 3850 bp, 3300 bp, 1900bp and 1300 bp after digestion while vectors containing the reporter construct in the reverse orientation resulted in visible bands at approximately 5100 bp, 3850 bp and 1650 bp. This difference was easily distinguishable, with
Figure 2: The delivery vector, pUC18R6KT-mini-Tn7-Pacl, and helper plasmid, pHSG415-tnsABCD. A) pUC18R6KT-mini-Tn7T-Pacl is the delivery vector. DNA contained between the Tn7R and Tn7L sites (grey) is excised from the plasmid and inserted into the chromosome. A $\text{PacI}$ site was engineered into the MCS to facilitate the insertion of reporter constructs. An ampicillin resistance gene (red) is present to allow for antibiotic selection. B) pHSG415-tnsABCD is the helper plasmid containing the $\text{tnsABCD}$ operon (blue) encoding the transposition genes. An ampicillin resistance gene (red) is present to allow for antibiotic selection. It is temperature sensitive to allow for easy curing of the helper plasmid post-transposition.
Figure 3: Digests of pUC18R6KT-mini-Tn7T plasmids to screen for insert orientation. Plasmids were digested with EcoRV, NotI and XbaI and ran on a 1% agarose gel to screen the insert orientation of the lux construct. Gel was stained in 0.5 µg/mL ethidium bromide for 10 minutes. Plasmids containing the insert in the forward orientation contain visible bands at approximately 3850 bp, 3300 bp, 1900 bp and 1300 bp (seen in lanes 1, 2, 6, 8 and 10). Plasmids containing the insert in the reverse orientation contain visible bands at approximately 5100 bp, 3850 bp and 1650 bp (seen in lanes 3, 4, 5, 7 and 9). All band size estimates made using Geneious 6.1.7.

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the vectors in lanes 1, 2, 6, 8 and 10 having forward orientation reporter constructs and the vectors in lanes 3, 4, 5, 6 and 9 having reverse orientation reporter constructs.

*Salmonella* Typhimurium ΔcsgD electrocompetent cells harboring the pHSG415-tnsABCD plasmid were electroporated with the desired miniTn7T-*lux* reporter construct delivery vectors resulting in transposition of the reporter construct into the bacterial chromosome downstream of the *glmS* gene. Transposition in *Salmonella* Typhimurium using this system typically resulted in successful transposition with a success rate of approximately 80% (Table 1); depending on the experiment, it was successful in 55 – 100% of clones tested. Transformants were then screened by antibiotic resistance and light production, and P22 phage were used to move the reporter construct into a clean genetic background. The final reporter strains were sequenced for confirmation of successful transposition, amplifying both the promoter region (using the pZEO5/06 primers; see Appendix A) as well as the regions between the construct itself and the bacterial chromosome (using the glmSdetectFOR/REV primers; see Appendix A). *Salmonella* Typhimurium luciferase reporter strains were generated with constructs in both the forward and reverse orientations, in both wild-type and ΔcsgD genetic backgrounds and with both kanamycin and chloramphenicol resistance markers (these reporter strains are referred to as *Salmonella* Typhimurium luciferase reporters, with either KanR or CamR resistance).

### 4.1.2 Orientation of *lux* insert does not affect luciferase expression

To determine whether the construct orientation affected expression of the *luxCDABE* operon from the *Salmonella* chromosome we continuously monitored light production from each strain over a period of 24 hours. Large differences in the expression from these strains would have been undesirable as our goal was to have a library of reporters whose luciferase expression could be used to compare the reporters with each other in vivo. *Salmonella* Typhimurium ΔcsgD reporter strains containing the reporter construct in either orientation were grown in 96-well plates in LB broth at a temperature of 30° C (Figure 4A). Reporters containing reverse orientation constructs (grey) had higher levels of luciferase expression over the course of the experiment when compared to reporters with forward orientation constructs (red). The luciferase expression of forward orientation clones clustered tightly with peak luciferase expression of approximately $5 \times 10^4$ counts per second (CPS) while the luciferase expression of reverse orientation clones was more variable with a range of peak expression from $5 \times 10^4$ to $7 \times 10^4$ CPS.
### Table 1: Success rate of Tn7 transposition.

The success rate of clones tested after chromosomal insertion of the *lux* reporter construct using our Tn7 transposition system. The percentage of clones that tested positive for insertion of the reporter construct was recorded after three separate experiments. Success rate calculated as (# clones positive)/(# clones tested).

<table>
<thead>
<tr>
<th>Experiment</th>
<th># clones tested</th>
<th># clones positive</th>
<th>Success rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kan^R sig70-16 reporters</td>
<td>16</td>
<td>16</td>
<td>100</td>
</tr>
<tr>
<td>Cam^R sig70-16 reporters</td>
<td>16</td>
<td>13</td>
<td>81</td>
</tr>
<tr>
<td>sig70c10/c35 reporters</td>
<td>11</td>
<td>6</td>
<td>55</td>
</tr>
</tbody>
</table>
Figure 4: The insert orientation of the luxCDABE construct affects temporal luciferase expression.

A) Luciferase expression of Salmonella Typhimurium ΔcsgD reporter strains grown in LB broth at 30°C. Reporters containing the lux construct in both the forward (red) and reverse (grey) orientation were assayed during the same experiment. Luciferase expression (measured as counts per second [CPS]) was assayed over a 24-hour period. Each line represents the mean luciferase expression of three replicates from one individual clone. 4 forward orientation clones and 18 reverse orientation clones were assayed.

B) Growth of the reporter strains was monitored simultaneously by measuring the absorbance (OD$_{590}$) values of the cultures. Reporters containing the lux construct in both the forward (red) and reverse (grey) orientation were assayed during the same experiment. Each line represents the mean OD of three replicates of one individual clone.
The growth of the two strains was also monitored throughout the course of the experiment, measured as the absorbance (OD$_{590}$ values) of each well during growth (Figure 4B). Both strains had similar growth patterns and peak absorbance values throughout the experiment, as well as similar expression dynamics. The differences in luciferase expression between the reporters containing constructs in either orientation did not appear to be explained by differences in growth. It appeared that reporters containing the luciferase construct in the reverse orientation had higher expression during the course of the experiment. Because of this, we decided to use reporters containing reverse orientation constructs for all subsequent experiments.

4.1.3 The antibiotic marker affects luciferase expression

As described above, isogenic reporter strains were generated containing either a kanamycin resistance marker (Kan$^R$) or chloramphenicol resistance marker (Cam$^R$). The different markers were used in order to facilitate studies where two strains could be used and then differentiated later on using antibiotic selection. To determine whether the antibiotic resistance marker affected the luciferase expression of these strains we performed a luciferase assay monitoring light production over a period of 48 hours. *Salmonella Typhimurium ΔcsgD* reporter strains containing either resistance marker were grown in 96-well plates in LB broth supplemented with either 50 µg/mL kanamycin or 10 µg/mL chloramphenicol at a temperature of 30° C. The luciferase data collected indicated that the Kan$^R$ reporters (green) had significantly higher levels of expression compared to the Cam$^R$ reporters (blue), peaking at approximately $5.5 \times 10^4$ CPS versus approximately $4.5 \times 10^4$ CPS, respectively (Figure 5A). The growth of the two strains showed that the Kan$^R$ strain grew at elevated levels as well, reaching peak OD$_{590}$ values higher than the Cam$^R$ strain after 48 hours of growth (Figure 5B). These results suggest that the luciferase expression of the two strains was different, although the Kan$^R$ reporter was also able to grow more efficiently in the test media than the Cam$^R$ strain. Whether the differences in luciferase expression could be entirely attributed to differences in growth was unclear, but the expression dynamics of the two reporter strains did appear to be very similar.

4.1.4 The antibiotic marker does not affect plating efficiency

To test the plating efficiency of both the Kan$^R$ and Cam$^R$ reporter strains we examined their recovery after preparing and plating a test inoculum on various growth media. This was
Figure 5: The antibiotic marker affects luciferase expression. A) Luciferase expression of Salmonella Typhimurium ΔcsgD reporter strains grown in LB broth at 30° C supplemented with the either 50 µg/mL kanamycin or 10 µg/mL chloramphenicol. Reporters containing either a kanamycin resistance marker (green) or a chloramphenicol resistance marker (blue) were assayed during the same experiment. Luciferase expression (measured as counts per second [CPS]) was assayed over a 48-hour period. Each line represents the mean luciferase expression of three replicates from one individual clone. 4 KanR clones and 6 CamR orientation clones were assayed. Significance was determined using multiple t tests. B) Growth of the reporter strains was monitored simultaneously by measuring the absorbance (OD_{590}) values of the cultures. Reporters containing either a kanamycin (green) or chloramphenicol (blue) resistance marker were assayed during the same experiment.
done to test various concentrations of antibiotics and ensure that the concentrations used to plate samples during an experiment would not alter the recovery levels of our reporter strains. Two 50 mL cultures of LB were inoculated with 500 µL of either the KanR or CamR reporter and incubated at 37°C for approximately 3 hours. From these flasks, 1 mL 0.7 OD600 stocks (equaling approximately 7×10^8 CFU) were made of each strain, and these stocks were used to generate three different inocula: one containing the KanR reporter, one containing the CamR reporter and one containing both strains at an equal ratio (to test the recovery of both strains in a competitive infection). Each inocula was plated in triplicate on a series of LB plates supplemented with 7, 10 or 15 µg/mL chloramphenicol, 50 or 100 µg/mL kanamycin or no antibiotic and grown at 28°C for approximately 16 hours. Colony counts were performed, and the average CFU counts of each inocula on each media were calculated (Table 2).

The CamR reporter was recovered from LB + Cam plates at similar levels across the various antibiotic concentrations. Its control growth on LB plates was 4.2×10^7 CFU/mL, with similar levels on Cam7 (4.17×10^8 CFU/mL), Cam10 (4.75×10^7 CFU/mL) and Cam15 (4.79×10^7 CFU/mL) as well as no growth on any LB + Kan plates, suggesting the antibiotic concentration had no effect on the recovery of this strain. The KanR reporter had similar results with control growth of 3.75×10^8 CFU/mL, similar recovery on Kan50 (3.88×10^8 CFU/mL) and Kan100 (3.21×10^8 CFU/mL) plates as well as no growth on any LB + Cam plates. Each strain in the inoculum containing both strains was expected to be recovered at approximately half the levels of each single-strain inocula. This was the case on all plates tested, with mean levels on Cam7 (2.42×10^8 CFU/mL), Cam10 (2.42×10^8 CFU/mL), Cam15 (2.33×10^8 CFU/mL), Kan50 (1.71×10^8 CFU/mL) and Kan100 (1.71×10^8 CFU/mL). This suggested that even when grown in the same inoculum both strains could be recovered at expected levels on all media tested. For our subsequent experiments we used LB + Cam10 and LB + Kan50 plates for the recovery of Salmonella from both in vitro and in vivo experiments.

4.1.5 *Salmonella* Typhimurium reporter strains are susceptible to streptomycin

Many of our animal experiments used streptomycin as a method to disrupt the resident microbiota of a mouse prior to challenge with *Salmonella*. We wanted to test whether our reporter strains were susceptible to streptomycin in vitro or if we could continuously supplement our mice with streptomycin. We set up a 96-well plate containing LB media supplemented with
Table 2: Plating efficiency of *Salmonella Typhimurium* reporters on various media. Inocula containing *Salmonella* Typhimurium reporter strains were plated on various media and the recovery of each strain was calculated. Inocula contained either a single strain (Cam\(^R\) or Kan\(^R\)) or both strains in the same inoculum (Cam\(^R\)/Kan\(^R\)). Each value represents the mean value of an inoculum plated in triplicate.
streptomycin at concentrations from 512 – 0.5 mg/L and inoculated these with the *Salmonella Typhimurium Kan*\(^R\) reporter. The plate was incubated at 37° C for 16 hours and afterwards the wells were checked for growth through their OD\(_{590}\) values. The minimum inhibitory concentration of streptomycin (MIC) for our *Salmonella Typhimurium* reporter strain was 256 mg/L (Table 3); at 128 mg/L two wells were positive for growth of *Salmonella* and from 64 – 0.5 mg/L all of the wells tested were positive for growth. Since streptomycin at these levels would be sufficient to prevent the growth of our reporter strains, we determined that any streptomycin treatments would need to be single treatments at the start of an experiment rather than sustained treatment over its entire time course.

4.1.6 *Salmonella Typhimurium* reporter strains are detectable in feces, organs and live mice

The *Salmonella* reporter strains were designed for use in *in vivo* bioluminescent studies using a bioluminescent imager (BLI). To test the utility of these strains and ensure that they could be detected in a variety of *in vivo* applications we monitored them in different situations using a BLI. Mice were challenged with *Salmonella Typhimurium* reporters and imaged in different situations; representative images from different experiments were taken (Figure 6). Mice that had been pre-treated with streptomycin prior to challenge had detectable *Salmonella* in their digestive tracts at one day post-challenge (Figure 6A). Regions of high luciferase counts appeared as red and yellow, while regions of lower luciferase counts appeared as blue and green. This suggested that *Salmonella* was present in these mice at high levels. Fecal pellets that had been shed from mice challenged with *Salmonella Typhimurium* contained detectable *Salmonella*, suggesting that the luciferase expression of our reporters persisted throughout the mice and continued after being shed (Figure 6B); *Salmonella* was detectable in each individual pellet. Once the mice began to show signs of systemic *Salmonella* infection and were euthanized, their organs (spleen, liver, digestive tract [including cecum] and mesenteric lymph nodes [MLNs]) were arranged and imaged (shown in Figure 6C). The spleen, livers, digestive tract and MLNs were all colonized with *Salmonella* and could be detected reliably at high levels. Finally, organ samples that had been processed by homogenization and plated on Kan\(^R\) or Cam\(^R\) media to select for *Salmonella* were imaged for detection (Figure 6D). Drop dilutions were performed and individual colonies could be detected using the BLI, demonstrating the power of this technology and the utility of our reporter strains *in vivo*. 
<table>
<thead>
<tr>
<th>Streptomycin concentration (mg/L)</th>
<th>0</th>
<th>512</th>
<th>256</th>
<th>128</th>
<th>64</th>
<th>32</th>
<th>16</th>
<th>8</th>
<th>4</th>
<th>2</th>
<th>1</th>
<th>0.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wells with positive growth</td>
<td>8</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>8</td>
</tr>
</tbody>
</table>

Table 3: Streptomycin MIC for a *Salmonella Typhimurium Kan*<sup>R</sup> reporter strain. A 96 well plate containing LB media supplemented with varying concentrations of streptomycin was inoculated with a *Salmonella* Typhimurium Kan<sup>R</sup> reporter strain. The plate was incubated at 37° C for 16 hours, and the absorbance (OD<sub>590</sub>) of each well was assayed as measure of growth.
Figure 6: Luciferase reporter strains are detectable using a bioluminescent imager (BLI). Female C57BL/6 mice were challenged with *Salmonella* Typhimurium luciferase reporter strains and were detected in: A) live mice anesthetized and imaged in the BLI; B) fecal pellets shed from these mice and imaged in the BLI; C) the spleen, liver, digestive tract (including cecum) and mesenteric lymph nodes (MLNs) of these mice once euthanized and imaged in the BLI; and D) homogenized organ samples from these mice that were plated on selective media, grown overnight and imaged in the BLI. Color scale illustrates the levels of luciferase detected, as measured by luminescence.
4.1.7 Generation of new sig70-16-based promoters

Many of the images generated using the BLI were taken using reporters containing the sig70-16 promoter (Figure 6). One problem we encountered using these strains was that they were difficult to detect in live mice and were typically only detectable immediately after challenge or just prior to the time of euthanization. To determine whether the magnitude of luciferase expression from this promoter was leading to this difficulty we designed new, modified promoters that resulted in elevated levels of luciferase expression. As explained in Section 4.1.1, the reporter system was designed to be a modular system allowing for the insertion of new promoters, resulting in a large number of possible reporter constructs (Figure 7). The base sig70-16 promoter sequence was designed based on the consensus sequences of many different σ70-dependent promoters in *E. coli* (86,153). The σ70 sigma factor (encoded by the gene *rpoD*) is the major sigma factor in *Salmonella* and *E. coli* and is expressed largely during exponential growth (76,130). The sig70-16 promoter was one of several σ70-dependent promoters that was selected from a library of synthetic promoters, containing slight degeneracies from the σ70 consensus sequence; its promoter sequence was 5'-CTCGAGAATAATTCTTACATTTATGCTTCCGGCTCGTATTCTACGTGCAATTGGATCC-3' (possible degenerate positions underlined) (86). We reasoned that restoring the sig70-16 promoter sequence back to either the consensus -10 or -35 sequences might increase the promoter strength. These regions, as well as the spacer sequence between them, are recognized by the σ70 sigma factor and play a role in the initiation of transcription (130), more efficient recognition should result in greater expression from the new promoter. We did not return both regions to the consensus sequence to try and avoid expression levels that would have negative impacts on other cellular processes. The two new promoters were termed sig70c10, in which we returned the -10 region to the consensus sequence and left the -35 sequence degenerate, and sig70c35, in which we returned the -35 region to the consensus sequence and left the -10 sequence degenerate (Figure 8A). Both were achieved by altering one or two nucleotides from the original sig70-16 promoter sequence.

The luciferase expression from pCS26 harboring the sig70c10 or sig70c35 promoters was monitored in *E. coli* DH10B cells during a 48-hour growth period (Figures 8B and 8C). pCS26 containing the original sig70-16 promoter (red) had expression levels that peaked around $1 \times 10^4$ CPS, while sig70c10 (blue) and sig70c35 (green) promoters resulted in luciferase expression levels that peaked around $3 \times 10^4$ CPS and $7 \times 10^4$ CPS, respectively (Figure 8B). The new
Figure 7: Tn7 transposition system allows for a suite of reporter constructs. A) The general reporter construct has three main features: an antibiotic resistance marker, a promoter that controls the expression of the reporter construct and the reporter genes themselves. B) Constructs can be customized to the needs of an experiment. Both kanamycin-resistant (green) and chloramphenicol-resistant (blue) reporters are available. Luciferase reporters (yellow) are available currently, with the ability to add other reporters in the future. Three varying levels of reporter gene expression are available with $1 \times$ (red), $3 \times$ (blue) and $10 \times$ (green) expression levels, as well as the ability to insert any other promoter of interest.
Figure 8: Small nucleotide alterations in the -10 and -35 promoter regions result in higher luciferase expression in pCS26. A) The plasmid pCS26 contains the luxCDABE operon under the control of a synthetic, σ^70-driven promoter (sig70-16). Slight alterations in the -10 or -35 sequences in the promoter (bolded nucleotides) were introduced, bringing these regions back to the consensus sequence. The sig70c10 promoter contains the -10 consensus sequence, while the sig70c35 promoter contains the -35 consensus sequence. B) Peak luciferase expression from pCS26 plasmids containing the sig70-16 (red), sig70c10 (blue) or sig70c35 (green) promoters in E. coli DH10B cells grown in LB broth at 37°C. Peak luciferase expression (measured as counts per second [CPS]) was determined to occur approximately 10 hours into a 48 hour assay (see 8C). Each bar is the result of the mean of multiple biological replicates. C) Luciferase expression of E. coli DH10B cells harboring the pCS26 plasmid with the sig70-16 (red), sig70c10 (blue) or sig70c35 (green) promoter. Reporters were grown in LB broth at 37°C. Luciferase expression (measured as counts per second [CPS]) was assayed over a 48-hour period. D) Peak luciferase expression from 8A, separated by antibiotic resistance marker. Expression was measure from both Kan^R (solid bars) and Cam^R (hatched bars) reporters.
promoters resulted in expression levels that were approximately 4 (sig70c10) or 10 (sig70c35) times higher than the sig70-16 promoter (Figure 8B and 8C), meaning that small nucleotide alterations (either one or two nucleotides) in the -10 and -35 regions of the promoter were sufficient to significantly increase the expression of downstream genes while retaining the original expression dynamics. We did notice that *E. coli* cells harboring pCS26 CamR reporter plasmids tended to have higher expression than cells harboring pCS26 KanR reporter plasmids containing either the sig70c10 and sig70c35 promoter (Figure 8D); there was a significant difference in the expression levels between the KanR and CamR vectors with these promoters.

To test the luciferase expression of reporter plasmids containing these new promoters in *Salmonella* we electroporated the pCS26 vectors containing the modified promoters into *Salmonella Typhimurium* ΔcsgD cells and performed the same luciferase assay to monitor their expression over 48-hours (Figure 9A). Both KanR and CamR versions of the pCS26 vector were tested. The same general trend was seen in *Salmonella Typhimurium*, with the new promoters having elevated expression levels, but unlike *E. coli*, the expression levels of the three promoters did not separate into discrete groups. Instead, the three groups appeared as more of a gradual progression from low expression to high expression. We separated the pCS26 KanR and CamR data into separate groups and observed that the pCS26 KanR peak expression profiles were similar to *E. coli*, with peak sig70-16 expression of approximately $0.5 \times 10^4$ CPS, peak expression from sig70c10 (blue) at $\sim 2 \times 10^4$ CPS (approximately 3x higher) and expression from sig70c35 (green) at $\sim 3.0 \times 10^4$ CPS (approximately 5x higher) (Figure 9B). In contrast, the pCS26 CamR expression profiles peaked around $1 - 1.5 \times 10^4$ CPS with either of the new promoters (Figure 9B); these values did not change even when expression was assayed in media supplemented with less chloramphenicol (i.e. 7, 9 and 10 µg/mL; data not shown). This data suggested that expression from the pCS26 CamR vector was impaired in *Salmonella* when compared to the KanR reporters. However, when the new luciferase reporter constructs containing the sig70c10 and sig70c35 promoters were inserted into the *Salmonella* chromosome through transposition we did not observe the same defect in luciferase expression (Figures 9C and 9D), suggesting it could be an artifact of expression from the pCS26 CamR plasmid in *Salmonella Typhimurium*.
Figure 9: The same promoter alterations produce varying levels of luciferase expression in Salmonella Typhimurium. A) Luciferase expression of Salmonella Typhimurium ΔcsgD cells harboring the pCS26 plasmid with the sig70-16 (red), sig70c10 (blue) or sig70c35 (green) promoter controlling luciferase expression. Grown in LB broth at 37° C supplemented with either 50 µg/mL kanamycin or 10 µg/mL chloramphenicol. Luciferase expression (measured as counts per second [CPS]) was assayed over a 48-hour period. B) Peak luciferase expression from pCS26 plasmids containing the sig70-16 (red), sig70c10 (blue) or sig70c35 (green) promoters controlling luciferase expression in Salmonella Typhimurium ΔcsgD cells grown in LB broth at 37° C, supplemented with antibiotic. Expression was measured from both pCS26 KanR (solid bars) and pCS26 CamR (hatched bars). Cells harboring pCS26 CamR were grown in media supplemented with 5, 7 and 10 µg/mL chloramphenicol, and this data is representative. Peak luciferase expression (measured as counts per second [CPS]) was determined to occur approximately 11 hours into a 48-hour assay (see 9A). Each is the result of the mean of multiple biological replicates. C) Luciferase expression of Salmonella Typhimurium ΔcsgD reporter strains after chromosomal insertion of the lux reporter construct. Reporters with the sig70-16 (red), sig70c10 (blue) or sig70c35 (green) promoters were measured, grown in LB broth at 37° C supplemented with either 50 µg/mL kanamycin or 10 µg/mL chloramphenicol. Luciferase expression (measured as counts per second [CPS]) was assayed over a 48-hour period. D) Peak luciferase expression from Salmonella Typhimurium reporters containing the sig70-16 (red), sig70c10 (blue) or sig70c35 (green) promoters controlling luciferase expression grown in LB broth at 37° C, supplemented with antibiotic. Expression was measured from both KanR (solid bars) and CamR (hatched bars) reporters. Peak luciferase expression (measured as counts per second [CPS]) was determined to occur approximately 8.5 hours into a 48-hour assay (see B). Each is the result of the mean of multiple biological replicates.
4.1.8 Comparing luciferase detection of reporter strains in C57BL/6 mice

To determine whether the sig70c10 and sig70c35 promoters increased the visibility of our reporter strains in vivo we performed a series of animal experiments using a BLI for detection. Mice were pre-treated with streptomycin, challenged with Salmonella Typhimurium reporters containing the sig70-16, sig70c10 or sig70c35 promoter and imaged one day post-challenge. We were able to detect the reporters containing each promoter in most mice one day post-challenge (Figures 10A – C). Mice challenged with the sig70-16 reporter had peak luciferase expression levels of approximately 400 CPS (Figure 10A), while those challenged with the sig70c10 reporter had peak levels of 1,200 CPS (Figure 10B) and sig70c35 reporters had peak levels of 3,000 CPS (Figure 10C). Similar to our in vitro findings, the peak luciferase levels represented approximately 3 and 7.5 times higher levels than the sig70-16 promoter. Mice were euthanized after the onset of severe clinical signs and their organs (spleen, liver, digestive tract and MLNs) were examined in the BLI as well. Mice that were colonized with the sig70-16 reporter had peak luciferase expression levels of approximately 600 CPS (Figure 10D) while those colonized with sig70c10 had peak levels of 1,000 CPS (Figure 10E) and sig70c35 had peak levels of 50,000 CPS (Figure 10F). This proved that the modified promoters allow for increased Salmonella detection in vivo, both in live mice as well as in their internal organs. The peak detection levels did not appear to follow the same trend in the organs, with the sig70c10 promoter resulting in <2 times the expression as the sig70-16 promoter and the sig70c35 promoter resulting in >80 times the expression as the sig70-16 promoter; it is possible that this was correlated with the bacterial load in each organ.

As mentioned previously many of our animal experiments involved pre-treating mice with streptomycin prior to challenge with Salmonella. We therefore also compared mice that had been pre-treated with streptomycin to those that had not, and observed whether there was a difference in detection. In the previous experiment we also used groups of mice that did not receive pre-treatment with streptomycin and were challenged with the sig70-16, sig70c10 or sig70c35 reporters. A representative image of the untreated group was taken (Figure 11A); we were unable to detect any luciferase from our reporters in untreated mice, even when using the sig70c35 reporters, which had the highest levels of luciferase expression. In contrast, we detected luciferase expression from our reporters in all streptomycin pre-treated mice on day 1 post-challenge (Figure 11B; sig70-16 reporter shown); however detection of these strains tapered off
Figure 10: *Salmonella* Typhimurium reporter strains have varying levels of luciferase expression *in vivo*. Female C57BL/6 mice were pre-treated with streptomycin then challenged with the *Salmonella* Typhimurium reporters containing the following promoters: sig70-16 (A, D), sig70c10 (B, E) or sig70c35 (C, F). Mice were anesthetized 1 day post-challenge and imaged on a bioluminescent imager. 4 – 5 days post-challenge, mice were euthanized and their organs (spleen, liver, cecum and MLNs) were recovered and imaged. Peak luciferase values (measured in CPS) are indicated.
Figure 11: Pre-treatment with streptomycin results in greater \textit{in vivo} detection of \textit{Salmonella Typhimurium} luciferase reporter strains. \textbf{A)} Female C57BL/6 mice were challenged with $1 \times 10^7$ CFU of a \textit{Salmonella Typhimurium} luciferase reporter strain. Mice were then anesthetized and imaged using a bioluminescent imager 1 day post-challenge. \textbf{B)} Female C57BL/6 mice were challenged with $1 \times 10^5$ CFU of a \textit{Salmonella Typhimurium} luciferase reporter strain after pre-treatment with 20 mg of oral streptomycin. Mice were then anesthetized and imaged using a bioluminescent imager 1 day post-challenge.
after day 1. Upon the onset of severe clinical signs, the reporters in some mice of both groups were detected, typically the day before euthanization, but not in all the mice that were tested. It appeared as though untreated mice had low detection of our modified reporters as well as the original sig70-16 reporters, suggesting that the difficulty in detection was due to some inherent property in the mice that streptomycin treatment overcame, regardless of the reporter used.

4.1.9 Generation and testing of the tetracycline-resistant pCS26 sig70-16 vector

To further strengthen our reporter library we generated a tetracycline-resistant (Tet\textsuperscript{R}) version of the pCS26 plasmid with the intent of generating Tet\textsuperscript{R} reporter strains. The Tet\textsuperscript{R} gene was PCR amplified from the pACYC184 plasmid and the resulting PCR product was digested using PstI and EcoRI. The digested Tet\textsuperscript{R} was then ligated into a PstI- and EcoRI-digested pCS26 sig70-16 plasmid to generate a pCS26 sig70-16 Tet\textsuperscript{R} vector encoding a tetracycline resistance marker as well as the luxCDABE luciferase operon. To test luciferase expression from this plasmid in Salmonella we electroporated the pCS26 sig70-16 Tet\textsuperscript{R} vector into Salmonella Typhimurium \textDelta{csgD} cells and monitored the expression over a 48-hour growth period. Luciferase expression from the pCS26 Tet\textsuperscript{R} vector was highly elevated compared to the Kan\textsuperscript{R} and Cam\textsuperscript{R} vectors (Figure 12A). The variability in expression was also greater than the Kan\textsuperscript{R} and Cam\textsuperscript{R} vectors, resulting in a large spread in the data. When growth of the reporter strains was measured, clones containing the Tet\textsuperscript{R} vector appeared to have depressed growth levels compared to clones containing the Kan\textsuperscript{R} and Cam\textsuperscript{R} vectors (Figure 12B). This indicated that an elevated growth rate was not the cause of elevated luciferase expression. In an attempt to normalize the growth of the Tet\textsuperscript{R} clones, we repeated the experiment in LB media supplemented with 5, 7, 9 or 10 µg/mL tetracycline; however this did not appreciably change the expression parameters of the Tet\textsuperscript{R} pCS26 sig70-16 vector (Shivak and White, unpublished data). The pCS26 Tet\textsuperscript{R} peak expression levels were approximately $8.5 \times 10^4$ CPS, which was approximately 6 times the peak expression from the Kan\textsuperscript{R} vector (green) and approximately 9 times the peak expression from the Cam\textsuperscript{R} vector (blue) (Figure 12C). Although the standard deviation in peak expression of the Tet\textsuperscript{R} clones was large, the expression was significantly higher than pCS26 vectors containing either Kan\textsuperscript{R} or Cam\textsuperscript{R} markers. For this reason we stopped the development of any Tet\textsuperscript{R} luciferase reporters.
Figure 12: pCS26 sig70-16 TetR has elevated luciferase expression and reduced growth. A) Luciferase expression of Salmonella Typhimurium ΔcsgD cells harboring the pCS26 sig70-16 plasmid with a KanR (green), CamR (blue) or TetR (black) antibiotic resistance marker. Grown in LB broth at 37°C supplemented with 50 µg/mL kanamycin, 10 µg/mL chloramphenicol or 7 µg/mL tetracycline. Luciferase expression (measured as counts per second [CPS]) was assayed over a 48-hour period. B) Growth of the reporter strains was monitored simultaneously by measuring the absorbance (OD_{590}) values of the cultures. Reporters containing KanR (green), CamR (blue) or TetR (black) antibiotic resistance marker were assayed during the same experiment. C) Peak luciferase expression from pCS26 plasmids containing the KanR (green), CamR (blue) or TetR (black) antibiotic resistance marker in Salmonella Typhimurium cells grown in LB broth at 37°C. Peak luciferase expression (measured as counts per second [CPS]) was determined to occur approximately 11.5 hours into a 48 hour assay. Each bar represents the mean of 32 biological replicates.
4.2 Preliminary animal trials characterizing the murine transmission model

4.2.1 The antibiotic marker does not affect the virulence of the Salmonella Typhimurium reporter strains in C57BL/6 mice

We designed the Salmonella Typhimurium reporter strains for use in animal experiments. To determine if either the Kan\textsuperscript{R} or Cam\textsuperscript{R} reporter strains had a significant difference in virulence, we designed an experiment where 12 mice were competitively challenged with WT Salmonella Typhimurium Kan\textsuperscript{R} and Cam\textsuperscript{R} reporters containing the sig70-16 promoter controlling luxCDABE expression (Figure 13). The challenge inoculum consisted of both strains at an approximately 1:1 ratio with each mouse receiving $1 \times 10^6$ CFU of each strain. At 5 – 6 days post-infection, the mice were euthanized and their organs (spleen, liver, cecum and MLNs) collected, homogenized and serially diluted onto LB agar supplemented with 50 $\mu$g/mL kanamycin or 10 $\mu$g/mL chloramphenicol. The number of Salmonella Typhimurium colony forming units from each strain was determined for the spleen (Figure 14A), liver (Figure 14B), cecum (Figure 14C) and MLNs (Figure 14D). In some individual mice the Kan\textsuperscript{R} reporter (green) was recovered at higher levels while in other mice the Cam\textsuperscript{R} reporter (blue) was recovered at higher levels. Typically, the same strain was recovered at higher levels in all organs from a single mouse, suggesting that the outcome of the competition between the strains was decided early on in infection, prior to systemic dissemination of Salmonella throughout the mouse. If either strain were more virulent than the other we would have expected it to be recovered from the majority of the mice at higher levels than the other. The final tally showed that the Kan\textsuperscript{R} reporter was recovered at higher levels in five mice while the Cam\textsuperscript{R} reporter was recovered at higher levels in seven mice, suggesting no difference in virulence. When we compared the competitive index values for each mouse we saw that in none of the organs tested were the competitive index (CI) values significantly different than 1 (Figure 14E). The CI values were calculated by comparing the input ratio of the two strains at the time of infection of the mice (an approximately 1:1 ratio) and the output ratio of the two strains recovered from the mice after infection, based on the CFU numbers recovered from each organ. The CI values calculated from each organ were not significantly different from 1, which indicated neither strain consistently outcompeted the other. This confirmed that there was no significant virulence difference between the Kan\textsuperscript{R} and Cam\textsuperscript{R} Salmonella Typhimurium reporter strains. Therefore, all subsequent animal experiments were performed with the sig70-16 versions of these Kan\textsuperscript{R} and Cam\textsuperscript{R} reporter strains. 

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Figure 13: Competitive infection of C57BL/6 mice with *Salmonella Typhimurium* Kan<sup>R</sup> and Cam<sup>R</sup> reporters. Twelve female C57BL/6 mice were challenged with a 1x10<sup>7</sup> CFU, 1:1 challenge of both strains. After 5 – 6 days post-challenge mice were euthanized and their organs were harvested (liver, spleen, cecum, mesenteric lymph nodes [MLNs]) and homogenized. Homogenized organs were plated on selective media to determine bacterial loads of each strain in each organ.
Figure 14: Bacterial counts and competitive indices from C57BL/6 mice after competitive infection with *Salmonella* Typhimurium. A to D) CFU counts of *Salmonella* Typhimurium Kan\(^R\) (green) and Cam\(^R\) (blue) reporters recovered from murine organs. Organs were homogenized and processed after euthanization and plated on both LB + Kan\(_{50}\) and LB + Cam\(_{10}\) plates to determine the levels of each strain present (measured as CFU per whole organ). E) Competitive indices were calculated for each mouse in each organ; horizontal lines represent the median in each organ. Competitive indices were calculated as follows: 
\[
\frac{[\text{CFU Kan}^{R}_{\text{OUT}}/\text{CFU Cam}^{R}_{\text{OUT}}]}{[\text{CFU Kan}^{R}_{\text{IN}}/\text{CFU Cam}^{R}_{\text{IN}}]},
\]
Dotted horizontal line indicates a CI value of 1, representing no difference. Significance was determined using a Wilcoxon Signed Rank Test, testing whether each organ was significantly different than 1.
4.2.2 C57BL/6 mice challenged with *Salmonella* Typhimurium shed low levels of bacteria in their feces

The main reason for generating the *Salmonella* reporter strains was for their use in a model of murine transmission that we had designed. The model involved the co-housing of both infected, seeder mice and naive, uninfected mice. After being infected with *Salmonella*, the seeder mice began shedding bacteria into the cage, causing infection of naive mice. The reporter strains were to be used as biological markers that we could monitor and use to track the spread of *Salmonella* throughout the mouse population using a BLI. Unfortunately, the reporters did not prove to be successful for this due to the limits in their detection *in vivo*, but the transmission model remained a useful tool. We hypothesized that the main route of transmission in the model would be through the fecal-oral route: seeder mice shedding *Salmonella* in their feces and spreading the infection to naive mice through contaminated feces. To determine whether this would be a realistic scenario we first needed to determine if mice challenged with *Salmonella* Typhimurium shed bacteria in their feces. We orally challenged ten mice with $1 \times 10^7$ CFU of a *Salmonella* Typhimurium reporter strain; five mice were challenged with the WT and five were challenged with the $\Delta csgD$ mutant (Figure 15). Each day these mice were isolated and four fecal pellets were collected from each individual mouse. These pellets were homogenized and processed, then plated on LB media supplemented with either 50 $\mu$g/mL kanamycin or 10 $\mu$g/mL chloramphenicol to recover and quantify the bacteria shed in the pellets.

Mice challenged with *Salmonella* Typhimurium shed no detectable bacteria in their feces (limit of detection [LoD] = 10 CFU/fecal pellet) until 5 days post-challenge, where they began shedding low to moderate levels until their euthanization on day 7 post-challenge (Figure 16). On day 5, two mice began shedding low levels of *Salmonella*, with bacteria counts of 20 and 30 CFU, respectively, detected in a single fecal pellet. By day 6, four mice were shedding moderate amounts of *Salmonella* in their feces with CFU counts ranging from 20 – 5000 CFU/pellet. On day 7 six mice were shedding *Salmonella* at levels ranging from 10 CFU – $2.6 \times 10^8$ CFU/pellet, with only two mice shedding at the high end of the range, while two mice continued to shed no detectable bacteria. Despite their variable shedding, all mice displayed severe clinical signs (i.e. hunched posture, ruffled fur, listlessness, weight loss >20% of their initial weight) and were euthanized by day 7 post-challenge. These results suggested that the majority of mice challenged with *Salmonella* Typhimurium did not shed high levels of *Salmonella* in their feces, while a
Figure 15: Infection of C57BL/6 mice to monitor the levels of *Salmonella* Typhimurium shedding. 10 female C57BL/6 mice were challenged with $1 \times 10^7$ CFU of a *Salmonella* Typhimurium reporter strain (either WT or a $\Delta csgD$ mutant). Each day individual mice were isolated and 4 fecal pellets were collected from each mouse. Fecal pellets were homogenized and plated on selective media to determine the levels of shedding. After 5 – 6 days post-challenge mice were euthanized and their organs were harvested (liver, spleen, cecum, mesenteric lymph nodes [MLNs]) and homogenized. Homogenized organs were plated on selective media to determine bacterial loads in each organ.
Figure 16: C57BL/6 mice shed low levels of *Salmonella* Typhimurium beginning at 5 days post-challenge. Four fecal pellets shed from mice challenged with WT *Salmonella* Typhimurium were collected and processed daily for 7 days. Each point represents the bacterial load in a single fecal pellet. For mice that had no detectable *Salmonella* in their feces, one data point was used to reduce visual clutter. The CFU of *Salmonella* per individual pellet was determined. The horizontal line represents the limit of detection.
small subset of the population began shedding high levels of *Salmonella* 7 days post-challenge. A requirement for efficient fecal-oral transmission in our model would be the presence of high levels of shedding shortly after infection, to maximize the spread of *Salmonella* throughout the population of mice. The results of this experiment suggested that high shedding levels would be achieved only by a small number of mice and only for one or two days before the mice succumbed to illness, meaning that they would act as poor carriers of disease.

4.2.3 Pre-treating C57BL/6 mice with streptomycin leads to increased shedding

To achieve efficient transmission through the fecal-oral route, a method of inducing high levels of fecal shedding needed to be found. Streptomycin pre-treatment had been demonstrated to increase the shedding levels in mice that are orally infected with *Salmonella*. To test this in our infection model, 20 mice were randomly assigned to four groups; two groups (10 mice) were pre-treated with 20 mg of oral streptomycin prior to challenge and two groups were challenged similar to the previous experiments. Each individual mouse was challenged with $1 \times 10^7$ CFU of WT *Salmonella* Typhimurium (*Cam* reporter) and their shedding was monitored over the course of the experiment. Four fecal pellets from each streptomycin pre-treated mouse were collected daily and processed individually, while four fecal pellets from each untreated mouse were collected daily and pooled together in a single tube prior to being processed and plated. This was done to ensure that we were able to detect *Salmonella* in the feces of the untreated mice even though our previous experiment had demonstrated that levels in a single fecal pellet were often undetectable.

Similar to the previous experiment the untreated mice had moderate levels of shedding throughout the experiment, ranging from approximately $1 \times 10^2$ – $1 \times 10^5$ CFU/g of feces, with two samples on day 6 having levels $>1 \times 10^7$ CFU/g (Figure 17, grey). This suggested that the mice in the previous experiment were shedding low levels of *Salmonella* throughout the time course that were below the level of detection; the pooling of fecal samples in untreated mice resulted in the reliable detection of *Salmonella*. The streptomycin pre-treated group had significantly higher levels of shedding throughout the experiment, ranging from approximately $1 \times 10^7$ – $1 \times 10^{10}$ CFU/g of feces (Figure 17, red). In general, the shedding levels appeared to be quite uniform over the course of the experiment. Interestingly, for this experiment the streptomycin-treated
Figure 17: Pre-treating mice with streptomycin results in significantly higher levels of shedding. Four fecal pellets shed from mice challenged with WT *Salmonella Typhimurium* were collected and processed daily for 6 days. Mice were either treated with 20 mg of streptomycin (red) or left untreated (grey). Each point from the treated group represents the bacterial load in a single fecal pellet, while each point from the untreated group represents the pooled bacterial load from four fecal pellets. The CFU of *Salmonella* per gram of feces was determined. The mean of each group is represented by a black line, with standard deviation. The dotted horizontal line represents the limit of detection. Significance (* = p<0.05) was determined using multiple t tests.
mice had a more rapid disease progression and had to be euthanized at an earlier time point than the untreated mice. Pre-treatment with streptomycin significantly increased the shedding levels in mice challenged with *Salmonella* Typhimurium but shortened the length of time that these mice would likely be in a cage and spreading infection.

**4.2.4 Initial challenge dose does not affect shedding levels of streptomycin pre-treated C57BL/6 mice**

Ideally, mice challenged with *Salmonella* would be present in a cage with other, naive mice for prolonged periods of time in order to maximize their exposure to *Salmonella*. In an attempt to prolong the lifespan of our streptomycin pre-treated mice we lowered the challenge dose of *Salmonella* to observe whether both the shedding patterns and life spans of these mice were altered. Three groups of six mice were each challenged with different levels of *Salmonella* Typhimurium (1×10⁴ CFU, 1×10⁵ CFU or 1×10⁶ CFU) (Figure 18). The shedding of the mice was monitored over the course of the experiment by collecting and processing 4 fecal pellets from each mouse, and over the course of the experiment we monitored the mice’s clinical signs to determine if there was a difference in the disease progression between the three groups. The shedding levels seen in the mice challenged with the 1×10⁴ CFU challenge (blue), 1×10⁵ CFU challenge (green) and 1×10⁶ CFU challenge (red) were determined (Figure 19). Throughout the trial, the mean shedding levels of the three groups of mice stayed at approximately 1×10⁹ – 1×10¹⁰ CFU/g of feces, with no significant differences observed. The shedding levels recorded were similar to the levels measured in the mice challenged with 1x×10⁷ CFU in our previous experiment. This indicated that the challenge dose did not affect the shedding levels observed in the mice. This was not unexpected, and had been recorded previously in the literature. By day 5 of the experiment, however, 7 mice had been euthanized with 2 each from the 1×10⁵ CFU and 1×10⁶ CFU groups and 3 from the 1×10⁴ CFU group. On day 6 all of the remaining mice were euthanized due to the onset of severe clinical signs. This suggested that while the challenge dose did not alter the shedding levels seen in infected mice it also did not alter the expected lifespan of the mice.
Figure 18: Testing three challenge levels of *Salmonella* Typhimurium in streptomycin pre-treated mice. A total of 18 female C57BL/6 mice — in groups of 6 — were challenged with WT *Salmonella* Typhimurium after treatment with streptomycin. Six mice were challenged with $1 \times 10^4$ CFU of *Salmonella*, six mice with $1 \times 10^5$ CFU of *Salmonella* and six mice with $1 \times 10^6$ CFU of *Salmonella*. Each day, individual mice were isolated and 4 fecal pellets were collected from each mouse. Fecal pellets were homogenized and plated on selective media to determine the levels of shedding from the mice. After 5 – 6 days post-challenge mice were euthanized and their organs were harvested (spleen, liver, cecum and MLNs) and homogenized. Homogenized organs were plated on selective media to determine bacterial loads in each organ.
Figure 19: The challenge dose does not alter the shedding or lifespan of C57BL/6 mice. Fecal pellets shed from mice challenged with *Salmonella* Typhimurium were collected and processed daily for 6 days. Mice were challenged with $1 \times 10^4$ CFU (blue), $1 \times 10^5$ CFU (green) or $1 \times 10^6$ CFU (red). All mice were euthanized on day 6. Each point represents the bacterial load from a single fecal pellet, represented as CFU per gram of feces. The mean of each group is represented by a black line, with standard deviation. The dotted horizontal line represents the limit of detection. Significance was determined using multiple t-tests.
4.2.5 WT and ΔcsgD Salmonella Typhimurium are shed from and colonize mice at similar levels

As outlined in Section 2.0, we hypothesized that biofilm formation would contribute to the transmission success of Salmonella Typhimurium. Therefore, one of the first tests of our transmission model would be to compare the transmission efficiencies of both WT Salmonella Typhimurium and the isogenic, ΔcsgD mutant that is impaired in biofilm formation\textsuperscript{162}. To compare the transmission efficiencies of these two strains in our model, we would need to monitor their spread throughout a population of mice. If one of the strains were shed from the infected seeder mice at higher levels they would likely be spread throughout the cage and into the naive, uninfected population at higher rates. This apparent increase in transmission efficiency would be due to a difference in shedding levels. Similarly, if one of the two strains were to colonize naive mice to a greater extent it would appear as though it had transmitted from the seeder mice at higher levels instead of colonizing the naive mice at higher levels. To test these two possibilities, we designed an experiment where two groups of six mice were challenged with WT Salmonella Typhimurium and two groups of six mice were challenged with the ΔcsgD mutant; all mice were pre-treated with streptomycin before challenge. To monitor shedding, three fecal pellets were collected from each mouse daily and to measure colonization, the organs from euthanized mice were collected and processed to determine the bacterial load. Over the course of the experiment, mice challenged with either strain displayed the same shedding pattern of approximately $1 \times 10^9$ – $1 \times 10^{11}$ CFU/g of feces, with no significant differences between groups (Figure 20A). Colonization levels were also similar between the different groups of mice, with the bacterial load in each type of organ ranging from approximately $1 \times 10^5$ – $1 \times 10^9$ CFU (Figure 20B). Again, there were no significant differences in colonization between the two strains. These results indicated that both WT Salmonella Typhimurium and the isogenic, ΔcsgD mutant strain colonized and were shed from mice at similar levels when mice were challenged orally with a single strain.
Figure 20: WT and ΔcsgD Salmonella Typhimurium are shed from and colonize mice at similar levels. A) Fecal pellets shed from mice challenged with Salmonella Typhimurium were collected and processed daily for 5 days. Mice were challenged with WT Salmonella (grey) or with the isogenic ΔcsgD mutant (red). Each point represents the bacteria shed in a single fecal pellet, represented as CFU per gram of feces. The mean of each group is represented by a black line, with standard deviation. The dotted horizontal line represents the limit of detection. B) Colonization levels from mice challenged with Salmonella Typhimurium, represented by the CFU per gram of organ. Mice were challenged with WT Salmonella (grey) or with the isogenic ΔcsgD mutant (red). Each point represents the bacteria in an individual mouse’s organs. The mean of each group is represented by a black line, with standard deviation. The dotted horizontal line represents the limit of detection.
4.3 Analysis of fecal survival and recovery of *Salmonella* Typhimurium

4.3.1 WT *Salmonella* Typhimurium have greater short-term survival potential in fecal pellets

Previous studies had shown that cellular aggregation and biofilm formation in *Salmonella* were not virulence adaptations, suggesting that these physiologies played a role in another aspect of the *Salmonella* life cycle. Due to a large body of work examining the resistance properties of biofilms, we hypothesized that cellular aggregation could be involved in the environmental persistence and survival of biofilm-associated cells. To test this we challenged streptomycin pre-treated mice with either WT *Salmonella* Typhimurium or the ΔcsgD mutant and collected fecal pellets from these mice daily (Figure 21). The fecal pellets were stored in 96-well plates after collection and left to dry at room temperature and in direct light to mimic environmental conditions. Each week, 6–10 fecal pellets from each strain were randomly selected, processed and plated on LB agar supplemented with antibiotics to determine the levels of *Salmonella* that could be recovered from the pellets after long-term storage.

On day 0 (the day of collection) there was no significant difference in the recovery levels of either strain, with levels of approximately $1 \times 10^8$ CFU/fecal pellet (Figure 22). After one week of storage we observed that the WT strain was recovered from fecal pellets at significantly higher levels than the ΔcsgD mutant, suggesting it was better able to persist inside of these fecal pellets. After 2 weeks we observed the same general trend with WT cells recovered at generally higher levels; however, the results lost significance ($p = 0.053$), likely due to the presence of a few outliers. The same general trend was present after 3 weeks. From 4–6 weeks the trend disappeared, with both the WT and ΔcsgD mutant cells recovered at a large range of levels and with no significant differences between the two groups. The results demonstrated that WT *Salmonella* Typhimurium had higher survival potential than the ΔcsgD mutant in the short term (a period of 1–2 weeks) under environmental conditions when shed in fecal pellets.
Figure 21: Testing the survival potential of WT and ΔcsgD Salmonella Typhimurium. Female C57BL/6 were challenged with either WT or ΔcsgD Salmonella Typhimurium after treatment with streptomycin. Each day individual mice were isolated and fecal pellets were collected from each mouse. These fecal pellets were stored in 96-well plates in direct light and at room temperature. Each week 6 – 10 fecal pellets were randomly selected, homogenized and plated on selective media to determine the levels of each strain that could be recovered.
Figure 22: WT *Salmonella* Typhimurium have greater short-term survival potential in fecal pellets. Fecal pellets shed from mice challenged with *Salmonella* Typhimurium were collected and processed weekly for 6 weeks. Fecal pellets were shed from mice challenged with WT *Salmonella* (grey) or the isogenic Δ*csgD* mutant (red). On day 0 four fecal pellets from each group were sampled, on weeks 1 – 4 six fecal pellets from each group were sampled and on weeks 5 – 6 10 fecal pellets from each group were sampled. Each point represents the bacteria recovered from a single fecal pellet. The mean of each group is represented by a black line, with standard deviation. The dotted horizontal line represents the limit of detection of 100 CFU/pellet (weeks 0 – 3) or 10 CFU/pellet (weeks 4 – 6). Significance (*) was determined by multiple t tests (p value <0.05).
4.3.2 Fresh fecal pellets allow for the recovery of higher levels of *Salmonella* Typhimurium

To maximize the number of fecal pellets we could test in future *Salmonella* survival experiments we collected fecal pellets in large quantities. Two groups of mice pre-treated with streptomycin were challenged with either WT *Salmonella* Typhimurium or the $\Delta csgD$ mutant and monitored over the course of the experiment. Instead of isolating each mouse and collecting fecal pellets daily, we moved the mice to new cages each day and collected all the fecal pellets shed into the cage over the previous day. Typically, we collected 96 fecal pellets (one 96-well plate) of each strain daily during the course of the experiment. The shedding levels we observed using this method (Figure 23; July and August) were significantly lower than the levels of shedding seen in previous trials (Figure 23; March). In our previous experiments, for the first three days post-challenge we measured shedding of approximately $1 \times 10^9$ CFU/g of feces, while the two mass collection experiments resulted in levels ranging from $1 \times 10^5$ – $1 \times 10^8$ CFU/g, with the recovery from some individual fecal pellets near the LoD of 10 CFU/g. It was clear that the recovery of *Salmonella* from fecal pellets in bulk was different than our initial experiment. We hypothesized that the variable period of time of up to one day that fecal pellets sat in a cage prior to being processed could account for the reduction in recovery levels from these fecal pellets.

To test if there was a difference in CFU recovered between fresh (collected and processed immediately) and desiccated (collected and processed up to one day post-shedding) fecal pellets, we designed an experiment to compare the recovery of *Salmonella* from both. Similar to the previous experiment, mice were challenged with either WT *Salmonella* Typhimurium or the $\Delta csgD$ mutant after pre-treatment with streptomycin, and fecal pellets were collected from the cage each day. We also collected three fresh pellets from two randomly selected mice per cage in order to compare these levels to the desiccated pellets (Figure 24). After processing, we observed that the fresh pellets (red) resulted in significantly higher recovery levels of *Salmonella* Typhimurium than desiccated pellets (grey) for the duration of the experiment. We were unable to collect fresh pellets from the mice on day 5 due to the progression of disease. Fresh fecal pellets collected in this trial resulted in *Salmonella* recovery levels of approximately $1 \times 10^6$ – $1 \times 10^7$ CFU/pellet, similar to the levels observed in previous trials. In contrast, the desiccated fecal pellets resulted in recovery levels of approximately $1 \times 10^4$ – $1 \times 10^5$ CFU/pellet, which were similar to those seen in the July and August trials (Figure 23). Considering that both groups of fecal pellets were collected from the same groups of mice, it appeared that a period of
Figure 23: Mass collection leads to reduced recovery of *Salmonella* Typhimurium in fecal pellets. Fecal pellets shed from mice challenged with *Salmonella* Typhimurium were collected and 6 pellets per cage were processed daily for 5 days to monitor the shedding levels. WT and ΔcsgD data has been pooled together. Data from a previous experiment (March; blue) was compared to a new method for the mass collection of pellets (July and August; green and red). Each point represents the bacteria shed in a single fecal pellet, represented as CFU per gram of feces. The mean of each group is represented by a black line, with standard deviation. The dotted horizontal line represents the limit of detection.
Figure 24: Fresh fecal pellets allow for significantly higher recovery of *Salmonella Typhimurium*. Fresh and desiccated fecal pellets shed from mice challenged with *Salmonella Typhimurium* were collected and 6 pellets of each type per cage were processed daily for 5 days to monitor the recovery of *Salmonella*. WT and ΔcsgD data was pooled together. The recovery of *Salmonella* from fresh fecal pellets (red) was compared to the recovery from desiccated fecal pellets (grey). Each point represents the bacteria recovered from a single fecal pellet, represented as CFU per pellet. The mean of each group is represented by a black line, with standard deviation. The dotted horizontal line represents the limit of detection of 10 CFU/pellet. Significance (*) was determined by multiple t tests (p value <0.05).
desiccation of ≤1 day was sufficient to significantly reduce the recovery levels of *Salmonella* from fecal pellets.

### 4.4 Testing the transmission potential of WT and Δ*csgD* *Salmonella* Typhimurium

#### 4.4.1 C57BL/6 seeder mice can transmit *Salmonella* Typhimurium infection to naive mice pre-treated with streptomycin

To begin testing the transmission potential of different strains of *Salmonella* Typhimurium we first needed to test if transmission from infected seeder mice to uninfected naive mice could occur. Our first experiment involved challenging seeder mice with *Salmonella* Typhimurium, co-housing them with naive mice and monitoring the spread of infection (Figure 25). Mice were randomly assigned to three groups with each group consisting of two seeder mice and five naive mice. The seeder mice were pre-treated with streptomycin and challenged with 1×10^5 CFU of WT *Salmonella* Typhimurium prior to co-housing. After challenge, the seeder mice were co-housed with the naive mice, with each group of naive mice manipulated in a different manner. Naive mice in group 1 were co-housed with the seeder mice and not manipulated, naive mice in group 2 were pre-treated with streptomycin – in the same manner as the seeder mice – prior to co-housing, and naive mice in group 3 were fasted for 16 hours prior to and 4 hours after co-housing in order to increase the chance of coprophagy. The clinical signs of the mice were monitored to track the spread of *Salmonella* infection; when naive mice began showing signs of infection transmission was deemed to have occurred.

At 10 days post-challenge, all mice were euthanized and their organs were collected and processed to determine whether *Salmonella* had transmitted from the infected mice to naive mice. All seeder mice were colonized successfully with *Salmonella* (Figure 26); each of these mice had been euthanized 4 to 5 days post-challenge. However, out of the three groups of naive mice, only mice in group 2 had detectable colonization with *Salmonella*. All naive mice from group 2 (streptomycin pre-treated) had high levels of *Salmonella* recovered from their organs, similar to the levels seen in the seeder mice. None of the naive mice from groups 1 (no manipulation) and 3 (fasted) had detectable *Salmonella* in any of their organs even though the seeder mice co-housed with these groups were all infected and shedding high levels of *Salmonella* into the cages. Looking at the transmission efficiency of the seeder mice (the percentage of naive mice infected with *Salmonella*) we observed that seeder mice in group 2 had
Figure 25: Testing the transmission potential of seeder mice challenged with *Salmonella Typhimurium*. Six female C57BL/6 were challenged with $1 \times 10^5$ CFU of *Salmonella Typhimurium* after treatment with streptomycin. Seeder mice were split into 3 groups of 2, with each group containing 5 naive mice. Group 1 was not manipulated. Group 2 naive mice were pre-treated with streptomycin before the addition of seeder mice. Group 3 naive mice were fasted for 20 hours (16 hours pre-mingling and 4 hours post-mingling) to promote coprophagy. The clinical signs of all mice were checked daily to monitor any transmission from the seeder mice to the naive mice. Mice were euthanized after the appearance of severe clinical signs (weight loss of >20%) or 10 days post-mingling and their organs were homogenized and processed to determine whether *Salmonella* had been transmitted.
Figure 26: Seeder mice can transmit *Salmonella* to naive mice pre-treated with streptomycin. After collection all organs were processed and plated on selective media to determine whether *Salmonella* had been transmitted. Seeder mice are marked S1 – S6 while naive mice are marked 1 – 15, and were divided into 3 groups. The levels of *Salmonella* in their organs (spleen [blue], liver [red], cecum [green] and MLNs [grey]) were tested. Mouse 9 was found dead in its cage overnight and no organ counts were determined. Transmission efficiency was calculated as the percentage of naive mice in each group with detectable levels of *Salmonella* in any organ.
a transmission efficiency of 100% while seeder mice in groups 1 and 3 had a transmission efficiency of 0%. The presence of an intact gut microbiota likely had a strong influence on whether or not transmission occurred in these mice.

4.4.2 Developing a working model of transmission for Salmonella Typhimurium in C57BL/6 mice

Based on the data we had collected up to this point, we thought we could design a standard transmission experiment (Figure 27). This model would consist of seven mice co-housed in a single cage: two seeder mice that were infected with Salmonella and five naive mice who remained uninfected. All of the naive mice would be pre-treated with streptomycin. Such a model would allow us to compare the transmission efficiencies of both the WT strain and the mutant strain and compare whether this mutation had a significant effect on transmission. If the mutant experienced a reduction in transmission efficiency, it could be inferred that the gene in question played a role in the transmission cycle of Salmonella.

Experiments based on this model were divided into two general groups: experiments examining short-term transmission and experiments examining long-term transmission (Figure 28). This differentiation was made in an attempt to probe two different aspects of transmission in a model resembling a real-world environmental scenario. Short-term transmission was primarily focused on the direct, host-to-host transmission of infection from seeder mice to naive mice through the fecal-oral route. The seeder mice, once infected with Salmonella, shed bacteria into the cage at high levels and naive mice would be exposed to these bacteria after a short period of time and become infected with Salmonella. Long-term transmission was focused on a prolonged environmental stage, such as fecal pellets that were shed into the environment and were encountered by naive mice after a significant period of time. The stage between shedding of the bacteria and encountering another host would involve numerous environmental stresses and potentially require a more specialized subset of genes to maximize the transmission potential of Salmonella.
**Figure 27: Overview of transmission model.** The transmission model we designed involved two seeder mice co-housed in a cage with five naive mice. One seeder mouse would be challenged with WT *Salmonella Typhimurium* and the other with the mutant strain being tested. Over time, the seeder mice would shed *Salmonella* in their feces and expose the naive mice to infection. If the mutant strain was defective in an aspect of the transmission pathway it would be expected that it would transmit to fewer mice than the WT, or at lower levels. By euthanizing the mice and processing their organs we would be able to observe any differences in transmission efficiencies between the two strains.
Figure 28: Distinction between short- and long-term transmission pathways in our model. To test transmission in both short- and long-term scenarios, we tested different transmission pathways. Short-term transmission was the result of direct transmission from seeder mice, with contaminated fecal pellets shed into the cage and naive mice exposed to *Salmonella*. It represented a more direct transmission pathway, with little environmental influence. Long-term transmission attempted to more accurately represent environmental transmission. Fecal pellets shed from seeder mice five weeks previously were desiccated and added to a cage containing naive mice. The long-term desiccation step put environmental pressure on the bacteria in the fecal pellets before they could be transmitted to naive mice.
4.4.3 *Salmonella Typhimurium* Δ*csgD* is not impaired in short-term transmission efficiency

To test the short-term transmission potential of WT *Salmonella Typhimurium* and the biofilm-negative Δ*csgD* mutant we performed an experiment comparing both strains’ ability to transmit to naive mice (Figure 29). Four groups of mice – each consisting of two seeder mice and five naive mice – were pre-treated with streptomycin and co-housed. The seeder mice were challenged with $1 \times 10^5$ CFU of *Salmonella Typhimurium* prior to co-housing; two groups had seeder mice that were challenged with WT *Salmonella* and two groups had seeder mice that were challenged with the Δ*csgD* mutant. Reciprocal challenges were performed, with one cage receiving Kan$^R$ WT *Salmonella* Typhimurium and another cage receiving Cam$^R$ WT *Salmonella* Typhimurium (and the same with the Δ*csgD* mutant) to ensure there were no differences in transmission due to the different antibiotic resistance markers. This design also allowed us to perform the experiments with blinding, so that we did not know which cage received each strain. After co-housing, the naive mice were monitored for the onset of clinical signs, which indicated the transmission of infection. At the conclusion of the experiment all of the mice were euthanized and their organs were processed to determine if they had been infected with *Salmonella Typhimurium*.

At 7 days after co-mingling, the naive mice from all four groups had become ill and were euthanized; infection with *Salmonella Typhimurium* was confirmed after their organs were collected and processed (Figure 30A). This indicated that both WT and Δ*csgD* *Salmonella Typhimurium* had 100% short-term transmission efficiency in this model, and were able to transmit to all of the naive mice in a cage. Interestingly, the *Salmonella* colonization levels of naive mice infected through the fecal-oral route were similar to those seen in seeder mice, suggesting that the route of infection did not affect the total colonization levels of *Salmonella*. The spread of infection throughout the mouse population was monitored through the weight loss of naive mice. After becoming infected with *Salmonella* the disease caused rapid weight loss until a point when the mice were euthanized. The weight loss of the naive mice is displayed with the critical point of 20% of initial weight lost represented as a dotted line (Figure 30B). Naive mice that were co-housed with WT-infected seeder mice (grey) generally experienced weight loss beginning at day 4 and underwent a gradual decline until day 7 when they were euthanized. One mouse from this group began rapidly losing weight on day 3 and did not recover, leading to its euthanization early on day 5. Naive mice that were co-housed with Δ*csgD* -infected seeder
Figure 29: Testing the short-term transmission potential of WT and ΔcsgD Salmonella Typhimurium. 8 female C57BL/6 were pre-treated with streptomycin and challenged with Salmonella Typhimurium – 4 mice with the WT and 4 with the ΔcsgD mutant. These seeder mice were split into 4 groups of 2, with each group containing 5 naive mice. The clinical signs of all mice were checked daily to monitor any transmission from the seeder mice to the naive mice. Mice were euthanized after the appearance of severe clinical signs (weight loss of >20%) and their organs were homogenized and processed to determine whether Salmonella had been transmitted.
**Figure 30: Salmonella Typhimurium ΔcsgD is not impaired in short-term transmission.** A) Colonization levels of naive mice infected with Salmonella, represented as CFU per gram of organ. Naive mice were co-housed with seeder mice challenged with either WT (grey) Salmonella Typhimurium or the isogenic ΔcsgD mutant (red), and the colonization levels of Salmonella in their organs were examined after euthanization. Each point represents the bacteria in an individual mouse’s organs. The mean of each group is represented by a black line, with standard deviation. The dotted horizontal line represents the limit of detection. B) Weight loss of the naive mice was tracked over the course of the experiment as a measure of the percentage of initial weight lost. Mice were housed with wild-type infected seeders (grey) or ΔcsgD infected seeders (red) and their weight loss was monitored over the course of the experiment. The line at 80% represents severe weight loss of 80%, which is the point at which mice must be euthanized.
mice (red) experienced a similar weight loss trend, with most beginning to lose weight at day 4 post-mingling and trending downwards until day 7 and euthanization. Two of these mice experienced rapid weight loss on day 3, but were able to recover slightly until euthanization on day 7. Together this data suggested that there was no difference in the speed of transmission between the two strains, as well as no deficiency in overall short-term transmission by the ΔcsgD mutant.

4.4.4 *Salmonella Typhimurium ΔcsgD is not impaired in long-term transmission efficiency*

To test if the ΔcsgD mutant was instead deficient in long-term transmission we designed a similar experiment examining both strains’ ability to transmit to naive mice from desiccated fecal pellets (Figure 31). The idea underlying this experiment was that while both WT and ΔcsgD *Salmonella Typhimurium* were able to transmit at high efficiencies in the short-term, the biofilm-related processes and survival advantages of the WT may play a larger role after a period of imposed long-term environmental persistence and survival. In introducing long-term storage and desiccation of the fecal pellets, we attempted to mimic a long-term scenario where *Salmonella* would be shed into the environment in fecal matter and persist for a long period of time prior to encountering a new host. The experiment therefore involved two groups of naive mice, each pre-treated with streptomycin. Each cage was seeded with 200 fecal pellets (100 WT pellets and 100 ΔcsgD pellets) that were shed from *Salmonella*-infected seeder mice 5 weeks previously; the pellets had been placed in 96-well plates and stored at room temperature with exposure to direct light. Two groups of five naive mice that had been pre-treated with streptomycin were in the cages and monitored for the onset of clinical symptoms of *Salmonella* infection.

In the first 10 days, four mice became infected with *Salmonella* (Figure 32A). Two of these mice were euthanized prior to their death (318N and 318L), while two mice were found dead overnight (317L and 3182R). The organs from the euthanized mice were collected and processed, while only the livers from the dead mice could be recovered. Both of the euthanized mice and one of the dead mice were highly colonized with the ΔcsgD mutant strain of *Salmonella*, with low levels of the WT strain in some organs. The remaining dead mouse was highly colonized with the WT strain and had undetectable levels of the ΔcsgD mutant. We
2 groups of 5 naive mice were put into cages after treatment with streptomycin and exposed to fecal pellets contaminated with *Salmonella* Typhimurium. Each cage was seeded with 100 fecal pellets containing WT *Salmonella* and 100 fecal pellets containing *Salmonella* ΔcsgD that had been desiccated for a period of 5 weeks. The clinical signs of all mice were checked daily to monitor any transmission from the fecal pellets to the naive mice. Mice were euthanized after the appearance of severe clinical signs (weight loss of >20%) or after a period of 14 days, and their organs were homogenized and processed to determine whether *Salmonella* had been transmitted.
Figure 32: *Salmonella* Typhimurium ΔcsgD is not impaired in long-term transmission. After euthanization the organs from all mice were processed and plated on selective media to enumerate the numbers of *Salmonella* present. A) Four mice became infected with *Salmonella* in the first 10 days and were euthanized (318N and 318L) or found dead in their cages (317L and 3182R; only the livers from these mice could be recovered). B) After 14 days the remaining mice were euthanized. The levels of *Salmonella* in each organ were examined. The dotted horizontal line represents the limit of detection.
allowed the experiment to proceed for four additional days. At 14 days post-seeding, although the remaining six mice showed no outward signs of infection, they were euthanized and their organs were examined for *Salmonella* colonization. We recovered *Salmonella* from five of the six mice. Three mice (317NM, 317B and 3172R) were colonized with the ΔcsgD mutant, with no detectable levels of the WT strain, whereas two mice (318R and 318B) were colonized with the WT and had no detectable levels of the ΔcsgD mutant (Figure 32B). One mouse (317R) remained uninfected with either strain. The colonization levels in the five mice with detectable *Salmonella* were lower than the four mice in the first 10 days of the experiment, suggesting it was possible that their infections would have progressed to symptomatic disease and death if given more time. Overall, the WT strain infected three mice at high levels while the ΔcsgD mutant infected six mice at high levels. Though taken from a small group of mice, these results suggested that the ΔcsgD mutant was not deficient in long-term transmission, as tested in our model.
5.0 DISCUSSION, FUTURE WORK AND CONCLUSIONS

5.1 Discussion

Though unsuccessful at demonstrating the role of *Salmonella* biofilm formation in the transmission process (through the mechanism of CsgD regulation of biofilm formation) our transmission model laid the foundation for future studies. Subsequent studies could attempt to assign roles to specific genes that are predicted to be involved in the transmission process. The model was well characterized over the course of this study and allows for modifications to be made at later stages to adapt to the needs of subsequent experiments. We were also successful in engineering a small library of *Salmonella* Typhimurium reporter strains for use in our experiments, and assembled a Tn7 transposition system to facilitate the generation of a large variety of further reporters. The reporters themselves could be used to detect *Salmonella* both *in vitro* and *in vivo* in streptomycin pre-treated mice, and potentially in future transmission experiments after further characterization of their luciferase expression *in vivo*. Regardless of their use *in vivo*, the Tn7 system we generated has numerous applications for *in vitro* experiments; the ease of promoter swapping allows for rapid comparisons of gene expression using a luminometer.

The modified Tn7 system we designed allows for the rapid and straightforward construction of various reporters in many bacterial species. The pUC18R6KT-miniTn7T delivery vector contains a R6K origin of replication, which means that its replication is supported in many bacterial species containing the *pir* gene. Tn7 transposition using our system could therefore be used in many different species to generate a diverse library of reporters. The requirement of the *pir* gene also allows for its successful use as a suicide vector, ensuring that it is cured from the cell after transposition. Tn7 transposition using the pUC18R6K miniTn7T vector had previously been successfully demonstrated in *P. aeruginosa*, *Y. pestis* and *B. thailandensis*, establishing its utility in a variety of bacterial species. Prior to using this method, we attempted to use another system utilizing a single plasmid-based system but did not have success in *Salmonella* Typhimurium, despite the authors stating its successful use. The vector (pGRG25) contained a pSC101 origin of replication, suggesting that its replication would be supported in *Salmonella*. Ligation of our reporter construct proved to be difficult, possibly due to the large size of both our construct (~8 kb) and the vector itself (~12.5 kb), though the authors had confirmed the successful transposition of the *lux* operon using the pGRG25 vector. Once we
had successfully ligated our construct into the vector, we were unable to initiate transposition in the recipient *Salmonella* Typhimurium cells, even after optimizing the expression of the *tnsABCD* operon from the P_{BAD} promoter (i.e. glucose concentration in the media).

The reporters that we constructed had similar yet not identical levels of luciferase expression depending on the orientation of the reporter construct (Figure 4A). Both the forward and reverse constructs were genetically identical and were inserted into the chromosome at identical sites, yet reporters with the construct in the reverse orientation had elevated luciferase expression. This could be due to slight differences in promoter recognition or efficiency of translation due to the change in reporter orientation, shifting the spatial location of sites on the chromosome. We were unable to determine the cause of the slight differences in expression, and decided not to pursue the cause of this effect. We found that the Kan^{R} and Cam^{R} luciferase constructs had different levels of luciferase expression when expression was compared in either the pCS26 vector or in the chromosomal reporters (Figures 5A, 8D and 9B/D). The *Salmonella* Typhimurium Kan^{R} reporters had elevated levels of luciferase expression compared to the Cam^{R} reporters, suggesting that the resistance marker or growth in the presence of kanamycin or chloramphenicol affected the expression of luciferase from these reporters (Figure 5A). When the expression of the various pCS26 reporter constructs was compared we observed that the Cam^{R} vectors had significantly lower levels of expression in *Salmonella* (Figure 9B). Furthermore, the expression of both the sig70c10 and sig70c35 promoters was similar in the Cam^{R} vector regardless of the promoter, with expression that plateaued at similar peak levels.

When we took the same reporter constructs and inserted them into the *Salmonella* Typhimurium chromosome we saw that there was no difference between the Kan^{R} and Cam^{R} reporters with the sig70-16 and sig70c10 promoters (Figure 9D). The Kan^{R} and Cam^{R} sig70c35 promoters remained significantly different. The apparent loss of significant differences between the differentially marked reporter constructs from vector to chromosome in *Salmonella* could possibly be due to complications caused by the growth of Salmonella in chloramphenicol, which has been shown to interfere with various cellular processes through the upregulation of cellular proteases triggered by the overproduction of chloramphenicol acetyltransferase. In contrast, we observed that the Cam^{R} vector in *E. coli* had significantly elevated luciferase expression compared to the Kan^{R} vector, suggesting that this was a *Salmonella*-related phenomenon (Figure 8D). Differences in σ^{70}-regulated promoter expression or polymerase transcription in *E. coli* and
Salmonella could explain the differences observed. Overall, these results suggest that quantitative comparisons of luciferase expression between Kan\textsuperscript{R} and Cam\textsuperscript{R} reporters is not possible at this time until these differences are characterized in greater depth.

Using the Tn7 transposition system and by introducing slight modifications to the promoter regions we were able to generate a suite of reporters with three levels of luciferase expression. Most importantly, we were able to generate this suite of reporter expression by altering only one or two nucleotides in the -10 or -35 regions of the sig70-16 promoter, bringing either of these regions to the $\sigma_{70}$ consensus sequence (Figure 8A). These small nucleotide changes resulted in expression that increased by up to 10 times \textit{in vitro} and almost 100 times \textit{in vivo}. The expression dynamics of the luciferase operon downstream of these promoters remained the same, demonstrating that only the magnitude of expression was altered (Figures 8C and 9A/C). The increased expression from these promoters resulted in somewhat better detection of luciferase \textit{in vivo} in mice pre-treated with streptomycin, as well as in the organs of these mice after euthanization (Figure 10). Unfortunately, the increased magnitude of luciferase expression did not increase the detection of these reporters beyond one-day post-challenge and prior to the onset of clinical signs. Our original intention with these reporter strains was to design a model of transmission that would use the reporters to track the spread of Salmonella in our mouse population. Ultimately, we hoped that we would be able to detect our Salmonella reporters using a BLI prior to the onset of clinical signs in our mice and better gauge the time of transmission; in practice our reporters were not detectable with enough accuracy and precision in most cases, regardless of the magnitude of expression. This could have been due to intrinsic factors like the absorption of bioluminescence by animal tissues or other factors affecting the bioluminescence of our reporters. The emission of bioluminescence is optimal in an aerobic environment, and the intestinal tract is largely anaerobic\textsuperscript{50}; this could explain why the reporters were detected early on after infection, when they had been recently exposed to an aerobic environment. Once Salmonella had reached levels high enough to cause severe clinical signs in an animal, the bacterial burden could have been high enough to overcome an emission threshold of detection. It has also been demonstrated that certain in mammalian tissues, hemoglobin absorbs a significant amount of bioluminescence at wavelengths shorter than 600 nm\textsuperscript{23,149}. The bacterial luciferase we utilized in our reporter strains produced a primary luminescence peak at 490 nm as well as a secondary peak at 590 nm, which could also help explain why their detection was limited \textit{in vivo}.
until a certain threshold was reached. Finally, the spatial position of each organ in the murine body as well as the position of a mouse on the imaging platform affects the ability to detect luciferase in a mouse using a BLI. It remains a possibility that slight differences in the organ depths of individual mice could result in non-uniform detection efficiencies of our reporter strains in vivo.

The differentially marked KanR and CamR reporter strains had similar virulence levels when tested in C57BL/6 mice (Figure 14). A large difference in the virulence of the two strains would have appeared as a large difference in the number of mice colonized by each strain, and CI values significantly different than 1. Each strain was represented at high levels in a similar number of mice (5 KanR vs. 7 CamR; Figures 14A – D), and the competitive index values we calculated were not significantly different than 1 in any of the organs we sampled (Figure 14E). It also appeared that each mouse had a dominant strain that was present in each of its organs, suggesting that the outcome of the competition between the strains was decided early on in the infection and that this strain colonized each organ at a higher level.

When mice were challenged with our reporter strains without streptomycin treatment, we saw very little shedding from these animals (Figure 16). No Salmonella was detected for the first four days of the experiment, and on day 5 only low levels were seen. This was similar to the results seen in previous studies, where C57BL/6 mice challenged with $1 \times 10^8$ CFU of Salmonella Typhimurium SL1344 had very low shedding levels on days 1 and 2 post-challenge. Salmonella Typhimurium infection in untreated mice typically results in a systemic infection with few of the hallmarks of human gastroenteritis, and a lasting, chronic infection in resistant (Slc11a1+) mice. Susceptible (Slc11a1-) mice, such as C57BL/6 mice, typically experience an acute, systemic infection. The absence of fecal shedding until the later stages of infection was therefore not surprising. Streptomycin treatment of mice results in pathology that more closely resembles gastroenteritis, with intestinal inflammation and high levels of shedding. The subsequent treatment of mice with streptomycin in our model followed the paradigms that had been established previously. C57BL/6 mice that had been pre-treated with a single 20 mg oral dose of streptomycin shed Salmonella in the range of $1 \times 10^7 – 1 \times 10^{10}$ CFU/fecal pellet (Figure 18) as reported in the literature. Histology of the mice used in our experiments was not performed, but macroscopic analysis of the ceca confirmed earlier reports that the cecum in mice treated with streptomycin became “shriveled to a small size, pale and filled with purulent exudate”. We also
observed that the initial inoculum dose did not impact the final shedding levels and colonization levels (Figure 20), as previously reported\textsuperscript{82}. That the challenge dose did not impact the colonization and life expectancy of the mice was not surprising; streptomycin treatment has been shown to increase the colonization potential of *Salmonella* and significantly reduce the oral 50\% infectious dose in mice\textsuperscript{7}. This treatment is thought to result in a transient disruption of the colonization resistance of mice, resulting in fast and efficient colonization by *Salmonella*\textsuperscript{7,53}. Such a large perturbation in the microbiota of these mice would allow for a significantly smaller *Salmonella* challenge to colonize to similar levels as a larger challenge.

Mice challenged with either WT or ΔcsgD *Salmonella* Typhimurium were colonized by and shed bacteria at the same levels (Figure 20). This suggested that the ΔcsgD mutation was not impacting the ability of *Salmonella* to colonize and be shed from a host, at least when challenged in isolation. CsgD has been implicated in many different biofilm-related processes, as it is a transcription regulator regulating many of the pathways involved in the production of the components of the biofilm matrix\textsuperscript{54}. One limitation of this experiment was that each infection was done in isolation: a mouse was challenged with either the WT or the ΔcsgD mutant. Recent work in our lab has shown that when WT and ΔcsgD *Salmonella* Typhimurium are given as competitive infections in C57BL/6 mice, the WT bacteria displayed a significant virulence advantage\textsuperscript{98}. The ΔcsgD mutant was also demonstrated to have depressed expression levels of different SPI-I genes, such as the effectors *sipD* and *spoE2*, when compared to WT planktonic cells. This suggests that CsgD may play an as of yet unknown regulatory role in *Salmonella* virulence, potentially through regulation of SPI-I-associated virulence, and that any future work comparing the transmission of WT and ΔcsgD *Salmonella* Typhimurium would need to be done using each strain in isolation.

Biofilm formation and cellular aggregation have been associated with environmental survival and persistence in previous studies due to the increased survival in many environmental conditions they bring. We tested the ability of WT and ΔcsgD *Salmonella* Typhimurium (strains that both aggregated and did not, respectively) to survive in the environment after being shed from a host (Figure 24). We observed that WT *Salmonella* had a significant advantage over the ΔcsgD mutant after one week of desiccation and storage, while the difference in recovery was lost after this period of time. After one week there was a significant drop in the recovery of both cell types, but the WT cells were recovered at significantly higher levels than the ΔcsgD mutant.
After week 3, the recovery of both cell types became quite sporadic, resulting in some pellets with high CFU counts and some pellets with extremely low CFU counts. The $\Delta csgD$ mutant in particular had a significant increase in the levels recovered after week 2. It is possible that this was due to the large variability in the recovery levels of bacteria from fecal pellets. The range of cell counts we observed in fecal pellets was typically anywhere from $1 \times 10^7$ CFU/g to $1 \times 10^{11}$ CFU/g (Figure 19). If a pellet was collected and stored and contained bacteria at the high end of the range it would be expected to result in the recovery of more bacteria at week 4 than pellets that contained bacteria at the low end of the range. The large variability in the levels of *Salmonella* in each individual fecal pellet could explain the variability in the recovery of bacteria after a period of 6 weeks.

We observed that a period of $\leq$ one day was sufficient to reduce the recovery of *Salmonella* in feces (Figures 23 and 24). Though cages were changed each day and all fecal samples collected and processed that day, the period between shedding and processed resulted in a significant decrease in the levels of *Salmonella* recovered from these fecal pellets. We had observed previously that a period of one week was sufficient to result in a drop in the recovery of *Salmonella* (Figure 22), but did not expect the same drop in recovery after less than one day. Clearly the short period of time after exiting a murine host exerts strong environmental pressures on *Salmonella*, resulting in the drop in recovery we observed.

We determined that the transmission of *Salmonella* Typhimurium from infected to naive C57BL/6 mice was possible if the naive mice were pre-treated with streptomycin (Figure 26). Transmission through this method was highly efficient, with all of the naive mice becoming infected and colonized with *Salmonella*. This had previously been demonstrated in 129X1/SvJ mice in a chronic *Salmonella* infection model, but to our knowledge this was the first acute infection model of *Salmonella* transmission. According to this model, the majority of the mice in our model would be termed moderate shedders or supershedders, shedding between $1 \times 10^7 - 1 \times 10^{11}$ CFU/g feces. Pre-treating the mice in our model with streptomycin essentially converted all of the mice to supershedders, ensuring highly efficient transmission. Mice that were fasted for 20 hours and mice that were not manipulated had undetectable levels of *Salmonella* in their organs, suggesting that transmission did not occur. In our transmission experiments we monitored the spread of *Salmonella* through CFU counts quantifying the colonization of the spleen, liver, cecum and MLNs of naive mice, as well as observing their clinical signs. It is
possible that this was not sensitive enough to detect transmission in some mice, or that a period of 14 days was not sufficient for transmission to occur in all scenarios. Other groups have looked for the presence of *Salmonella*-specific intestinal IgA and IgG\textsuperscript{94}, TNF-\(\alpha\) and IL-1\(\beta\) in mice colonized by *P. aeruginosa*\textsuperscript{83} and other immunological markers as indicators of transmission, and it is possible that using criteria such as these would result in a more sensitive detection of transmission. Our original intent was to monitor the transmission of *Salmonella* using the reporter strains we had generated. As *Salmonella* spread to mice, we had hoped to observe luminescence in naive mice before the onset of clinical signs, giving us a better view of transmission. Our reporters were not sensitive enough to facilitate these types of studies, which is why we used clinical signs and colonization levels as indicators of transmission. It may be useful in the future to confirm that transmission in this model occurs through the fecal-oral route. An experiment where the mice are separated from fecal pellets as they are shed (i.e. with raised mesh flooring) would be simple to perform; if transmission rates dropped, it would suggest that access to fecal pellets is required for optimal transmission, confirming the fecal-oral route of infection.

Using our model of transmission we modeled both short-term and long-term transmission scenarios. Upon testing the short-term transmission potential of WT and \(\Delta csgD\) *Salmonella* Typhimurium we observed no difference in their abilities to transmit to naive mice (Figure 30). All of the naive mice were colonized and infected by *Salmonella*, regardless of the strain, and at identical rates. Thinking that a difference in transmission would only become apparent in a prolonged scenario, we tested the long-term transmission potential of both WT and \(\Delta csgD\) *Salmonella* Typhimurium as well (Figure 32). Again, we saw no difference in the ability of these strains to infect naive mice, suggesting that CsgD was not vital in the transmission process as it existed in our model. Recent work has demonstrated that a population of *Salmonella* Typhimurium splits into both planktonic cells and multicellular aggregates and that this process involves the bistable expression of *csgD*\textsuperscript{98}. WT *Salmonella* Typhimurium differentiates into two distinct phenotypes; one phenotype (planktonic cells) primed for host invasion and the other (multicellular aggregates) primed for environmental persistence. The *csgD* mutant, not demonstrating the aggregative phenotype, demonstrated a phenotype resembling the WT planktonic cells. As both populations would be primed for host invasion and virulence, it may not be surprising that we did not observe a difference in short-term transmission. We also did not
observe a large difference in the recovery of WT *Salmonella* and the *csgD* mutant from fecal pellets after a period of 5 weeks (Figure 22), suggesting the lack of difference between the two strains in long-term transmission was also not surprising. However, the number of mice used in these transmission experiments was not large, meaning that it is difficult to draw firm conclusions. Subsequent experiments should be performed with larger groups of mice so that stronger conclusions could be drawn; our initial plans for this transmission model involved a total number of 50 mice per experiment in order to be able to discern significance from the data. Some compromise between the ease of performing such transmission experiments and the power of the results would need to be found. In the long-term transmission experiment we utilized 10 mice split into 2 groups. In previous trials we had used upwards of 20 mice in order to strengthen our results and ensure significance could be found. Depending on the number of different exposures (i.e. whether each cage receives the same treatment or not) it would be beneficial to have at least 3 cages of 5 mice per treatment in the future; a modified long-term transmission experiment would therefore include at least 15 mice. Performing experiments such as these would strengthen the results obtained from our model and help to solidify the conclusions that we could draw.

### 5.2 Future work

Other uses for our model of transmission remain. The gene *shdA*, encoding a fibronectin- and collagen-binding effector, has been implicated in regulating the shedding of *Salmonella* from warm-blooded hosts and is another potential target related to transmission.  

Δ*shdA* mutants experienced significantly reduced quantity and duration of shedding from murine hosts in a typhoid model of infection, while having no impact on the levels of colonization. It was also found to be absent in *S. enterica* subspecies other than subspecies *enterica*, who typically have an expanded host range. Expression of *shdA* was only detected *in vivo*, and not when *Salmonella* were grown in liquid culture. This suggests that it plays a role in the host-to-host transmission of *Salmonella* in serovars that are considered host-generalists. MisL is another fibronectin-binding protein that has been observed to play a role in the shedding of *Salmonella*. Similar to *shdA*, Δ*misL* mutants were shed from mice at reduced rates and the expression of *misL* was not detected when grown in standard laboratory broth. That both ShdA and MisL are implicated in the binding of extracellular components like fibronectin and collagen suggests that
their role in shedding may involve adherence to the intestinal wall of a host, ensuring that *Salmonella* are able to effectively persist in the intestinal tract \(^{106}\). Their apparent importance in the shedding of *Salmonella* suggests that they may play a role in the transmission process as well, meaning they are likely candidates for testing in our model. \(\Delta shdA\) and \(\Delta misL\) mutants could be tested against the wild-type in our transmission model, and the impact of these mutations on transmission could be observed.

Now that our transmission model is established, it is also possible to also begin testing other conditions under which transmission may occur, and whether or not it would be possible to view the differences between strains. Using the model we attempted to mimic both short- and long-term transmission scenarios. In our vision of long-term transmission we tested the effects of a prolonged period of desiccation on the ability of both WT and an isogenic \(\Delta csgD\) strain of *Salmonella* to transmit to naive mice. In reality, other environmental stresses and conditions would be present and exerting survival pressures on the bacteria. As mentioned in in Section 1.2.3, *Salmonella* biofilms have been shown to provide increased resistance to other non-host conditions as well. An immediate application for our model as it exists would be to adapt the long-term transmission experiment for alternate environmental stresses. Fluctuations in pH, temperature and nutrient availability could all be introduced to mimic an environmental situation where the resistance properties of biofilms could be beneficial. It is possible that the increased persistence afforded by a biofilm could lead to more efficient transmission after encountering adverse environmental conditions such as these. Alternatively, it would also be possible to examine alternate methods of transmission. As demonstrated in Section 1.1.2, water sources that have become contaminated with *Salmonella*, often from fecal shedding, are an important environmental reservoir of these bacteria. It is possible that the introduction of an environmental stage involving a period of survival in water would lead to differences in the ability of *Salmonella* to transmit. Instead of delivering *Salmonella* as an oral challenge or through contaminated fecal pellets we could inoculate the water supply of a cage of mice with a specified quantity of both WT and \(\Delta csgD\) *Salmonella*. It would also be possible to “contaminate” the water supplies using fecal pellets that we had collected previously. Other experiments could also involve altering the types of *Salmonella* delivered to the mice. Competing differentiated WT *Salmonella* populations (ie. planktonic and aggregative) rather than different mutant strains could be performed by contaminating fecal pellets with either planktonic or aggregated population of
Salmonella and left desiccated for a period of time. The appearance of a transmission difference in these subpopulations would suggest a role for the aggregative phenotype and biofilm formation in persistence and survival through differences in a single population of bacteria. Situations like these would adapt our transmission model to mimic other potential transmission scenarios.

In our model, we typically observed all-or-nothing transmission efficiency; in all cases, either all of the mice in a group became infected with Salmonella or no mice did. One of the concerns we had in using streptomycin treatment was that it may lower the barrier of entry for a pathogen to such a degree that even avirulent pathogens could successfully infect a mouse due its lack of colonization resistance. It has been demonstrated that intestinal inflammation is sufficient to enhance the colonization of the murine gut by avirulent Salmonella strains (such as strains lacking the two virulence-associated T3SS’s) that would be unable to colonize a typical mouse intestine\textsuperscript{136}; it has also been shown that treatment with streptomycin is sufficient to cause inflammation in the murine gut\textsuperscript{134}. It is possible that this was sufficient to reduce the impact of the $\Delta$cs$\text{gD}$ mutation on transmission. Other methods that disrupt the microbiota in a less drastic manner could potentially bring the benefits of efficient Salmonella colonization while retaining some measure of colonization resistance to prevent severely attenuated pathogens from colonizing the intestine. One method that could accomplish this is the use of mice with a low-complexity microbiota (LCM), which consists of an altered microbiota of 8 strains typically found in the rodent intestine (i.e. Bacteroides spp., Clostridium spp., Lactobacilli etc.)\textsuperscript{135}. It has been shown that LCM mice are colonized by Salmonella Typhimurium at similar levels to conventional mice (mice with a conventional microbiota) that have been pre-treated with streptomycin\textsuperscript{135}. An avirulent mutant was able to colonize the intestine of LCM mice, but was unable to cause intestinal inflammation and disease. It is possible that by using other techniques which aim to reduce the colonization resistance of mice in less drastic methods it would be possible to increase the sensitivity of our model, and be able to observe degrees of transmission efficiency rather than an all-or-nothing response. Alternatively, it is also possible that the large quantities of streptomycin given to the mice in each trial to ensure efficient shedding persisted at high levels for the duration of the experiment and impacted the growth and survival of our reporter strains. Viable bacteria did, however, continue to be shed from the mice throughout the course of our experiments, suggesting that this would not likely be a significant problem. Further
developing alternative methods that do not utilize streptomycin would help to allay any concerns regarding the survival of our reporter strains in a mouse that had been pre-treated with streptomycin.

### 5.3 Conclusions

The establishment of our transmission model means that subsequent studies examining the role of specific genetic factors in the transmission process can be performed. It is possible to test other individual genes, other transmission methods as well as adapting the model to support high throughput methods such as Tn-seq, allowing large portions of the *Salmonella* genome to be tested in transmission at once. Alternatively, utilizing mice from different genetic backgrounds could test the role of host factors and the immune response in transmission. Using a modified Tn7 transposition system we were able to generate a modular system allowing for the generation of a variety of *Salmonella* reporter strains. We confirmed previous reports that pre-treating mice with streptomycin prior to challenge with *Salmonella* increases the levels of bacterial shedding, and we characterized the shedding characteristics of mice challenged with our reporter strains. Using mice pre-treated with streptomycin, we also demonstrated that highly efficient transmission could occur from seeder to naive mice. Finally, we demonstrated that *Salmonella Typhimurium ΔcsgD* mutants were not deficient in short- or long-term transmission in our model. The studies performed here helped lay the foundations for subsequent transmission experiments examining the role of genetic elements in the *Salmonella* genome.
6.0 REFERENCES


125. **Sarnovsky RJ, May EW, Craig NL** (1996). The Tn7 transposase is a heteromeric complex in which DNA breakage and joining activities are distributed between different gene products. *The EMBO Journal*; 15(22): 6348-6361.


APPENDIX A

PRIMERS/OLIGONUCLEOTIDES USED IN THIS STUDY

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Nucleotide sequence (5’ → 3’)</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>glmSdetectFOR</td>
<td>AACCACCCGTTCAGGCTGGCTA</td>
<td>Confirmation of successful chromosomal insertion</td>
</tr>
<tr>
<td>glmSdetectREV</td>
<td>ACGTTGACCAGCCGCCTGAAC</td>
<td></td>
</tr>
<tr>
<td>pCS26_Pac_FOR</td>
<td>GATCTGCGATTCTGATAAC</td>
<td>PCR amplify the luciferase construct from pCS26</td>
</tr>
<tr>
<td>pCS26_Pac_REV</td>
<td>GGGCTAGTCAATGATAATTAC</td>
<td></td>
</tr>
<tr>
<td>pZE05</td>
<td>CCAGCTGGCAATTCCGA</td>
<td>PCR amplify promoter region of pCS26</td>
</tr>
<tr>
<td>pZE06</td>
<td>AATCATCACTTTTCGGAGA</td>
<td></td>
</tr>
<tr>
<td>Sac_Pac_Kpn1</td>
<td>CTAAATCAAAGGTAC</td>
<td>Generate PacI polylinker for pUC18R6K mini-Tn7</td>
</tr>
<tr>
<td>Sac_Pac_Kpn2</td>
<td>CTAAATCAAAGGTAGCT</td>
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</tr>
<tr>
<td>sig70-16-10c2F</td>
<td>TCGAGAATAATTCTTTACATTTATGCTT CCGGCTCGTATAATACGTGCAATTG</td>
<td>Generate phosphorylated sig70c10 promoter</td>
</tr>
<tr>
<td>sig70-16-10c2R</td>
<td>GATCCAAATTGCGACGTATTATACGAGCC GGAAGCATAAAATGTAAGAATTATTC</td>
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<tr>
<td>sig70-16-35c2F</td>
<td>TCGAGAATAATCTTTAGAACATTTTATGCTT TCCGGCTCGTATCTTACGTGCAATTG</td>
<td>Generate phosphorylated sig70c35 promoter</td>
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<tr>
<td>sig70-16-35c2R</td>
<td>GATCCAAATTGCGACGTAGAATACGAGGCC GGAAGCATAAAATGTAAGAATTATTC</td>
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<tr>
<td>TetExtractFor2</td>
<td>GATCGAATTTCTCATGTTGGACA</td>
<td>PCR amplify TetR gene from pACYC184</td>
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<tr>
<td>TetExtractRev2</td>
<td>GATCCCTGCAGGAGGTTGGTTTGT</td>
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Table 4: Primer/oligonucleotide used in this study. A list of all the primers and oligonucleotides used over the course of this study, with sequence information and a description of their purpose. Important restriction sites are underlined.