Investigation of Non-typhoidal *Salmonella* pathogenesis, Biofilm and Vaccine development

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AKOSIEREREM SENIBO SOKARIBO

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Canada

OR

Dean
College of Graduate and Postdoctoral Studies
University of Saskatchewan
116 Thorvaldson Building, 110 Science Place
Saskatoon, Saskatchewan S7N 5C9
Canada

Abstract

Salmonella are a diverse group of pathogenic bacteria that remains a serious public health concern worldwide. Understanding the mechanism of pathogenesis and transmission are important for the development of effective vaccines, and strategies to mitigate *Salmonella* infections. In this thesis I investigate different aspects of *Salmonella* biology.

Non-typhoidal *Salmonella* (NTS) associated with gastroenteritis worldwide are the leading cause of bloodstream infections in sub-Saharan Africa. The invasive NTS (iNTS) differ from gastroenteritis causing isolates by more than 700 single nucleotide polymorphisms (SNP). I identified a conserved SNP in invasive *S.* Typhimurium D23580 that results in a missense mutation in the sensory domain of a diguanylate cyclase enzyme, STM1987. STM1987 catalyzes the formation of c-di-GMP, which positively regulates cellulose production. Previous studies have shown that *Salmonella* produces cellulose inside macrophages as an antivirulence factor. The mutation in STM1987 results in a 10-fold drop in cellulose production, and increased survival inside human and murine macrophage cell lines. Using competitive index experiments, I showed that compared to wildtype, *S.* Typhimurium with SNP in *stm1987* have increased virulence in mice. My results showed that STM1987 plays a role in *Salmonella* virulence during infection. Due to the high mortality rate associated with infections a vaccine is urgently needed to reduce incidence of iNTS.

With the rise in antibiotic resistant isolates, there is a growing need for an effective vaccine to reduce the prevalence of diseases caused by NTS and iNTS. Current vaccine development strategies are focued on extracellular polysaccharides (EPS) present on bacterial surface. My first objective was to boost the biosynthesis of EPS O-Antigen capsule and purify large quantities for immunization trials in mice. Using random mutagenesis, colanic acid production was increased instead of O-Antigen capsule. Immunization with colanic acid alone or colanic acid conjugated to carrier proteins did not induce a protective response in mice against *Salmonella*. However, generalized modules for membrane antigens (GMMAs) purified from colanic acid overproducing strains induced a partially protective response against a lethal *Salmonella* challenge in mice. My work shows that GMMAs can be developed as potential vaccine candidates against NTS and

iNTS infections. In addition to vaccine development, strategies to reduce *Salmonella* transmission will also reduce the global prevalence of NTS and iNTS.

Biofilm formation is important for the long-term survival of *Salmonella* in the environment. Similar to sporulation, biofilm formation is induced as a survival strategy under conditions of starvation, however it has not been determined if biofilm formation is also a committed (point of no return) process like sporulation. *Salmonella* biofilm formation is subject to tight and complex regulation through transcription factor CsgD. Using luciferase reporter assays, I examined the regulation of *csgD* expression in response to different environmental signals, introduced before and after *csgD* expression has been induced. In the presence of high osmolarity *csgD* expression is repressed, however when introduced after induction, increased osmolarity has no effect on *csgD* expression. In contrast, the introduction of glucose and elevated temperatures represses *csgD* expression before and after induction. My results showed the existence of a regulatory hierarchy among signals that regulate biofilm formation in *Salmonella*. I conclude that in the presence of changing osmolarity biofilm formation is a committed process similar to sporulation, however under certain conditions such as increased nutrient availability and elevated temperature, biofilm formation is a reversible process.

When *S.* Typhimurium is grown under biofilm inducing conditions, the bistable synthesis of CsgD results in the formation of two distinct cell types: multicellular aggregates and planktonic cells. Transcriptome comparison showed that multicellular aggregates had higher expression of genes associated with environmental persistence, while planktonic cells had higher expression of genes involved in virulence. About 798 function unknown (FUN) genes are differentially expressed between multicellular aggregates and planktonic cells. I hypothesized that a proportion of the identified FUN genes are involved in *Salmonella* environmental persistence and/or virulence. I mapped FUN genes operons and analyzed 23 operons with potential roles in *Salmonella* virulence and or persistence. Using these FUN operons, I highlight the difficulties associated with the identification of the roles of FUN genes and propose a framework that can aid in this process.

My research provides new insights into NTS pathogenesis, transmission, and vaccines development. Understanding different aspects of *Salmonella* biology is essential for the better management of diseases caused by this pathogen.

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Dedication

I dedicate this thesis to my parents Sokaribo and Ibiada
Thank you for always believing in me, encouraging me, and giving me everything I need to achieve my dreams.

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List of Abbreviations

ATR	Acid tolerance response
c-di-GMP	Bis-(3’ – 5’)-cyclic dimeric guanosine monophosphate
CI	Competitive index
CFU	Colony forming unit
DGC	Diguanylate cyclase
EPS	Extracellular polysaccharide
FUN	Function unknown
GMMA	Generalized modules for membrane antigens
GO	Gene ontology
H-NS	Histone-like nucleoid structuring
IHF	Integration host factor
IL	Interleukin
iNTS	Invasive nontyphoidal *Salmonella*
KEGG	Kyoto Encyclopedia of Genes and Genomes
LPS	Lipopolysaccharide
M	Microfold
MOI	Multiplicity of infection
NTS	Nontyphoidal *Salmonella*
NK	Natural killer
OMV	Outer membrane vesicles
PAMP	Pathogen associated molecular pathogen
PBS	Phosphate buffered saline
PDE	Phosphodiesterase
PMV	Polymorphonuclear lymphocytes
Rdar	Red, dry, and rough
RNS	Reactive nitrogen species
ROS	Reactive oxygen species

SCV	*Salmonella* containing vacuole
SNP	Single nucleotide polymerase
SPI	*Salmonella* pathogenicity Island
STM14	*Salmonella* typhimurium 14028
T3SS	Type three secretion system
TN	Transposon
TS	Typhoidal *Salmonella*

1.0 LITERATURE REVIEW

1.1 *Salmonella*

Salmonella species are one of the leading causes of foodborne diseases, with significant number of morbidity and mortality worldwide [1]. It was discovered in 1885 by an America pathologist Dr. Daniel Elmer Salmon and his assistant Theobald Smith, who isolated *Salmonella* from the intestine of pigs infected with classical swine fever [2]. *Salmonella* is a Gram-negative motile facultative anaerobe, that belongs to the family Enterobacteriaceae. *Salmonella* is a major public health concern worldwide due to the large medical and economic burden associated with infections.

1.1.1 *Salmonella* Classification

Salmonella is a highly diverse bacteria that is divided into species, subspecies and serovars, using a combination of genomic relatedness, serology, geographical origin, and disease syndrome. *Salmonella* is divided into two species: *bongori* and *enterica*, based on genomic relatedness determined using DNA-DNA hybridization [3]. Using biochemical analysis, genomic relatedness, and geographical origin, *S.* enterica is further divided into six subspecies: *enterica* (I), *salamae* (II), *arizonae* (IIIa), *diarizonae* (IIIb), *houtenae* (IV) and *indica* (VI) [3]. *Salmonella* species and subspecies are further divided into serovars, using the White-Kauffmann-Le minor scheme which is based on serology to three surface antigens: flagellar (H), capsular polysaccharide (K) and the oligosaccharide portion of lipopolysaccharide (O). Over 2600 *Salmonella* serovars have been identified (Fig 1.1) [4]. About 60% of the identified serovars belong to *S. enterica* subspecies *enterica* mainly found in warm blooded animals. Serovars in *S. bongori* and other *S. enterica* subspecies are found in cold blooded animals [3]. *S. enterica* subspecies *enterica* (*S. enterica*) causes 99% of human infections and based on disease syndrome is divided into two groups: Typhoidal *Salmonella* (TS) and Non-typhoidal *Salmonella* (NTS) [5]. In general, NTS are ubiquitous and cause disease in a wide range of host, while TS are usually host restricted [5].

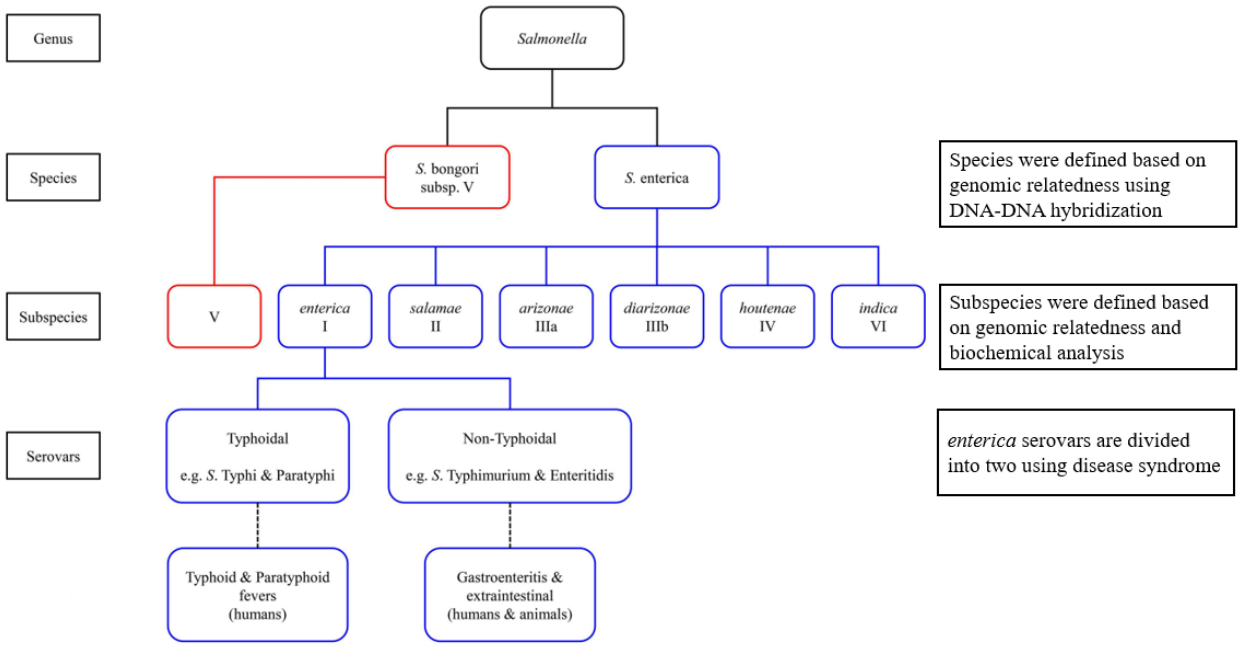

Figure 1.1: *Salmonella* classification. Using genomic relatedness *Salmonella* is divided into two species: *enterica* and *bongori*. *S. enterica* is further divided into six subspecies based on genomic relatedness and biochemical analysis. *S. enterica* subspecies *enterica* serovars is divided into typhoidal and Non-typhoidal *Salmonella* using disease syndrome. Figure adopted with some modification from Hurley et al., [6].

1.1.2 Clinical Manifestation

Salmonella enterica serovars cause three main types of diseases: enteric fever, gastroenteritis, and invasive bloodstream infections [1]. Enteric fever is a life-threatening disease cause by TS serovars: Typhi and Paratyphi. After ingestion TS serovars have an asymptomatic period of 7 to 14 days and once developed symptoms can persist for up to 3 weeks. The predominant symptom is fever due a gradual rise in temperature up to 40°C. Other reported symptoms include, chills, abdominal pain, hepatosplenomegaly, rash (rose spots), nausea, anorexia, diarrhea or constipation, headache, and dry cough [5]. Enteric fever can be treated with antibiotic, however, isolates resistant to chloramphenicol, ampicillin, trimethoprim-sulfamethoxazole, ciprofloxacin, or azithromycin have been reported in multiple countries [7].

Gastroenteritis is predominately caused by NTS serovars: Typhimurium and Enteriditis. Symptoms appear 6 – 12 hours after ingestion and persist for less than 10 days. The most common initial symptoms reported are nausea, vomiting and watery diarrhea. Other symptoms include fever, chills, abdominal pain, and headaches [5]. NTS infection is usually limited to the gastrointestinal tract, however, about 5% of patients develop secondary bacteremia when

bacteria disseminate to other parts of the body [5]. NTS gastroenteritis is usually self-limiting in immunocompetent individuals, and severe cases can be treated with antibiotic. However, antibiotic treatment is often discouraged because it increases the risk of NTS persisting in the gastrointestinal tract and the spread of multidrug resistant isolates [8,9].

In sub-Saharan Africa, NTS serovars associated with gastroenteritis are the leading cause of invasive bloodstream infections. These isolates are referred to as invasive Non-typhoidal *Salmonella* (iNTS) and have a fatality rate of 20 – 25%. iNTS disease is prominent in HIV infected adults and in children with HIV infection, malnutrition, or malaria [10]. The clinical presentation of iNTS disease are diverse and nonspecific: fever, pneumonia, diarrhea, anaemia, splenomegaly, and hepatomegaly [10]. Due to the clinical similarities between iNTS disease, pneumonia, and malaria, iNTS infection is often diagnosed with microbiological test. Antibiotics can be used to treat iNTS infection, however multidrug resistant isolates have been reported [11,12]. In addition, recurrent iNTS disease in HIV infected individual declines with the start of antiretroviral therapy [13].

1.1.3 Epidemiology and Transmission

S. enterica serovars are important foodborne pathogens that cause significant number of morbidity and mortality worldwide [1]. Annually, TS serovars cause an estimated 22 million cases of enteric fever with 200, 000 deaths globally [14]. The incidence and mortality rate associated with enteric fever vary from country to country. In high income countries, the number of TS infections are low with an annual incidence rate of <10 per 100, 000 people. In contrast, TS infections are endemic in low income countries with a high incidence rate of >100 per 100, 000 people annually. In low income countries, the lack of clean water and adequate sanitation facilitates the spread of TS through the fecal oral route [14]. While in high income countries, TS infections are mainly associated with travel to endemic regions, or consumption of food contaminated by chronic carriers. About 1 to 4% of individuals infected with TS become asymptomatic chronic carriers and shed bacteria for years [5]. The role of chronic carriers in the transmission of TS is demonstrated by Mary Mallon (Typhoid Mary) and Mr. N. Mary Mallon was an Irish cook who infected between 26 – 54 people through her food. She was tracked down by sanitary engineer George Soper and was ordered to stop cooking by public health authorities. Due to her lack of compliance, and to protect the public she was forced to live in isolation for 23

years [15]. Mr. N, a milker was responsible for about 207 causes of TS infections between 1896 – 1909. He was detected by R W Johnstone, who concluded that for 14 years enteric fever outbreak in Folkestone Urban district could be connected to milk infected by Mr. N [16]. Chronic carriage is associated with biofilm formation by TS in the gallbladder. It is difficult to treat with antibiotics, hence surgical procedures to remove the gallbladder is the only effective treatment currently available for chronic carriers [17].

The most common *Salmonella* infection is gastroenteritis with an estimated 94 million cases with 155, 000 deaths annually [18]. About 80.3 million cases of NTS gastroenteritis are acquired through the consumption of contaminated foods [18]. The predominant NTS serovars associated with gastroenteritis are *S.* Typhimurium and *S.* Enteriditis. Endemic worldwide NTS serovars are primarily transmitted through the consumption of contaminated food produces such as fruits, vegetables, or process foods, and animal produce such as poultry, dairy and eggs [19–21].

In 2017, an estimated 535, 000 cases of iNTS infection was recorded with 77, 500 deaths [22,23]. About 80% of reported iNTS infections occurred in sub-Saharan Africa, with majority of the cases associated with HIV-infected adults and children <5 years old [23]. The transmission of iNTS serovars is not well understood. However, the isolation of invasive *S.* Typhimurium from the household members of index cases, but not from livestock living in the same household nor from their drinking water, supports an anthroponotic mode of transmission [24].

1.1.4 Pathogenesis

Salmonella pathogenesis is mainly attributed to the function of two type three secretion systems (T3SS-1 and T3SS-2) encoded on *Salmonella* pathogenicity Islands 1 and 2 (SPI-1 and SPI-2) [25]. The T3SSs are needle-like complexes used by bacteria to deliver effectors directly into the cytoplasm of host cells. SPI-1 present in all serovars of *S. bongori* and *S. enterica,* encode the T3SS-1 genes important for *Salmonella* invasion of intestinal epithelial cells. While SPI-2 present exclusively in *S. enterica* serovars, encode T3SS-2 genes important for intracellular survival inside host cells [25,26].

Salmonella infections begins with the ingestion of the organism in contaminated food or water. To survive the acidic pH of the stomach, *Salmonella* induces the acid tolerance response (ATR)

using about fifty heat shock proteins, which promotes survival, by inducing a pH homeostasis that maintains the bacteria internal pH at values higher that the extracellular environment, [27].

After surviving the acidic condition of the stomach, *Salmonella* travels to the small intestine where it uses flagella motility and chemotaxis to identify cells (at the apical surface of intestinal epithelia) that are permissive for invasion [28]. *Salmonella* preferentially penetrates the intestinal epithelial through microfold (M) cells. However, it can also penetrate via dendritic cells, intestinal epithelia cells and through breaches in the epithelia lining [14]. *Salmonella* initiates contact with permissive cells using adhesive proteins such as fimbriae. This contact is further stabilized by SPI-1 T3SS effectors which also induce the formation of membrane ruffles at the site of attachment and the internalization of *Salmonella* through micropinocytosis [26].

Once engulfed *Salmonella* is internalized into large vesicles called *Salmonella*-containing vacuoles (SCV). SCV is the only intracellular compartment in which *Salmonella* survives and replicate, in eukaryotic cells [26]. Inside SCV, effectors secreted by SPI-2 T3SS prevents the fusion of SCV with lysosome, promote SCV maturation, and manipulate the host cell environment to promote *Salmonella* survival inside SCV. After traversing the epithelia cells, *Salmonella* are taken up by phagocytic cells at the submucosa [14].

Unlike TS serovars which disseminate to systemic site through phagocytic cells, NTS infection is restricted to the gut [5,29]. During invasion and intracellular survival, effectors secreted by NTS induce an inflammatory response which leads to the recruitment of polymorphonuclear lymphocytes (PMNs) and phagocytic cells, that are effective in eliminating NTS using mechanisms such as: activating infected macrophages, production of reactive oxygen and nitrogen species (ROS and RNS), and stimulating epithelia cells to release antimicrobial peptides [14]. TS are distinct from NTS serovars in that they transverse the intestinal epithelia without inducing inflammatoion. TS taken up by phagocytic cells at the submucosa are carried to other sites in the body such as the liver, spleen, MLN, and gall bladder, where they can establish chronic infection [29].

1.1.5 Factors that mediate the difference between TS and NTS pathogenesis

TS serovars are human restricted pathogens that cause enteric fever, while NTS serovars are host generalist pathogens that cause self-limiting gastroenteritis in immunocompetent individuals [5].

The main difference between enteric fever and gastroenteritis is the inflammatory response induced by NTS serovars. TS and NTS share about 90% of their genes, conserved genes are likely the core genome of *Salmonella* important for proliferation, survival, infection, and transmission. Almost 500 genes are unique to NTS while over 600 genes are unique to TS [29]. The presence of unique genes such as *viaB* locus that encodes the Vi antigen, has be attributed to the lack of an inflammatory response during TS infection. Approximately 5% and 1% of TS and NTS genes are pseudogenes, respectively [29]. The high number of pseudogenes in TS, has also be correlated to the diffecence between TS and NTS pathogenesis [29].

The lack of inflammatory response associated with enteric fever can be correlated to the production of Vi antigen. Vi antigen is a capsular polysaccharide encoded by the *viaB* locus, present in SPI-7. The *viaB* locus encode genes involved in the regulation (*tviA*), biosynthesis (*tviBCDE*), and transport (*vexABCDE*) of the Vi antigen [30]. The *viaB* locus is present in *S.* Typhi and most TS serovars, but absent in NTS serovars: *S.* Typhimurium and *S.* Enteriditis [30]. The expression of Vi antigen is upregulated during *Salmonella* invasion of epithelial cells and downregulated during intracellular survival and systemic dissemination [31,32]. It has been proposed that the Vi antigen prevents inflammation by shielding antigens for antibody binding and downregulating the expression of TLR antagonist such as flagella and LPS [33]. Deletion of the *viaB* locus has been shown to increase the inflammatory response induced by TS serovar *S.* Typhi. While introduction of the *viaB* locus has been shown to reduce the inflammatory response induced by NTS serovar *S.* Typhimurium in bovine ligated ileal loop [34].

The pseudogenization of genes present in the SPIs, and genes required for survival in the inflamed gut, also contributes to the lack of inflammatory response induced by TS. SPI-1 and SPI-2 T3SSs are present in both TS and NTS serovars; however, several genes such as *sopA*, *sopE2* and *sseJ* are present as pseudogenes in TS [35,36]. SopA is a ubiquitin ligase involved in the induction of an inflammatory response during infection [37]. *S.* Typhi trans complemented with *S.* Typhimurium *sopA* induced Hep-2 cells to secrete increased amounts of inflammaroty cytokines, IL-8 and IL-18 [35]. SseJ interacts with SipA which is important for actin rearrangement during *Salmonella* invasion [38]. *S.* Typhi trans complemented with *S.* Typhimurium *sseJ*, exhibits increased cytotoxicity and proliferation in cultured epithelial cells [36].

In contrast to TS serovars, NTS can survive in the inflamed gut, due to their ability to respire using tetrathionate [39]. During inflammation, the oxygen content in the intestinal lumen is low, and this promotes the use of fermentation to obtain energy from amino acids and complex polysaccharides. Hydrogen sulfide produced as a by product of fermentation is converted to thiosulfate by epithelia cells. Neutrophils recruited to the site of inflammation release reactive oxygen species (ROS), which interact with thiosulfate to form tetrathionate [7,26]. Tetrathionate is an alternative electron acceptor, which can be used by NTS for anaerobic respiration [39]. The *ttrRS* and *ttrBCA* operons required for the tetrathionate respiration are pseudogene in TS [40,41].

Typhoid toxin encoded on SPI-11 is exclusively produced when *S.* Typhi localized within SCV [29]. NTS serovars: *S.* Typhimurium and *S.* Enteriditis do not produce the typhoid toxin. The typhoid toxin is an atypical AB toxin, consisting of two enzymatically active (A) subunits (CdtB and PltA) and one binding (B) subunit (PltB) [42]. CdtB is a homolog to the A subunit of cytolethal distending toxin, as well as to DNase I protein families, whereas PltA (which has ADP-ribosyl transferase activity) and PltB share similarities with subunits of pertussis toxin [43]. The role of typhoid toxin in *S.* Typhi pathogenesis is not well understood. However, it is speculated to be involved in promoting chronic *S.* Typhi infection [29].

They are potentially several unidentified factors that contribute to the difference in TS and NTS pathogenesis. The identification of these factors will contribute to the development of effective antimicrobials and vaccines required to combat *Salmonella* infections. The rest of this thesis will be primarily focused on NTS serovars.

1.2 Non-typhoidal *Salmonella* vaccines

1.2.1 Immune response induced by Non-typhoidal *Salmonella*

Understanding the host immune response is critical for the development of protective vaccines against NTS. Based on pathogenesis, the immune response to NTS can be divided into two categories: intracellular immunity which eliminate pathogens inside host cells, and extracellular immunity which eliminates pathogens outside host cells.

Salmonella is an intracellular pathogen, and this suggests that the innate immunity is critical for controlling NTS infection. During invasion, the initial interaction between NTS and epithelia cells induce an inflammatory response; because epithelia cells have receptors that recognize

pathogen-associated molecular patterns (PAMPs) [44]. *Salmonella* that invade the intestinal epithelia through M cells are taken up by phagocytic cells such as macrophages in Peyer's patches [45]. Neutrophils, macrophages, NK cells, and inflammatory monocytes recruited to the intestinal epithelia, control intracellular *Salmonella* infection by producing cytokines, chemokines, and antimicrobials such inducible nitric oxide synthase (iNOS), reactive oxygen and nitrogen species (ROS and RNS) [28].

The type of cytokine produced is important for controlling NTS infection. Cytokine analysis of recovered patients suggest that a Th-1 type cytokine response is important for eliminating NTS. Th-1 and Th-1 inducing cytokines such as IFN-γ, TNF-α, IL-12, IL-15, and IL-18 were found to be significantly elevated in patients with NTS gastroenteritis [46]. A cytokine profile analysis showed that IFN-γ, TNF-α, and IL-12 are present at elevated levels in patients that clear NTS infection compared to non-clearance group [47]. The importance of IFN-γ and IL-12 has been demonstrated by the increased susceptibility of patients with defects in IL-12 and IFN-γ production to NTS infections [48,49]. Adaptive immunity mediated through CD4 T-cells is also important for eliminating intracellular NTS, through the activation of infected macrophages [44]. The importance of CD 4 T-cells in resolving NTS infection is illustrated by the high motility rate associated with iNTS infection in HIV infected individuals [10].

Although NTS is an intracellular pathogen, it exists outside the host before invasion and when it moves from cell to cell. Therefore, antibodies also play a role in eliminating NTS infection. Mucosal immunity against invading pathogens is initiated by the identification of *Salmonella* PAMPs by antigen presenting cells such as dendritic cells (DCs) present in the tissues surrounding the intestinal lumen [44]. Antigens processed by DCs are presented to T cells which induce the production of secretory IgA (SIgA) antibodies. SIgA antibodies travel to the intestinal lumen and prevent further *Salmonella* migration and invasion [44]. In addition to SIgA, IgM and IgG antibodies are also produced and are involved in the elimination of extracellular NTS [44,50].

A protective vaccine against NTS infection will induce a combination of innate immunity, adaptive immunity, and antibodies production. Immunological studies in mice suggests that a combination of innate and T-cell mediated immunity play a bigger role in the resolution of *S.* Typhimurium infection compared to antibodies [50]. However, patients with

immunodeficiencies in antibodies production have increased susceptibility to NTS gastroenteritis [50].

1.2.2 Non-typhoidal *Salmonella* vaccine development

Although several vaccines against *S.* Typhi have been successfully developed and are presently been administered to millions of people [51,52], there are currently no licensed vaccines against NTS. The primary challenge associated with developing a vaccine, is the numerous NTS serovars that cause disease in humans. However, evidence from human studies supports the feasibility of developing effective NTS vaccines. Reduced incidence of age related iNTS disease in sub-Saharan Africa, has been correlated with the production of NTS specific antibodies [53,54]. In addition, serum collected from healthy individuals have been shown to have bactericidal activities against *S.* Typhimurium [55]. Potential vaccines against NTS can be divided into two categories: live attenuated, and subunit vaccines [56,57].

Attenuated strains are excellent vaccine candidates because, they can induce both innate and adaptive immunity. They can also be used to deliver multiply antigens, rising the possibility of inducing cross-protective immunity against several NTS serovars. Attenuated strains currently been developed as live attenuated vaccines against NTS, have mutations in genes involved in amino acid biosynthesis, divalent metal cation transport, outer membrane proteins, LPS biosynthesis genes or SPI T3SS genes [57,58]. Deletion of genes involved in the synthesis of aromatic amino acids (*aroC*, *aroA* and *aroD*) have been used to generate attenuated *S.* Typhimurium mutants, that induce protective immune response against a lethal *S.* Typhimurium challenge in mice [58]. *S.* Typhimurium Δ*ssaV* Δ*fur* attenuated by deletion of *ssaV* (which encodes part of the SPI-2 T3SS needle) and *fur* (a ferric uptake regulator), induced a protective immune response in mice against *S.* Typhimurium. *S.* Typhimurium WT05 attenuated by deletion of *ssaV* and *aroC* is the only NTS vaccine that has been tested in a phase 1 clinical trial. However, it was discontinued because healthy volunteers had prolonged stool shedding of the vaccine strain for up to 23 days [59]. Immunization with *S.* Typhimurium SA186 attenuated by deletion of *znuABC* involved in zinc transport, reduced *S.* Typhimurium colonization in pigs [60], and protected mice against a lethal *S.* Typhimurium challenge [61]. Deletion of *tolA* (part of the Tol-Pal system that maintains the integrity of the outer membrane) attenuated *S.* Typhimurium in mice infected via the oral and intravenous route, and provided protection

against subsequent *S*. Typhimurium challenge [62]. *S*. Typhimurium or *S*. Enteriditis attenuated by mutation of *hfq* (encoding a small RNA chaperone involved in post transcriptional gene regulation), *clpP* (which encodes a master protease regulator), *guaBA* (involved in guanine synthesis) or LPS biosynthesis have also been shown to induce protective immune responses in mice [63–66]. The major challenge associated with live attenuated vaccines, is the probability that they can cause disease in immunocompromised individuals. A live attenuated *Salmonella* vaccine with mutation in *aroA* or *htrA* has been shown to cause lethal infection in mice deficient in T cells [67], IL-12 [68] or IFN-γ production [69]. However, *S*. Typhimurium CVD1921 attenuated by deletion of *guaBA*, has been shown to be safe in rhesus macaques chronically infected with simian immunodeficiency virus (SIV) [70]. Due to safety concerns, live attenuated vaccines will likely be administered only to immuocompetent individuals.

Glycoconjugates are the basis of most subunit vaccines currently been developed against NTS. The O-antigen portion of lipopolysaccharide (LPS O-Ag) has been implicated as the target of protective immunity [71,72], hence LPS O-Ag form the bases of most glycoconjugate vaccines against NTS. LPS O-Ag has been conjugated to tetanus toxoid (TT) [73], porins [74,75], inactivated diptheria toxin, CRM197 [76], and flagellin [77]. Although most of the conjugate vaccine induced a protective immune response against a lethal NTS challenge in mice, more research is required to determine their safety and efficacy in humans.

Generalized Modules for Membrane Antigens (GMMAs) are outer membrane vesicles (OMVs), produced by genetically modified bacteria. Compared to glycoconjugates, GMMAs induce a higher and more diverse immune response with greater serum bactericidal activity [78,79]. Immunization with GMMAs reduced bacterial colonization of mice infected with *S*. Typhimurium [78]. GMMAs isolated from *S*. Typhimurium deficient in flagellin production, induced cross-protective immunity against a heterologous NTS challenge [80]. Although several vaccines are currently been developed, none has gone beyond phase 1 clinical trials. A protective vaccine against NTS is needed to reduce the global incidence of gastroenteritis and iNTS infections.

1.2.3 Polysaccharide vaccines

Polysaccharides present on bacterial surface play important roles in pathogenesis and are often targets of protective immunity. Hence, they are the targets for vaccine development against

several pathogens. However, one of the problems of polysaccharide vaccines is their inability to induce a T-cell mediated immune response required for isotype switching [81,82]. The binding of antigens to B-cell receptors induce B-cells to produce high avidity but low affinity IgM antibodies. However, to induce the production of high affinity IgG antibodies (isotype switching), B-cells have to be stimulated by T-helper cells [83,84]. To activate T-helper cells, antigens internalized and processed by APCs (B-cells) bind to major histocompatibility complex II (MHC II), and are presented to the α, β-T cell receptors on the surface of T helper cell, which stimulate B-cells to proliferate and differentiate to antibody (IgG) secreting plasma cells with immune memory [83,84]. Unlike proteins, polysaccharides are not processed and presented on MHC II, hence there are poor immunogens because, they fail to induce isotype switching and the production of memory B-cells [83,84].

In the late 1920, it was discovered that polysaccharides conjugated to carrier proteins induce isotype switching and the formation of memory B-cells [85], and this has formed the bases of most polysaccharide vaccines. The first polysaccharide conjugate vaccine was developed against *Haemophilus influenza* group b (Hib). Hib capsular polysaccharide, polyribosyl-ribitol phosphate (PRP) conjugated to diphtheria toxoid, inactivated diphtheria toxin (CRM197), *N. meningitidis* outer membrane protein, and tetanus toxoid, has been successfully administered to millions of people [83,84,86]. Polysaccharide conjugate vaccines have also been developed against *N. meningitidis*, *S. pneumoni*a, *S. agalactiae* and *S.* Typhi [84,86]. These polysaccharide-based vaccines have been successfully introduced into the routine immunization programs of multiple countries and have significantly reduced the global burden of disease associated with these pathogens. NTS contain several polysaccharides such as colanic acid, O-Ag capsule and LPS-OAg which are currently being developed as polysaccharide vaccines against NTS.

1.3 *Salmonella* biofilms

Biofilms are the predominant mode of bacterial life and are associated with approximately 80% of all bacterial infections [87]. Biofilms are structured communities of bacterial cells within a self produced extracellular matrix adherent to biotic or abiotic surfaces [87]. *Salmonella* forms biofilm at temperatures below 30°C, under conditions of low osmolarity and nutrient limitation [88,89]. The ability to form biofilms on abiotic surfaces such as plastic, cement, glass, and stainless stain, has be associated with *Salmonella* resistance to environmental stress factors such

as desiccation, disinfectant, and nutrient starvation [87]. Biofilm formation has also been associated with persistence on plants and is correlated to numerous *Salmonella* outbreaks with contaminated fruits and vegetables. In the presence of bile *S.* Typhimurium, Enteriditis and Typhi have been shown to form biofilms on gallstones [90]. Pellicle formation and rdar (red, dry and rough) morphotype are the most studied form of *Salmonella* biofilms. When grown in liquid culture pellicles are the ring of adherent materials attached to the glass wall at the air-liquid interface. While the rdar morphotype is the appearance of *Salmonella* biofilms on Congo red dye containing agar [91]. *Salmonella* serovars positive for pellicle formation and rdar morphotype often form biofilms on abiotic surfaces, and plants.

1.3.1 Composition of *Salmonella* biofilms

The components found in most *Salmonella* biofilms include proteins such as curli fimbriae and BapA, and extracellular polysaccharides such as cellulose, capsular polysaccharides and lipopolysaccharides [87]. Although the extracellular matrix of biofilms can vary with environmental conditions, in most cases curli fimbriae and cellulose are indispensable for *Salmonella* biofilm formation. Curli fimbriae, a functional amyloid provides short range interactions between cells, while cellulose, a β 1 – 4 linked glucose polymer provides long range interactions over the distance of the entire colony [92]. When grown on agar, the three-dimensional structure formed by *Salmonella* is primarily determined by the presence of curli fimbriae and cellulose. *Salmonella* morphotypes on Congo red containing agar is often used to identify curli and cellulose producing isolates [91]. The rdar morphotype indicates curli and cellulose production. The brown dry and rough (bdar) morphotype indicates lack of cellulose production, while the pink dry and rough morphotype indicates lack of curli production. *Salmonella* isolates negative for both curli and cellulose production have a smooth and white morphology (Fig 1.2).

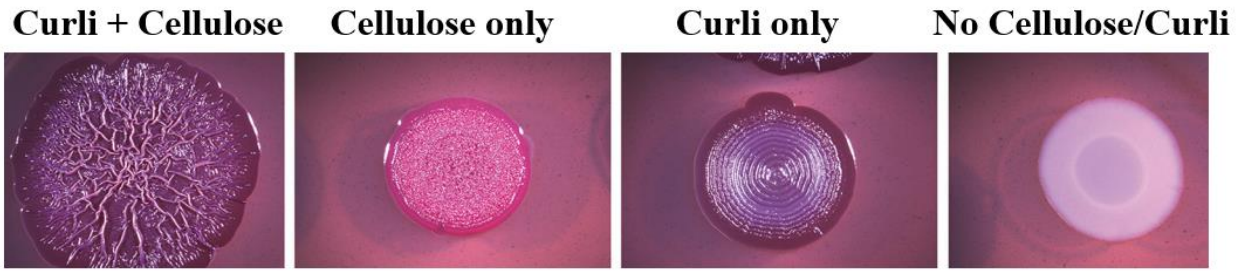

Figure 1.2: *Salmonella* morphology on Congo Red agar. *Salmonella* positive for curli and cellulose production have a red, dry and rough morphology. Isolate that produce only cellulose have a pink, dry and rough morphotype, while isolates that produce only curli have brown, dry and rough morphotype. Isolates negative for both cellulose and curli production have a smooth and white morphology. Adopted with some modification from Serra et al., [93].

1.3.2 Regulation of biofilm formation through CsgD

Salmonella biofilm formation is regulated by a highly complex regulatory network through transcription factor CsgD. CsgD is a LuxR helix-turn-helix (HTH) type transcription factor that positively regulates the expression of most biofilm components [94]. It directly regulates curli fimbriae biosynthesis operons, *csgBAC* and *csgDEFG* [95], BapA biosynthesis operon *bapABCD* and O-Antigen capsule biosynthesis operons *yihUTSRQPO* and *yihVW* [96]. CsgD indirectly regulates cellulose production by activating the expression of diguanylate cyclase *adrA*, which synthesis c-di-GMP required for cellulose production [87].

The intergenic region between *csgBAC* and *csgDEFG* operons is one of the largest in *Salmonella* with 754 bp (in *S*. Typhimurium 14028), allowing for the binding of multiple transcription factors, and making it a hub for integrating different environmental and host signals [87,97]. CsgD expression is regulated at the transcriptional, post transcriptional, translation and post translation levels by environmental signals such as temperature, osmolarity, nutrient availability, pH, oxygen tension, and ethanol. It is also regulated by regulatory proteins such as H-NS, OmpR, IHF, CpxR and MlrA and by bacterial secondary messenger, c-di-GMP [87–89].

CsgD expression is activated during stationary phase growth by sigma factor RpoS (σ^s), which forms a holoenzyme by binding to RNA polymerase (E-σ^s) [98]. E-σ^s activates the expression of genes involved in general stress response and is required for survival during stationary phase growth [99]. The activation of *csgD* expression by E-σ^s is enhanced by Crl which facilitates open complex formation at the *csgD* promoter [100,101]. Crl accumulates at temperatures below

30°C. It is proposed to be a thermosensor because *Salmonella* forms biofilms at temperatures below 30°C.

The effect of changing osmolarity on *csgD* expression is mediated through two component systems EnvZ/OmpR and CpxA/CpxR [102,103]. Under conditions of low osmolarity, low levels of OmpR phosphorylated (OmpR-P) by membrane bound sensor kinase EnvZ, binds to a high affinity site (D1) -50.5 bp upstream of the *csgD* transcription start site (TSS), and activates transcription [102]. Under conditions of high osmolarity *csgD* expression is repressed by high cellular levels of OmpR-P which binds to low affinity sites D2 and D3-D6 further upstream of D1 and repress *csgD* expression. The CpxA/CpxR system also represses *csgD* expression under conditions of high osmolarity, through the binding of phosphorylated CpxR to multiple sites on the *csgD* promoter, some of which overlaps with OmpR-P binding site [103]. Whether CpxR binds simultaneously or competes with OmpR for DNA binding still needs to the evaluated. The EnvZ/OmpR two component system has also been implicated in ethanol induced up-regulation of *csgD* expression [89]. Under conditions of high osmolarity due to high sucrose levels, the repression of *csgD* expression is mediated through H-NS [103].

Other regulatory proteins that have been found to regulate *csgD* expression are IHF and MlrA [89]. Integration host factor (IHF) is a histone-like architectural protein that regulates gene expression by changing DNA topology. Changes in DNA topology can bring distantly located elements in close proximity to each other. Using transcriptional fusions *csgD* expression was found to be reduced by 3-fold in a *S.* Typhimurium *ihf* mutant under microaerophilic growth conditions, whereas no change was observed under aerobic conditions [89]. Therefore, it has been proposed that IHF is involved in the activation of *csgD* expression under microaerophilic growth conditions. MlrA (MerR-like regulator) was identified as a positive regulator of *csgD* expression, during a complementation screen to recover *S.* Typhimurium and *E. coli* curli expressions [104]. Although the environmental signals and mechanism of MlrA-mediated *csgD* expression is not known, MlrA is speculated to regulate *csgD* expression in response to metal ions [87,89].

CsgD expression is also regulated by c-di-GMP, which promotes biofilm formation by upregulating *csgD* expression, and activating cellulose synthase required for cellulose biosynthesis. C-di-GMP is synthesized by diguanylate cyclases (DGCs) containing a GGDEF

domain and degraded by phosphodiesterase (PDEs) containing a EAL or HD-GYP domain. *Salmonella* contains several GGDEF and EAL proteins that regulate different stages of biofilm formation [91]. Among the 12 GGDEF proteins present in *Salmonella*, STM2123 and STM3388 upregulate, while among the fifteen EAL proteins STM1703, STM4264, STM3611 and STM1827 downregulate *csgD* expression [105]. GGDEF containing proteins STM1987 and ArdA are primarily required for cellulose production in *Salmonella* [106]. Several sRNAs such as MarC, RprA, and OmrA/OmrB are also involved in regulating *csgD* expression [98].

CsgD is the master regulator of biofilm formation and its expression is regulated through a complex network of transcription factors, sRNAs and bacterial messengers [87]. How these factors are integrated to fine tune biofilm formation under different conditions still needs to be elucidated.

2.0 Hypotheses and Objectives

This thesis is comprised of four sections, the hypothesis, and objectives for each section are outlined below.

2.1 A SNP in the cache 1 signaling domain of diguanylate cyclase STM1987 leads to increased virulence of invasive *Salmonella* strains

The genetic and phenotypic difference between gastroenteritis- and invasive- *S.* Typhimurium strains can largely be attributed to >700 single nucleotide polymorphisms (SNPs). We identified a conserved SNP in invasive *S.* Typhimurium, that results in a missense mutation in the sensory domain of a diguanylate cyclase enzyme, STM1987. STM1987 catalyzes the formation of c-di-GMP, which positively regulates cellulose production. Previous studies have shown that *Salmonella* produces cellulose inside macrophages as an antivirulence factor [107].

Hypothesis

Reduced cellulose production due to reduced STM1987 activity, increases *Salmonella* survival inside macrophages, and virulence in a mouse model of infection

Objectives

1. Determine the effects of SNP in *stm1987* on cellulose production
2. Evaluate the effects of SNP in *stm1987* on the virulence of invasive *S.* Typhimurium D23580
3. Evaluate the effects of reduced cellulose production on *Salmonella* survival inside macrophages.

2.2 A GMMA-EPS based vaccine for Non-typhoidal *Salmonella*

There are currently no vaccines against NTS serovars that cause gastroenteritis and invasive bloodstream infections. Extracellular polysaccharide (EPS) present on the bacterial surface is the target of protective immunity against NTS [53, 54].

Hypothesis

Immunization with purified EPS can induce a protective immune response against *S.* Typhimurium and *S.* Enteriditis, the predominant NTS serovars associated with gastroenteritis.

Objectives

1. To develop a vaccine against NTS
2. Use random mutagenesis to boost EPS biosynthesis in *S.* Typhimurium
3. Conjugate purified EPS to carrier proteins
4. Determine if immunization with EPS alone or conjugated to carrier proteins induce a protective immune response in mice.

2.3 Metabolic activation of CsgD in the regulation of Salmonella biofilms

Transcription factor CsgD is the master regulator of *Salmonella* biofilm formation. Similar to sporulation, biofilm formation (*csgD* expression) is induced under conditions of starvation. Sporulation is an irreversible process that is unresponsive to known regulatory inputs once activated [108]. How CsgD responds to known regulatory inputs once activated has not been extensively investigated.

Hypothesis

CsgD responds differently to known regulatory inputs once activated

Objectives

To determine the effects of osmolarity, temperature and glucose on *csgD* expression after induction.

2.4 The challenges of identifying the roles of function unknown genes

S. Typhimurium exposed to environmental stresses differentiate into two distinct cell types: multicellular aggregate associated with environmental persistence, and planktonic cell associated with virulence. RNAseq comparison identified 798 function unknown (FUN) genes differentially expressed between both cell types.

Hypothesis

A proportion of FUN genes highly expressed in multicellular aggregates and/or planktonic cells have roles in *Salmonella* virulence or persistence.

Objectives

1. Develop a framework for the analysis of FUN genes identified during genetic screens.
2. Identify novel genes with roles in *Salmonella* virulence and/or persistence.

3.0 A SNP in the cache 1 signaling domain of diguanylate cyclase STM1987 leads to increased virulence of invasive *Salmonella* strains.

Akosiererem S. Sokaribo[1,2], Keith D. MacKenzie[3,4], Lindsay R. Balezantis[2], Yejun Wang[5], Melissa B. Palmer[1,2], Beatrice Chung[1,2], Nancy J. Herman[2], Madeline C. McCarthy[1,2], Jeffrey M. Chen[1,2], and Aaron P. White[1,2*]

Affiliations:
[1] Vaccine and Infectious Disease Organization-International Vaccine Centre, Saskatoon, Saskatchewan, Canada
[2] Department of Biochemistry Microbiology and Immunology, University of Saskatchewan, Saskatoon, Saskatchewan, Canada
[3] Present address: Department of Biology, Faculty of Science, University of Regina, Regina, Saskatchewan, Canada
[4] Institute for Microbial Systems and Society, Faculty of Science, University of Regina, Regina, Saskatchewan, Canada
[5] Department of Cell Biology and Genetics, School of Basic Medicine, Shenzhen University Health Science, Shenzhen, China

* Corresponding author, E-mail: aaron.white@usask.ca

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Authors contribution

Akosiererem S. Sokaribo: Designed and performed experiments, analyzed data, and wrote manuscript.

Keith D. MacKenzie: Assisted in experimental design

Lindsay R. Balezantis and Madeline C. McCarthy: Assisted with animal trial experiments

Yejun Wang: Performed bioinformatic analysis to identify SNP

Melissa B. Palmer: Generated *S.* Typhimurium Δ*stm1987* mutant

Beatrice Chung and Jeffrey M. Chen: Grew macrophages used for survival assays

Nancy J. Herman: Cloned *stm1987* gene into plasmid pBR322.

Aaron P. White: Designed experiment, analyzed data, edited manuscript, and obtained funding for this research

3.1 Abstract

Non-typhoidal *Salmonella* (NTS) strains are associated with gastroenteritis worldwide but are also the leading cause of bacterial bloodstream infections in sub-Saharan Africa. The invasive NTS (iNTS) strains that cause bloodstream infections differ from standard gastroenteritis causing strains, by more than 700 single nucleotide polymorphisms (SNPs). These SNPs are known to alter metabolic pathways, biofilm formation and contribute to serum resistance, and are thought to signify iNTS strains becoming human-adapted similar to typhoidal *Salmonella* serovars. Identifying SNPs that contribute to invasion or increased virulence have been more elusive. In this study, we identified a SNP in the cache 1 signaling domain of diguanylate cyclase STM1987 in the invasive *S.* Typhimurium type strain D23580. This SNP was conserved in 118 other iNTS strains analyzed, and comparatively absent in global *S.* Typhimurium isolates associated with gastroenteritis. STM1987 catalyzes the formation of cyclic-di-GMP and is proposed to stimulate production of cellulose independent of the master biofilm regulator, CsgD. We show that the amino acid change in STM1987 leads to a 10-fold drop in cellulose production and increased virulence in a mouse model of acute infection. Reduced cellulose production due to the SNP led to enhanced survival inside both murine and human macrophage cell lines. In contrast, inactivating CsgD-dependent cellulose production did not lead to any measurable change in virulence. We hypothesize that the SNP in *stm1987* represents a pathoadaptive mutation for iNTS strains.

Keywords: cellulose, diguanylate cyclase, STM1987, invasive *Salmonella*, CsgD-independent, virulence

3.2 Introduction

Salmonella enterica are important foodborne pathogens that cause a significant amount of morbidity and mortality worldwide [1]. From a clinical perspective, *S. enterica* cause two main types of diseases: typhoid fever and gastroenteritis [5]. Endemic in resource-poor settings, typhoid fever is a life-threatening systemic disease caused by typhoidal *Salmonella* (TS) serovars: primarily Typhi and Paratyphi [109]. Gastroenteritis infections worldwide are caused by strains from nontyphoidal *Salmonella* (NTS) serovars, with serovars Typhimurium and Enteritidis being the most common [18]. TS are human restricted while NTS can cause disease in a wide range of hosts. Genomic changes such as gene acquisition, single nucleotide polymorphisms (SNPs), and pseudogene accumulation have been associated with the restricted host range of TS serovars [110,111]. Over the past three decades, an invasive disease caused by NTS serovars has emerged as the third type of disease caused by *S. enterica*.

In sub-Saharan Africa, strains of the gastroenteritis associated NTS serovars Typhimurium and Enteritidis, are the leading cause of invasive bloodstream infections that are distinct from typhoid fever [23]. These isolates are referred to as invasive NTS (iNTS). In 2017, iNTS serovars caused an estimated 535, 000 cases of invasive bloodstream infections, resulting in 77, 500 deaths [112]. Bacteremia caused by iNTS serovars is common in children with severe anemia, HIV, malaria, or malnutrition, and in immunocompromised adults. Symptoms include non-specific fever, headaches, chills, malaise, and in rare cases, diarrhea [10]. Two closely related lineages of *S. enterica* serovar Typhimurium (*S.* Typhimurium) that belong to multilocus sequence type ST313 are the most common cause of bacteremia caused by iNTS [11]. Genome sequencing of *S.* Typhimurium D23580, a representative ST313 isolate that was isolated from a child in Malawi, identified >700 single nucleotide polymorphisms (SNPs) that were absent in non-ST313 isolates [11,113,114]. These SNPs contribute to the ongoing genomic changes of ST313 isolates, which are thought to be evolving similar to human-adapted typhoidal *Salmonella* serovars [115].

Cellulose, composed of β (1 - 4) linked D-glucose units, is the most abundant polymer on earth. It is the major component of plant cell walls and is the starting material for numerous medical, industrial, and agricultural products [116]. Cellulose is also one of the major components of the extracellular matrix of bacteria biofilms, and its production has been described in a wide variety

of bacteria, such as *Agrobacterium tumefaciens*, *Acetobacter xylinus*, *Rhizobium* spp. [117] in addition to clinically relevant *S. enterica* [118] and *E. coli* strains [91,119,120]. In bacteria, cellulose is synthesized by membrane-bound cellulose synthase complex comprised of BcsA and BcsB subunits [121]. BcsA is comprised of two domains: a transmembrane and a cytoplasmic domain. The transmembrane domain consists of eight transmembrane helices that form pores across the inner membrane, while the cytoplasmic domain is composed of a glycosyltransferase segment, and a C-terminal fragment that contains the PilZ domain, which binds bis-(3', 5')-cyclic dimeric guanosine monophosphate (cyclic-di-GMP or c-di-GMP) [91]. BcsB is in the periplasm and is anchored to one of the transmembrane helices of BcsA [121,122]. In the absence of c-di-GMP, BcsA is in an inactive state. Cyclic-di GMP allosterically activates BcsA by binding to the PilZ domain [123].

Cyclic-di-GMP is an important bacterial secondary messenger involved in the regulation of diverse processes such as biofilm formation, motility, and virulence [91,124]. C-di-GMP is synthesized from two molecules of guanosine triphosphate (GTP), by diguanylate cyclase (DGC) enzymes containing a GGDEF domain and degraded by specific phosphodiesterases (PDEs) [125]. DGCs are typically comprised of two domains: the highly conserved GGDEF motif required for c-di-GMP synthesis is present in the C-terminal domain [126], while the N-terminal domain typically consists of the sensory receptor that activates the GGDEF activity. The *S. enterica* genome encodes twelve GGDEF motif-containing proteins, many of which are constitutively expressed. Recombinant *S. enterica* strains lacking all twelve GGDEF motif-containing proteins are avirulent and impaired for biofilm formation [127,128].

Biofilms are structured communities of bacterial cells encased in a self-produced extracellular polysaccharide matrix. For *S. enterica*, cellulose and curli fimbriae are the main components and have been shown to contribute to the long-term survival and persistence of cells in the environment [118]. Recently, *S.* Typhimurium was also shown to produce cellulose inside macrophages as an anti-virulence factor [107]. These diverse and opposing functions illustrate a complex role for cellulose in the *Salmonella* life cycle. As described above, the most important factor for cellulose biosynthesis is the production of c-di-GMP by DGCs, which respond to a variety of different environmental signals [91,117,129]. The roles of DGCs AdrA and STM1987

in cellulose production have been well established in *Salmonella*. AdrA induces cellulose production in LB media at 28°C (biofilm inducing condition) in a CsgD-dependent manner, while STM1987 induces cellulose production at 37°C in nutrient-deficient media (ATM) independent of CsgD [106]. Transcription factor CsgD is the master regulator of biofilm formation in *S. enterica*, it positively regulates the biosynthesis of cellulose, curli fimbriae, and other biofilm components [87]. By treating the biofilm obtained under different conditions with cellulase, Solano et al.[130] showed that biofilm obtained at 28°C in LB media is composed of other extracellular matrix components besides cellulose, while biofilm obtained at 37°C in ATM media is predominantly composed of cellulose [106,130]. Recently it was shown that STM1987 can sense arginine, phytic acid, glucose, and GlcNAC at biologically relevant concentrations. Since arginine is thought to be a prominent signal inside macrophages [131–133], we wondered if STM1987 could control cellulose production by *S. enterica* inside macrophages. If so, STM1987 could be an important *Salmonella* virulence determinant.

In this study, we identified a missense mutation in *stm1987* in iNTS type strain *S.* Typhimurium D23580. *S.* Typhimurium strains possessing this altered STM1987 enzyme had reduced cellulose production and increased virulence in mice. Reduced cellulose production correlated with enhanced *S.* Typhimurium survival inside macrophages. In contrast, a mutation preventing CsgD-dependent cellulose production (via DGC AdrA) did not influence virulence.

3.3 Results

3.3.1 The presence of a SNP in *stm1987* in *S.* Typhimurium strain D23580 results in an enzyme with reduced activity.

From a list of SNP differences between gastroenteritis-causing *S.* Typhimurium ST19 strain 14028 and invasive *S.* Typhimurium ST313 strain D23580 (Y Wang, unpublished), we identified a C-to-G transversion SNP in *S.* Typhimurium D23580, located 566 bp downstream of the translational start site of *stm1987*. The mutation results in the replacement of threonine to arginine at residue 189 in the cache 1 sensory domain (Fig. 3.1), a region that was shown to be critical for sensing external signals and regulating the overall enzyme activity of STM1987 [134].

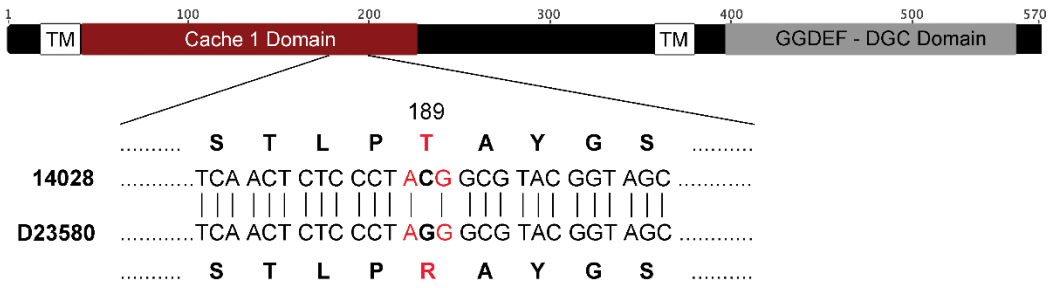

Figure 3.1: Schematic of *stm1987* allele in *S.* Typhimurium 14028 and D23580. The 570 amino acid STM1987 protein is represented by the black rectangle and is shown to scale, with transmembrane (TM), Cache 1 signaling, and GGDEF-diguanylate cyclase domains highlighted. The magnified region shows nucleotides between 553 and 579, with nucleotide 566 in bold (C in 14028 and G in D23580). Single-letter amino acids corresponding to each codon are shown above for *S.* Typhimurium 14028, and below for strain D23580. Amino acid residue 189 is shown in bold red (T for 14028 and R for D23580).

To determine if the amino acid change in STM1987 would influence enzyme activity, the *stm1987* alleles from *S.* Typhimurium strains 14028 and D23580 were cloned into pBR322 and used to complement a *S.* Typhimurium 14028 Δ*stm1987* mutant strain. Plasmid-based expression of *stm1987* or other DGCs has previously been demonstrated to result in the overproduction of cellulose [106,127,135]. When macrocolonies of each strain were grown on media containing calcofluor white, a fluorescent dye that stains cellulose [130], the strain complemented with the *stm1987*^ST14028 allele was noticeably brighter than the *stm1987*^D23580 complemented strain, and both were brighter than the pBR322 control (Fig 3.2A). Cellulose production was also visualized by growing strains in liquid culture and quantifying the amount of biofilm material deposited at the air-liquid interface of the culture vessel [106,130]. After 24 hours growth at 37ºC, the Δ*stm1987* strain complemented with the *stm1987*^ST14028 allele had a thick ring at the air-liquid interface, whereas the strain complemented with the *stm1987*^D23580 allele had a faint ring, and the pBR322 control strain had no visible ring (Fig. 3.2B). We confirmed that the curli biosynthesis operon (i.e., *csgBAC*) was not expressed under these conditions (Fig. S3.1), suggesting that cellulose was the primary component of the ring. Crystal violet staining showed that the *stm1987*^ST14028 complemented strain produced approximately 10-times more biomass attached to the glass as compared to the *stm1987*^D23580 complemented strain, which was just above background levels (Fig 3.2C). To confirm that cellulose was the primary component of the biomass at the air-liquid interface, we repeated the same experiment in a mutant strain that was unable to make cellulose (Δ*bcsA*). In this cellulose-deficient strain background, none of the

complemented strains produced a visible ring at the air-liquid interface (Fig. S3.2). Taken together, these experiments demonstrated that the single amino acid change in the cache 1 domain of STM1987 in *S.* Typhimurium D23580 resulted in reduced cellulose production.

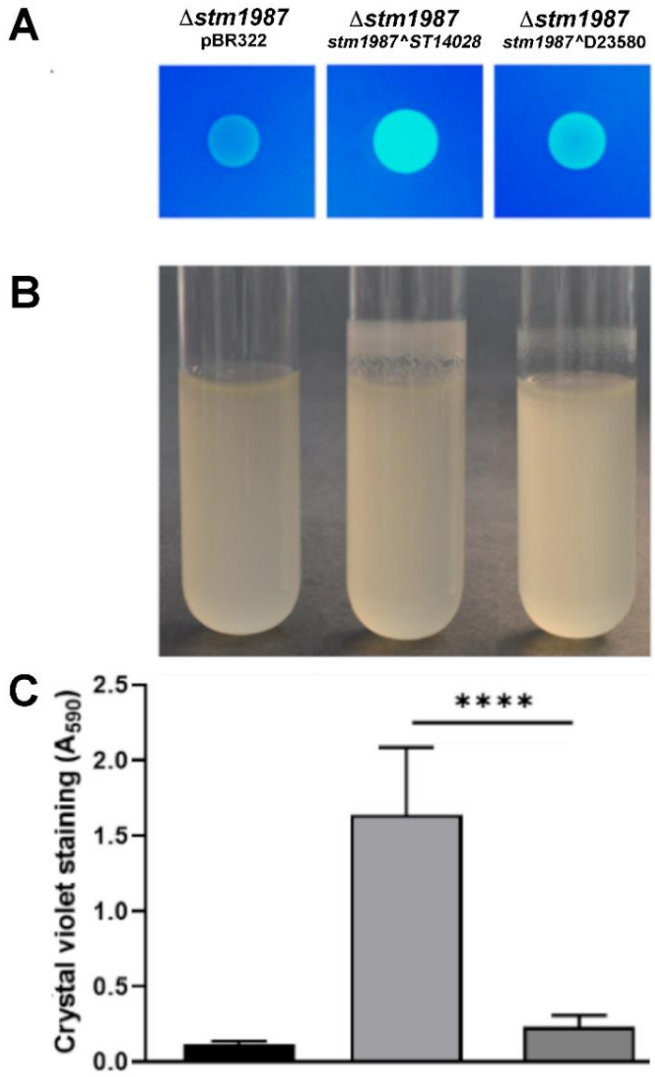

Figure 3.2: Phenotypic comparison of *stm1987* alleles from *S.* Typhimurium 14028 and D23580. *S.* Typhimurium 14028 Δ*stm1987* was transformed with pBR322 (control) or pBR322 harboring the *stm1987*^ST14028 or *stm1987*^D23580 alleles. (A). Cells from overnight cultures were grown on tryptone agar supplemented with calcofluor white and colonies were visualized under UV light after 12 h of growth at 28ºC. (B). Strains were grown in LB broth for 24 h at 37°C and tubes were visualized for the presence of surface-attached biomass at the air-liquid interface. (C). The amount of attached biomass was quantified using crystal violet staining, with the absorbance of the resulting solution measured at 590 nm (A_{590}). Bars represent the average values and error bars represent the standard deviations from 5 biological replicates, statistical significance ****, $p < 0.0001$.

3.3.2 SNP in *stm1987* is conserved in both invasive lineages of *S.* Typhimurium

To determine if the *stm1987* SNP identified in *S.* Typhimurium strain D23580 was conserved, we examined the gene sequences in 118 additional ST313 *S.* Typhimurium strains. Whole-genome sequencing-based phylogeny has previously been used to cluster the ST313 strains associated with invasive disease in sub-Saharan Africa, into closely related lineages I and II [11]. We overlaid the SNP information on a phylogenetic tree that was modeled after Okoro et al. [11] (Figure 3.3; [136]). All information pertaining to the strains in this tree, as well as additional isolates that were screened are listed in Table S12.1. The *stm1987* SNP was conserved in all lineage I isolates (44/44 shown in Figure 3.3; 50 total) and lineage II isolates (57/57 shown in Figure 3.3; 69 total). In contrast, the SNP was almost completely absent from a global clade of gastroenteritis-causing *S.* Typhimurium isolates of sequence types ST-19, -34, -128, and 568 (absent in 8/9 isolates shown in Figure 3.3: 54/56 isolates total). *S.* Typhimurium strains DT24, isolated from a patient in the UK, and 5634, isolated from a patient in Kenya, were the only global isolates analyzed that possessed the SNP (Table S12.1). The conservation of the *stm1987* SNP in invasive isolates from sub-Saharan Africa indicated that this mutation has been selectively maintained within this group of isolates.

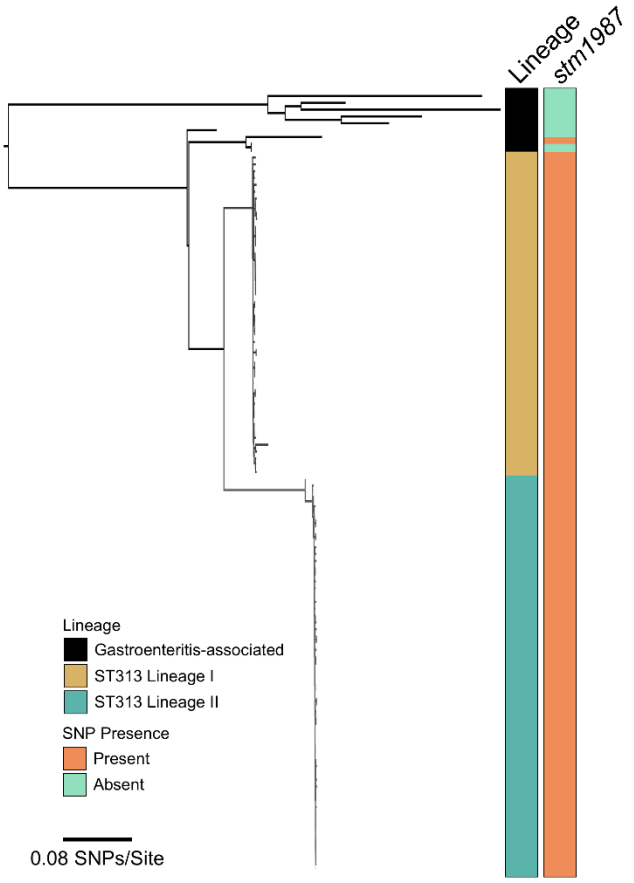

Figure 3.3: Conservation of *stm1987* single nucleotide polymorphism in invasive *S.* Typhimurium lineages. The maximum likelihood phylogenic tree was constructed from sequenced *S.* Typhimurium isolates [11], as previously reported [136]. The isolates analyzed were divided into *S.* Typhimurium groups associated with gastroenteritis or invasive disease (lineages I and II). The presence or absence of the STM1987 SNP is noted for each group.

3.3.3 Reduced STM1987 activity is associated with increased *S.* Typhimurium virulence in a mouse model of infection.

We hypothesized that reduced activity of STM1987 could be correlated with increased virulence and survival inside macrophages, due to reduced production of cellulose. To test this, we used genome engineering to generate a SNP-corrected strain of *S.* Typhimurium D23580 to contain the allele from *S.* Typhimurium 14028. To evaluate if there was a virulence difference between *S.* Typhimurium D23580 and SNP-corrected *S.* Typhimurium D23580 (*stm1987*^ST10428), we sought to test their ability to cause infections in susceptible (NRAMP-/-) mice after intraperitoneal (IP) infection. This model was based on vaccine trials by Cal MacLennan and colleagues [78]. We performed pilot experiments in C57BL/6 mice challenged IP with 10^4 CFU

of *S*. Typhimurium D23580, and *S. enterica* serovar Enteritidis (*S.* Enteritidis) D7795, another iNTS isolate. *Salmonella* CFU levels in internal organs and blood samples increased steadily from 24 h to 72 h post-infection (Fig. S3.3), with mice starting to show clinical signs of infection at 72 h. We concluded that 72 h was the optimal time point, where the colonization levels were potentially high enough to evaluate virulence differences between strains. C57BL/6 mice were challenged IP with 10^4 CFU of *S*. Typhimurium D23580 and the SNP-corrected strain. 72 h after infection, the colonization levels indicated that there was no virulence difference between both strains, as similar CFU numbers were recovered from the liver, spleen, kidneys, MLN, and blood of infected mice (Fig 3.4A). To explain this result, we reasoned that the presence of additional SNPs in *S*. Typhimurium D23580 could mask the effects of the *stm1987* mutation.

To isolate the potential effects of the *stm1987* SNP, we moved this mutation into the well-characterized *S*. Typhimurium 14028 strain. The use of *S*. Typhimurium 14028 also allowed us to generate antibiotic resistant, marked strains, which enabled us to perform randomized and blinded competitive index (CI) experiments. We performed two independent CI experiments in C57BL/6 mice challenged orally with 10^7 CFU of a 1:1 mixture of wild-type *S*. Typhimurium 14028 and *S*. Typhimurium 14028 (*stm1987*^D23580). At 4-7 days post-infection, mice were euthanized and the bacterial loads in the spleen, liver, cecum, and MLN were determined. *S*. Typhimurium 14028 (*stm1987*^D23580) was recovered at higher proportions than the wildtype strain from the MLN and cecum, but there was no colonization difference measured in the liver and spleen (Fig 3.4B).

To bypass small intestinal colonization and more directly assess systemic spread within the mouse, we performed a competitive index experiment between *S*. Typhimurium 14028 strains by IP injection. At 72 h post-infection, *S*. Typhimurium 14028 (*stm1987*^D23580) was recovered at a higher proportion than wildtype in all organs analyzed (Fig 3.4C). This gave a clear indication that the amino acid change in the cache 1 signaling domain of STM1987 could lead to increased invasiveness and virulence for *S*. Typhimurium strains.

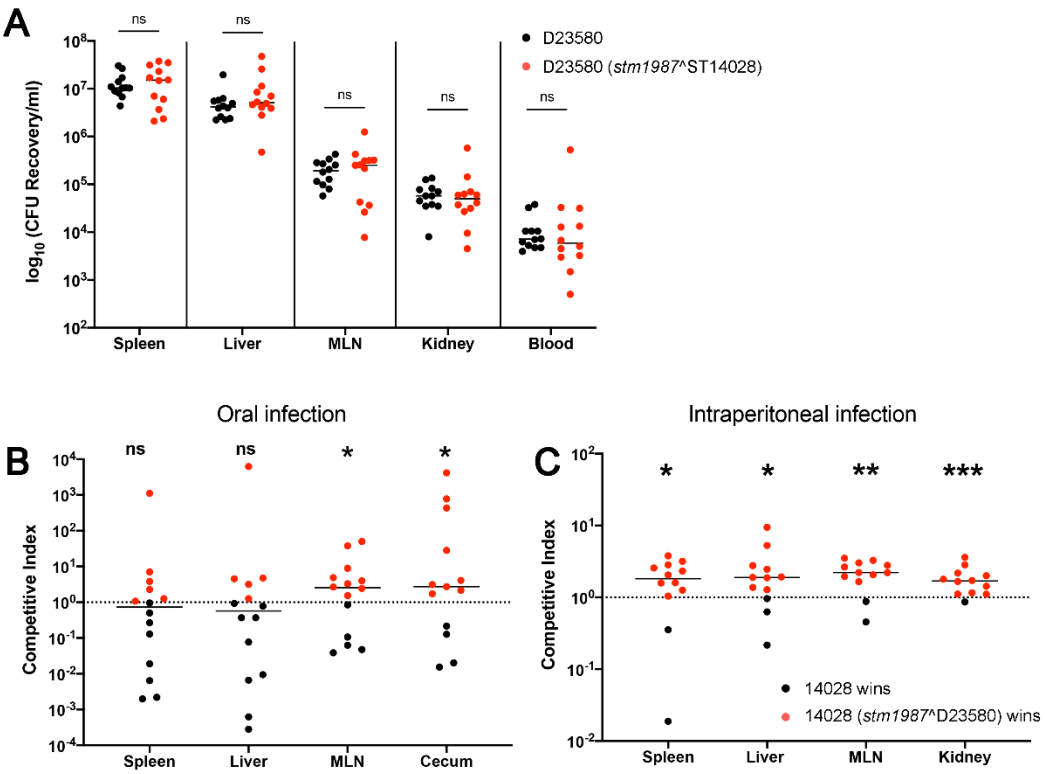

Figure 3.4: Evaluating the role of STM1987 in the virulence of *S.* Typhimurium strains D23580 and 14028. (A) C57BL/6 mice were injected IP with 10^4 CFU *S.* Typhimurium D23580 or *S.* Typhimurium D23580 (*stm1987*^ST14028) strains. At 72 h post-infection, the CFU levels were enumerated from the liver, spleen, mesenteric lymph nodes (MLN), kidney, and the blood. Each dot represents the CFU counts from the designated organ from a single mouse. (B and C) Competitive index (CI) infections were performed with C57BL/6 mice challenged orally with 10^7 CFU (B) or IP with 10^4 CFU (C) consisting of a 1:1 ratio of *S.* Typhimurium 14028 and *S.* Typhimurium 14028 (*stm1987*^D23580) strains. At 4-7 days (B) or 72 h (C) post-infection, bacteria were enumerated from each internal organ as shown. CI values were calculated from each organ: (CFU 14028 (*stm1987*^D23580)/ 14028)output/(CFU 14028 (*stm1987*^D23580)/ 14028)input. A CI value of 1, which represents a situation where strains are equally virulent, is represented by the horizontal dotted line in B and C. Red circles represent CI values where the S. Typhimurium 14028 (*stm1987*^D23580) won the competition. Statistical differences between groups of mice were noted as *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, or ns $p > 0.05$.

3.3.4 Reduced STM1987 activity increases *S.* Typhimurium survival inside macrophages

To determine if the SNP in *stm1987* was connected to intra-macrophage survival, murine RAW 264.7 and human THP-1 macrophages were infected with wild-type and SNP-corrected *S.* Typhimurium D23580. No difference in macrophage survival was observed between both strains (Fig 3.5 A and B). To evaluate macrophage survival in the absence of other mutations present in D23580, we performed macrophage assays with *S.* Typhimurium 14028. In both human and

murine macrophage *S*. Typhimurium (*stm1987*^D23580) with the SNP, have increased survival compared to wildtype, however the difference was not statistically significant (Fig 3.5 A and B).

To isolate the effect of the SNP and reduced cellulose production on intramacrophage survival, *S*. Typhimurium 14028 Δ*stm1987* was complemented with the *stm1987*^ST14028 or *stm1987*^D23580 alleles or the empty vector (pBR322). The pBR322 control strain displayed the highest level of survival in both types of macrophages, whereas the strain complemented with the *stm1987*^ST14028 allele had reduced survival similiar to background levels (Fig 3.6 A and B). In contrast, the *stm1987*^D23580 complemented strain had significantly higher survival in both human and murine macrophages compared to the *stm1987*^ST14028 complemented strain (Fig. 3.6 A and B). We repeated these same experiments with an *S.* Typhimurium 14028 strain that was also cellulose-deficient (Δ*bcsA*). With the ability to make cellulose removed, there was no statistical difference in survival of strains complemented with the vector only or either of the *stm1987* alleles (Fig 3.6 A and B). This confirmed that the difference in survival rate between strains complemented with different STM1987 enzymes was due to differing levels of cellulose production. We concluded that reduced cellulose production due to reduced STM1987 activity leads to increased survival inside macrophages. We hypothesized that this could explain the increased virulence of the *S*. Typhimurium 14028 (*stm1987*^D23580) strain measured in the mouse challenge experiments.

Recently it was discovered that *E. coli* and *S*. Typhimurium produce a chemically modified form of cellulose called phosphoethanolamine (pEtN) cellulose, and pEtN transferase BcsG was shown to be required for pEtN cellulose production [137]. Previously reduced cellulose production by *S*. Typhimurium D23580 was partially attributed to a premature stop codon in *bcsG* [136]. To determine if the lack of difference between wild-type and SNP-corrected *S*. Typhimurium D23580 was due to *bcsG* mutation, intramacrophage survival assays was repeated with *S*. Typhimurium 14028 with *bcsG* SNP (14028 (*bcsG*^D23580). *S*. Typhimurium 14028 *bcsG*^D23580 Δ*stm1987* was complemented with the *stm1987*^ST14028 or *stm1987*^D23580 alleles or the empty vector (pBR322). Similar to *bcsA* mutation, no ring of adherent material was attached to the culture vessel (data not shown) and there was no difference in intramacrophage survival between *S*. Typhimurium 14028 *bcsG*^D23580 Δ*stm1987* complemented with the empty

vector or either of the *stm1987* alleles (Fig 3.6 A and B). Our results suggest that the effect of *stm1987* SNP on the intramacrophage survival of *S.* Typhimurium D23580 is masked by mutation in *bcsG*. We hypothesized that this could explain the lack of virulence different between *S.* Typhimurium D23580 and the SNP-corrected strain measured in the mouse challenge experiment.

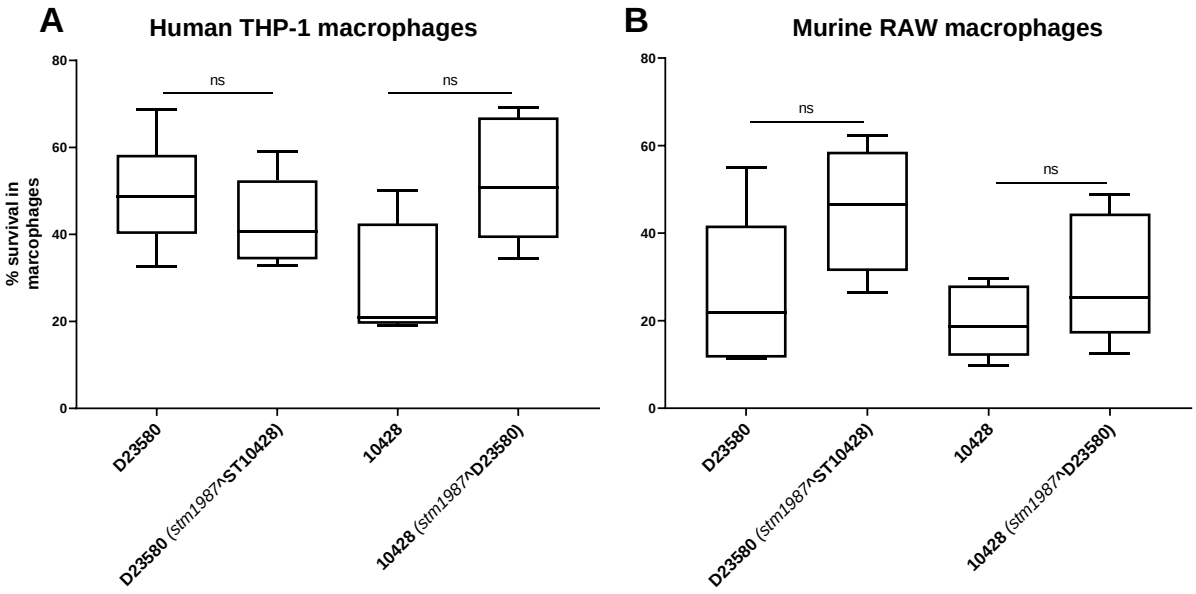

Figure 3.5: *S.* Typhimurium survival inside human and murine macrophages. A. Human THP-1 and B. murine RAW 264.7 macrophages were infected with *S.* Typhimurium D2350 wildtype or SNP corrected strain (D23580 (*stm1987*^ST10428) or *S.* Typhimurium 14028 wildtype or 14028 with *stm1987* SNP (14028 (*stm1987*^D23580). Percent survival was determined as the proportion of intracellular CFU remaining at 18 h post-infection, as compared to the initial CFU values measured after 30 min. Statistical significance between groups was noted as ns $p > 0.05$.

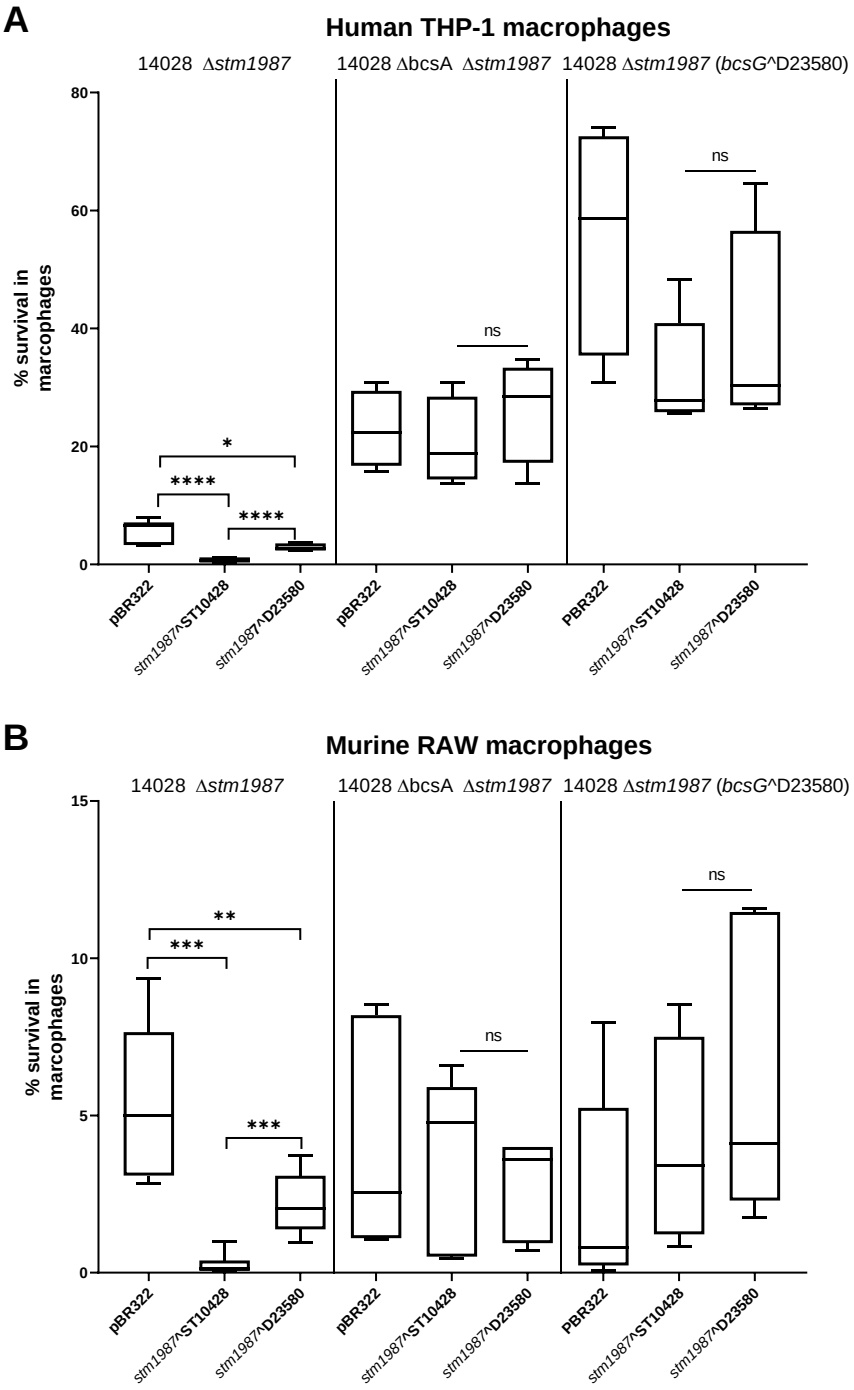

Figure 3.6: Survival of *S*. Typhimurium complemented strains inside human and murine macrophages. A. Human THP-1 and B. murine RAW 264.7 macrophages were infected with *S*. Typhimurium Δ*stm1987* or *S*. Typhimurium Δ*bcsA* Δ*stm1987* or *S*. Typhimurium (*bcsG*^D23580) Δ*stm1987* strains transformed with pBR322, pBR322/*stm1987*^ST14028 or pBR322/*stm1987*^D23580. Percent survival was determined as the proportion of intracellular CFU remaining at 18 h post-infection, as compared to the initial CFU values measured after 30 min. Statistical significance between groups was noted as *$p < 0.05$, ***$p < 0.001$, ****$p < 0.0001$, or ns $p > 0.05$.

3.3.5 Expression of *stm1987* is not regulated by transcription factor CsgD

In the context of *Salmonella* biofilm formation, cellulose production is regulated by transcription factor CsgD, which activates the expression of *adrA* [91,106]. YedQ, which is an *E. coli* homolog of STM1987, has been shown to participate in a CsgD-independent pathway for cellulose production [138]. Compared to *S.* Typhimurium 14028, *csgD* expression in D23580 is reduced due to SNPs in the promoter region [136]. To determine if *stm1987* expression in *S.* Typhimurium can occur independently of CsgD activity, we tested promoter activity using a luciferase reporter assay. The expression of *stm1987* was slightly reduced (1.3-fold) in D23580 compared to 14028 (Fig 3.7A). While no difference in *stm1987* expression was observed between *S.* Typhimurium 14028 wild-type and Δ*csgD* mutant strains (Fig 3.7B). The CsgD-independent regulation of *stm1987* was in contrast to *adrA*, which was reduced by 6-fold in D23580 compared to 14028 and remained at background expression levels in the Δ*csgD* strains (Fig 3.7C and D).

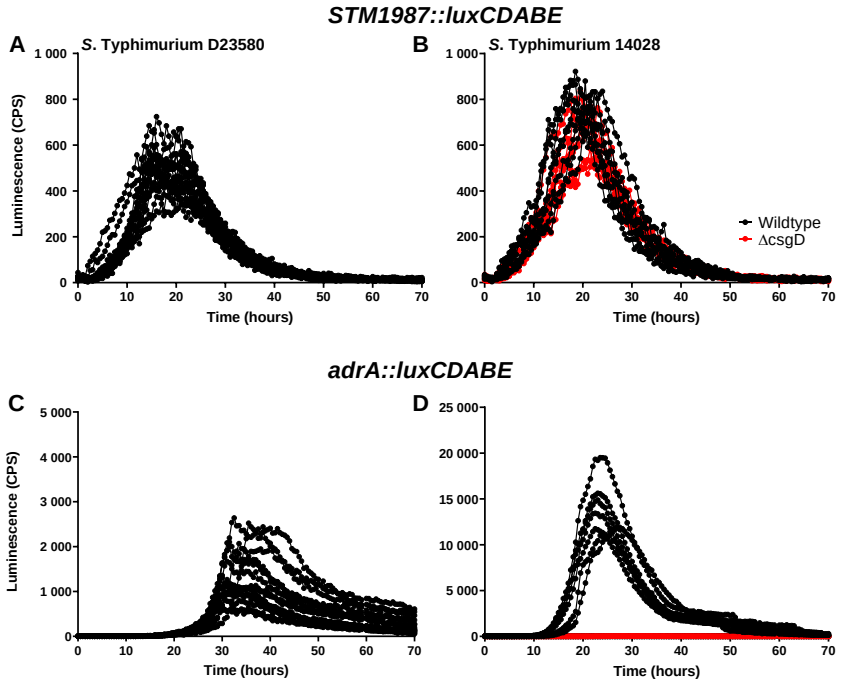

Figure 3.7: Transcription of *stm1987* is not regulated by CsgD in *S.* Typhimurium. A. and C. *S.* Typhimurium D23580, B and D. 14028 wildtype (black) or *csgD* mutant (red) strain contains a *stm1987*::luxCDABE (A, B) or *adrA*::luxCDABE (C, D) promoter fusion designed to measure gene expression by light production. For each reporter, shown are the luminescence values (counts per second, CPS) plotted as a function of time, with measurements recorded every 30 min during growth at 28ºC in 1% tryptone (A, B) or in LB without salt (C,D). Each expression curve corresponds to an individual biological replicate.

3.3.6 The CsgD-dependent pathway for cellulose production has no role in *S.* Typhimurium virulence.

We recently characterized several SNPs in invasive *S.* enterica isolates that reduced or prevented biofilm formation [136]. One of the conserved SNPs identified was a C to T transition in the promoter region of *csgD,* which abolished *csgD* expression in *S.* Enteritidis D7795. Correcting the *csgD* promoter SNP was enough to restore biofilm formation and cellulose production in this strain [136].

We wanted to investigate if lack of CsgD-dependent cellulose production would influence *S. enterica* virulence. Mice were challenged IP with *S.* Enteritidis D7795 or SNP-corrected *S.* Enteritidis D7795 (P*csgD*^ST14028). At 72 h post-infection, there was no colonization difference between the two strains in the organs tested (Fig 7A). However, similar to *S.* Typhimurium D23580, we hypothesized that it was difficult to determine the impact of a single SNP in the virulence of *S.* Enteritidis D7795 due to the presence of additional SNPs. To isolate the effects of this single mutation more carefully, we directly compared the virulence of *S.* Typhimurium 14028 and a strain carrying the inactivating *csgD* promoter SNP (i.e., *S.* Typhimurium 14028 (P*csgD*^D7795); [136]). We compared the virulence of these strains by performing CI infections in both oral and IP challenge models. There was no competitive difference between strains recovered from internal organs 4-7 days after oral challenge (Fig 7B) or 72 h after IP challenge (Fig. 7C). The overall conclusion from these experiments was that the ability of *S.* Typhimurium to form biofilms or to produce cellulose in a CsgD-dependent manner does not appear to affect virulence.

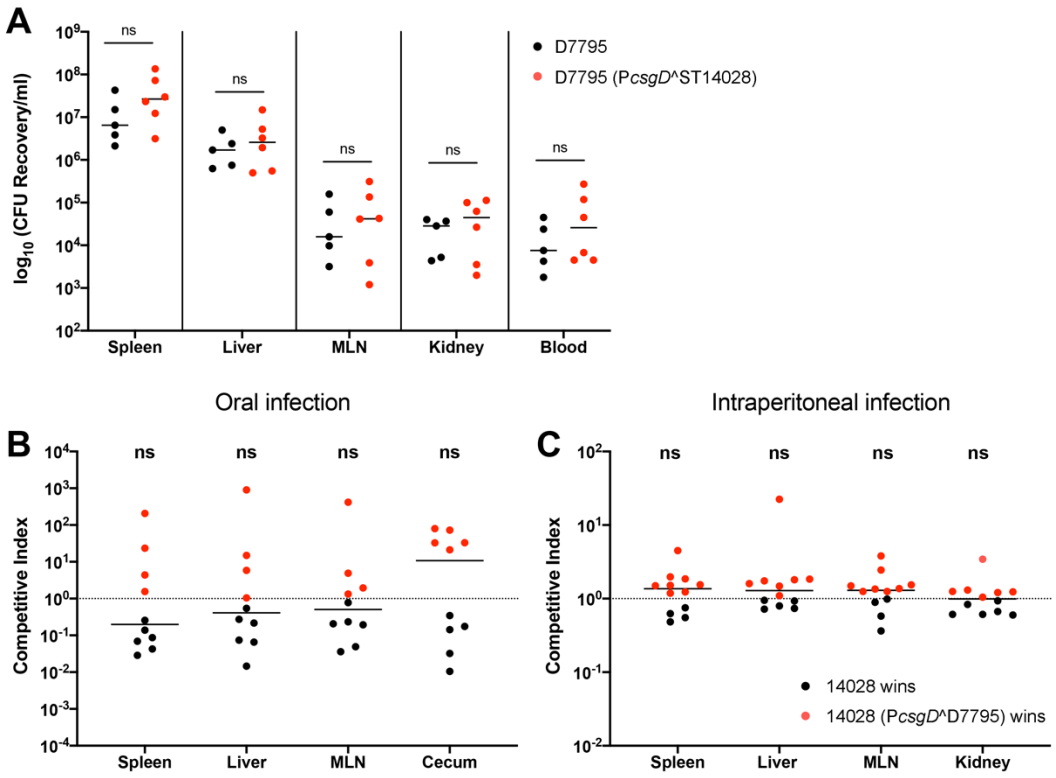

Figure 3.8: Examining the role of biofilm formation and CsgD-dependent cellulose production in *S.* Enteritidis and *S.* Typhimurium virulence. (A) C57BL/6 mice were infected IP with 10^4 CFU of *S.* Enteritidis D7795 or strain D7795 (P*csgD*^ST14028) and the CFU levels in liver, spleen, kidney, MLN, and blood were determined 72 h post-infection. (B and C) CI infections were performed in C57BL/6 mice challenged orally with 10^7 CFU (B) or IP with 10^4 CFU (C) of a 1:1 ratio of *S.* Typhimurium 14028 wild-type and 14028 (P*csgD*^D7795) strains. At 4-7 days (B) or 72 h (C) post-infection, bacteria were enumerated, and CI values were calculated for each mouse in each organ. CI = (CFU 14028 (P*csgD*^D7795)/14028)output/(CFU 14028 (P*csgD*^D7795)/14028)input. The horizontal dotted line represents a CI value of 1, or equal virulence between strains. Red circles represent mice where *S.* Typhimurium 14028 (PcsgD^D7795) won the competition. ns = not significant; p-value > 0.05.

3.4 Discussion

Invasive NTS serovars are thought to be evolving towards human adaptation through genomic degradation in the form of SNPs and the accumulation of pseudogenes, similar to typhoidal *Salmonella* serovars [48,115,139]. The difference between gastroenteritis- and invasive-*S.* Typhimurium strains reside in these genomic changes, some of which have been linked to the ability of invasive NTS serovars to cause systemic disease. In invasive *S.* Typhimurium D23580, we identified a SNP resulting in a missense mutation in the cache 1 signaling domain of DGC STM1987, that reduced cellulose biosynthesis and led to increased virulence in mouse models of infection. This SNP was conserved in both lineages of *S.* Typhimurium ST313 isolates that cause

invasive disease and was comparatively absent in global *S.* Typhimurium isolates that cause gastroenteritis.

There are at least two pathways for cellulose production in *Salmonella*: CsgD-dependent and CsgD-independent. Traditionally, cellulose production has been correlated with biofilm formation and persistence in non-host environments [140]. While initial studies indicated that cellulose had no role in virulence [118,120,140,141], it was recently shown that cellulose can be produced by *Salmonella* inside macrophages, serving as an anti-virulence factor [107,142]. These opposing functions imply a complex role for cellulose in *Salmonella* biology. In the context of biofilm formation, transcription factor CsgD promotes cellulose production by regulating the expression of the DGC AdrA [87,95,143]. Under some growth conditions, such as limiting nutrients, cellulose can be produced independently of CsgD [130]; this CsgD-independent pathway has been implicated in *S. enterica* colonization of plants [144] and biofilm formation on polystyrene surfaces [145]. A CsgD-independent pathway for cellulose production was first identified in *E. coli*, involving the STM1987 homologue YedQ [138]. All cellulose biosynthesis pathways converge at c-di-GMP-based activation of BcsA [123], so it makes sense that the uncoupling of cellulose production from CsgD regulation could occur at the level of c-di-GMP biosynthesis. Our gene expression assays showed that *stm1987* transcription was independent of CsgD in *S.* Typhimurium. Mills et al.[134] showed that STM1987 stimulates cellulose production in response to putative host signals such as arginine and the cache 1 signaling domain was critical for this response [134,146]. Since arginine is thought to be a key metabolic signal inside macrophages [131–133], this led us to investigate the effect of the amino acid change in the Cache 1 domain identified in *S.* Typhimurium D23580. The missense mutation in STM1987 decreased cellulose production and led to an increase in virulence, whereas a SNP that shut off CsgD-dependent cellulose production did not influence virulence. By regulating cellulose production via CsgD-dependent and CsgD-independent pathways, we speculate that *S. enterica* is able to separate cellulose production inside the macrophage and under biofilm inducing conditions.

It is challenging to identify changes that alter the pathogenesis of invasive *S.* Typhimurium isolates because of the numerous genomic changes they possess compared to standard

gastroenteritis-causing isolates [114,115,139]. Nevertheless, several changes have been associated with clear phenotypes. Hammarlof et al. [147] identified a SNP in the promoter region of *pgtE* that increased the expression of this outer membrane protease, which promoted survival and dissemination of ST313 isolates during infection [147]. Carden et al. [148] showed that pseudogenization of effector *sseI*, part of the *Salmonella* pathogenicity Isalnd-2 type 3 secretion system, contributes to the hyperdissemination of D23580 from the gut to systemic organs [148]. Ramachandran et al. [149] showed that ST313 isolates survive better inside murine and human macrophages compared to ST19 strains due to reduced flagellin production, although the specific genomic changes were not identified [149]. In our experiments with *S*. Typhimurium D23580, the effect of reduced STM1987 activity on virulence may be masked by the presence of other mutations. In particular, reduced cellulose production in strain D23580 has been linked to a premature stop codon in *bcsG* [136,150], which encodes a component of cellulose synthase that modifies the growing β-D- 1,4-glucan chain with phosphoethanolamine [137], and is required for cellulose production [151]. Based on the results of our intramacrophage survival assay we hypothesize that the existing mutation in *bcsG* masks any colonization advantage provided by the SNP in *stm1987* in *S*. Typhimurium D23580. The *stm1987* mutation is present in both lineage I and II ST313 isolates, whereas the *bcsG* mutation is only found in lineage II [136,150]. This indicates that the SNP in *stm1987* is the more ancient mutation and also suggests that adaptation could be a stepwise process, whereby the amount of cellulose produced by *S*. Typhimurium D23580 would be further reduced due to the presence of a *bcsG* mutation. A real-world example of adaptive evolution of iNTS strains was shown by Klemm et al., [48] who used comparative genomics to track changes over time in a *Salmonella* serovar Enteriditis strain causing recurrent infections in an immunocompromised, interleukin-12 β1 receptor-deficient patient [48] .

Several groups have attempted to establish animal models to evaluate the virulence of human adapted ST313 isolates. Following oral inoculation, ST313 isolates are able to colonize systemic organs in susceptible mice [115,152,153]. Carden et al.[148] used the streptomycin -treated mouse model to specifically analyze the rate of dissemination to systemic sites compared to *S*. Typhimurium ST19 strain SL1344 [148]. Parsons et al. [154] showed that following oral inoculation, ST313 isolates D23580 and Q456 cause a more invasive infection in chickens (i.e.,

spleen and liver) compared to ST19 isolates F98 and 4/74 [154]. However, their result was not reproduced by Lacharme-Lora et al., [155] who showed that ST313 strain D23580 was less virulent compared to ST19 strain 4/74 in both inbred and outbred chickens [155]. *S.* Typhimurium D23580 have also been shown to colonize chick embryos and *G. mellonella* larvae [155]. Rhesus macaques infected via the intragastric route developed only mild diarrhea, with no significant pathology in the liver, ileum, colon, and MLN [152]. Although these animal infection models demonstrate that ST313 strains can infect nonhuman hosts, it is not well known how each disease model relates to iNTS infection in humans, which is distinct from gastroenteritis and typhoid fever [10,23,112]. To evaluate the effect of *stm1987* SNP on virulence we infected susceptible C57BL/6 mice via the IP route. In this model, iNTS strains were able to rapidly colonize the systemic organs as well as disseminate to distant tissues via the bloodstream, confirming the results of other labs [78,115].

Due to better defined animal models and well characterized virulence properties of non-ST313 *S.* Typhimurium strains, we reasoned that moving iNTS mutations into these strains might be a better way to evaluate the effects of individual SNPs. In addition, because iNTS strains are typically resistant to multiple antibiotics [11,12,139], the use of sensitive non-ST313 strains allows for easier genomic modifications. In our case, we were able to show that the *stm1987* SNP conferred increased virulence compared to a wildtype strain, following both oral and IP routes of infection. We do not know why increased virulence was not observed in the spleen and liver after oral challenge. However, following oral ingestion it takes about 72 hours for *Salmonella* to reach the liver and spleen, when mice start to exhibit signs of infection [156]. It is possible that at later stages of infection the balance between virulence and host resistance to infection may favor *Salmonella* [157], hence replication may be largely unrestricted in a diseased mouse, making it more difficult to differentiate the colonization levels between strains. IP injection bypasses the oral invasion steps and is predicted to speed up access to macrophages and internal organs. For this reason, we concluded that the increased virulence of *S.* Typhimurium 14028 (*stm1987*^D23580) in all organs tested following IP injection was correlated with increased survival inside macrophages.

The importance of c-di-GMP in the lifecycle of pathogenic bacterial species is highlighted by our results. c-di-GMP has been implicated in diverse processes such as biofilm formation, motility,

cell cycle progression and virulence [146,158,159]. The domains for DGC activity (i.e., GGDEF) and PDE activity (i.e., EAL or HD-GYP), can be found separately or together in hybrid proteins that contain both domains; most *S.* Typhimurium strains encode 5 GGDEF, 7 GGDEF-EAL and 10 EAL domain proteins [160]. In general, low levels of c-di-GMP enhanced by the actions of PDEs promote *Salmonella* virulence in mice and increased survival inside macrophages [161], while high c-di-GMP levels enhanced by DGCs promotes biofilm formation and environmental persistence. The activities of most DGCs and PDEs are controlled by the sensory domains present in the N-terminal regions of the proteins [162]. Independent of transcription and translation, the sensory domains allow bacteria to quickly adapt to changing environmental conditions, such as oxygen, redox state, temperature, light, nutritional signals, and quorum sensing molecules. These sensory domains can be localized to the cytoplasm such as PAS (Per-Arnt-Sim), GAF and globin domains, associated with membranes such as MASE and MHYT domains, or in the periplasm such as CHASE, Cache 1 and CSS domains [162]. Sarenko et al. [163] showed that the DGC and PDE proteins in *E. coli* fall into two categories: 1) DGCs and PDEs that form a tightly interconnected network and regulate a particular phenotype such as biofilm formation, or 2) distinct enzymes that are not connected to other proteins but contribute to controlling the overall cellular pool of c-di-GMP under specific conditions [163]. The specificity of the signaling domain may be the main indicator of the conditions under which distinct DGCs and PDEs are induced. For example, the cache 1 domain of STM1987 appears to have a critical role for responding to intracellular signals inside macrophages, hypothesized to be arginine [134]. It was somewhat surprising that a single amino acid change in the cache 1 domain altered the overall enzymatic activity, independent of the GGDEF domain. This shows that for pathogenic bacteria, mutations in the signaling domains can alter the activity of cyclic-di-GMP associated enzymes. For sensory domains like PAS, that can detect a broad diversity of environmental signals, such as molecular oxygens, light, small metabolites and temperature [162], it is possible that mutations could increase or limit the types of signals detected. Such mutations or SNPs in signaling domains could contribute to bacterial evolution in terms of adaption to new ecological niches and/or hosts.

The lifecycle of *Salmonella* involves exposure to both host and non-host environments. Cellulose formation is crucial for the transmission of *Salmonella* because it functions with other biofilm

components to enable long-term survival in the non-host environment [133]. Similarly, we propose that *Salmonella* produces cellulose during infection to better survive the harsh intracellular environment of a macrophage. *Salmonella* can cause an acute or chronic infection in the host. An acute infection is characterized by hypervirulence, killing of the host, and transmission to a new host, while a chronic infection is characterized by reduced virulence and long-term survival in the host. It is likely that under certain conditions *Salmonella* establishes a chronic infection, by producing cellulose inside the macrophage to limit replication and prolong long-term survival. However since infection is a balance between pathogen virulence and host resistance to infection, we speculate that c-di-GMP and or cellulose are PAMPs that activate the host immune response, therefore *Salmonella* isolates that produce high levels of c-di-GMP/cellulose have reduced survival because they are killed by the host immune response. C-di-GMP is an immunostimulatory molecule that can activate the innate immune response [164,165]. It is possible that inside the macrophage invasive *S.* Typhimurium isolates have a survival advantage compared to gastroenteritis causing isolates, because reduced c-di-GMP/cellulose production due to mutations in *stm1987* and *bcsG*, decrease their ability to stimulate an immune response while maintaining their ability to survive the harsh intracellular environment of a macrophage. ST313 invasive isolates have been shown to be more resistant to killing by murine and human macrophages compared to gastroenteritis causing ST19 strains [150]. Several studies have also shown that the inflammatory response induced by iNTS isolates is reduced compared to NTS [113,149].

In conclusion, our results indicate that a major role for STM1987, and the CsgD-independent pathway for cellulose production, is the regulation of *S. enterica* survival and virulence inside macrophages. We have recently shown that CsgD-dependent biofilms are formed during acute infections in mice, with curli fimbriae detected in the distal GI tract [166]. Since curli fimbriae are a pathogen associated molecular pattern (PAMP) that activate toll-like receptors (TLR-1 and TLR-2) and intracellular NOD like receptors [167–169], it is tempting to speculate that cellulose could also play a role in this. How the CsgD-dependent and -independent pathways fit within the fine-tuned pathogenesis of *S. enterica* strains is still up for debate and requires further investigation.

3.5 Materials and Methods

3.5.1 Bacterial strains, media, and growth conditions

The bacterial strains used in this study are listed in Table S3.1. For standard growth, strains were inoculated from frozen stocks onto LB agar (lysogeny broth, 1% NaCl, 1.5% agar) supplemented with an appropriate antibiotic (50 µg mL^{-1} kanamycin (Kan), 34 µg mL^{-1} chloramphenicol (Cam) or 5 µg mL^{-1} tetracycline (Tet)) and grown overnight at 37˚C. Isolated colonies were used to inoculate 5 mL LB broth and the culture was incubated for 18 hours at 37˚C with agitation at 200 rpm. To visualize cellulose production, 2 µl aliquots of overnight culture were spotted on 1% tryptone agar supplemented with calcofluor white (fluorescent brightener 28; Sigma-Aldrich; 200 µg mL^{-1}) [130]. To evaluate cellulose production by crystal violet staining, 5 x 10^6 cells were inoculated into 5 mL LB broth and incubated at 37°C for 18 hours with agitation at 200 rpm. For macrophage survival assays, cells from overnight cultures were diluted 1:100 into fresh LB and grown at 37°C until the cultures reached an optical density of 0.7 at 600 nm. For murine infection experiments, overnight cultures grown in LB broth were diluted to the desired CFU concentration in 100 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), pH 8, and used to infect mice.

3.5.2 Generation of *S.* Typhimurium 14028 mutant strains

The *S.* Typhimurium 14028 Δ*stm1987* and *S.* Typhimurium 14028 Δ*stm1987* Δ*bcsA* were generated using the lambda red recombinase knockout procedure [170]. Primers STM14_2608-ko_sense and STM14_2608-ko_antisense or bcsA-ko_sense and bcsA-ko_antisense containing 50-nucleotide sequence on either side of the *stm1987* or *bcsA* gene (Table S3.2) were used to amplify the *cat* gene from pKD3 using Phusion High-Fidelity DNA polymerase (New England BioLabs). The PCR products were purified and electroporated into *S.* Typhimurium 14028 cells containing pKD46. Mutant strains were first selected by growth at 37˚C on LB agar supplemented with 7 µg mL^{-1} Cam, before re-streaking onto LB agar containing 34 µg mL^{-1} Cam. The mutations were moved into a clean strain background by transduction with P22 phage [171]. The *cat* gene was resolved from the chromosome using pCP20 [170]. Sanger DNA sequencing of PCR products amplified from the chromosome of the Δ*stm1987* strain using primers STM1987detect1 and STM1987detect2, confirmed the loss of the *stm1987* open reading

frame, while primers bcsAdetect1 and bcsAdetect2 were used to confirm the loss of *bcsA* open reading frame.

3.5.3 Cellulose quantification using crystal violet staining

In vitro production of cellulose by *S.* Typhimurium was visible as a ring of adherent cells at the air-liquid interface. After 24 h growth, the culture broth was discarded, and the culture tubes were washed twice with water to remove any non-adherent cells, before air drying for 10 min. 8 mL of crystal violet (CV) solution (0.1% wt/vol,) was added to each tube to completely cover the ring of adherent cells, and tubes were incubated for 10 min at room temperature (RT). The CV solution was removed, and tubes were washed twice with water, before air drying for 10 min. To dissolve bound CV, 8 mL of 95% ethanol was added to each tube and incubated at RT for 15 min. The optical density of the resulting solution was measured at 590 nm.

3.5.4 Reference genome sequences and identification of polymorphism in *S.* Typhimurium strains

Whole-genome sequences were obtained from the National Centre for Biotechnology Information (NCBI) via the following accession numbers: NC_016856 (*S.* Typhimurium 14028) and NC_016854 (*S.* Typhimurium D23580). Genome assemblies of *S.* Typhimurium isolates from sub-Saharan Africa and different parts of the world were investigated for a SNP in *stm1987* through in silico PCR. Names, accession numbers, and meta-data pertaining to all strains that were screened are listed in Table S3.

3.5.5 Generation of bacterial luciferase reporters and pBR322-STM1987 plasmid vectors

To generate a luciferase reporter to measure *stm1987* expression, the promoter-containing DNA region was PCR amplified from *S.* Typhimurium 14028 using primers STM14_2408for1 and STM14_2408rev2 (Table S2), and Phusion high-fidelity DNA polymerase (New England BioLabs), with reaction conditions outlined by the manufacturer. The resulting PCR product was purified, sequentially digested with *Xho*I and *Bam*HI (New England BioLabs), and ligated using T4 DNA ligase (New England BioLabs) into the pCS26 vector cut with *Xho*I and *Bam*HI. Primers pZE05 and pZE06 were used to verify the successful fusion of *stm1987* promoter region to *luxCDABE*. The generation of *csgBAC* or *adrA* luciferase fusions have been previously described [118,172].

To generate pBR322/*stm1987* plasmid vectors, DNA fragments containing *stm1987* with native promoters were PCR amplified from genomic DNA of *S.* Typhimurium 14028 or *S.* Typhimurium D23580, using primers STM1987forEco and STM1987revAatII (Table S2). PCR products were purified, digested with *Eco*RI and *Aat*II, and ligated into digested pBR322 using T4 DNA ligase.

3.5.6 Luciferase reporter assays

For bioluminescence assays, overnight cultures of reporter strains of *S.* Typhimurium were diluted 1 in 600 in 1% tryptone supplemented with 50 μg mL^{-1} Kan, to a final volume of 150 μl per well in 96 well clear bottom black plates (9520 Costar; Corning Inc). To minimize evaporation of the medium during the assay, cultures were overlaid with 50 μL of mineral oil. Cultures were assayed for absorbance (600 nm, 0.1 s) and luminescence (1s; in counts per second [CPS]), every 30 min during growth at 28˚C or 37°C with agitation in a Victor X3 multilabel plate reader (Perkin-Elmer).

3.5.7 Genome engineering in *Salmonella* Typhimurium 14028 and D23580

The I-*Sce*I suicide plasmid system [173] was used for genome engineering to either introduce or correct the *stm1987* SNP in *S.* Typhimurium strains. The *stm1987* gene was PCR-amplified from *S.* Typhimurium 14028 and D23580 using primers STM1987forEco and STM1987revHind (Table S2) and Phusion high-fidelity DNA polymerase (New England BioLabs). Purified PCR products were digested with *Bam*HI and *Pst*I (New England Biolabs) and ligated into *Bam*HI/*Pst*I-digested pSEVA212. Two clones were selected in *E. coli* S17-1 (λpir) and used for all subsequent experiments. Purified pSEVA212/*stm1987* constructs were transformed into *S.* Typhimurium 14028 or D23580 by mating, with selection for growth on M9 minimal agar supplemented with 1mM MgSO4, 0.2% glucose and 100 μg mL^{-1} Kan (M9-Glc-Kan100). Merodiploid strains with pSEVA212/*stm1987* plasmid inserted into the genome were confirmed by re-streaking onto M9-Glc-Kan100 agar. 200-300 ng of purified pSEVA628S [174] was transformed into merodiploid strains by electroporation with selection on LB agar supplemented with 1 mM m-toluate and 20 μg mL^{-1} gentamicin. Resulting colonies were re-streaked onto LB agar supplemented with 20 μg mL^{-1} gentamicin, streaked on LB agar supplemented with 50 μg mL^{-1} Kan to confirm the loss of the pSEVA212 plasmid, and streaked on TCR plates (1% tryptone, 1.5% agar, 40 μg mL^{-1} Congo red) to check the biofilm phenotype. Colonies were

selected from TCR plates and grown at 37°C for two overnight growth steps without gentamicin to generate cells that lack pSEVA628S. Final colonies were streaked onto (1) LB agar, (2) LB agar + 50 μg mL^{-1} Kan, (3) LB agar + 20 μg mL^{-1} gentamicin, and (4) TCR plates to select the desired phenotypes. At this stage, two colonies were chosen for further analysis. Primers STM1987checkFOR and STM1987checkREV (Table S2) were used to confirm the presence or absence of the SNP in *stm1987* at nucleotide 566. The *S.* Typhimurium 14028 and *S.* Enteritidis D7795 strains containing the *csgD* promoter SNP into genomes have been previously described [136].

3.5.8 Murine infection Experiments

Six to eight-week-old female C57BL/6 mice were purchased from Jackson Laboratory (Bar Harbor, ME) and assigned to cage groups using a randomization table prepared in Microsoft Excel; individual mice were marked with ear notches.

To determine the optimal time point for evaluation of iNTS colonization, two groups of six mice were challenged IP with ~10^4 CFU of *S.* Typhimurium D23580 or *S.* Enteritidis D7795 strains containing the Kan^r sig70_16-*luxCDAB*E construct. At 24 h, 48 h and 72 h post-infection, two mice from each group were humanly euthanized. In subsequent infections, groups of 6 mice were challenged intraperitoneally with ~10^4 CFU of *S.* Typhimurium D23580 (with or without *stm1987* SNP) or *S.* Enteritidis D7795 (with or without P*csgD* SNP), each containing the Kan-resistant, sig70_16-l*uxCDABE* construct [175].

For competitive index (CI) experiments, two groups of six mice were challenged with a mixed inoculum consisting of a 1:1 ratio of *S.* Typhimurium 14028 strains (i.e., wild-type vs. *stm1987*^D23580, wild-type vs. P*csgD*^D7795) containing either Kan^r or Cam^r sig70_16 *luxCDABE* constructs. Mice were challenged via oral gavage with 10^7 CFU or IP with 10^4 CFU; animal care technicians assigned challenges to groups of mice. The cage assignments were revealed to members of the White laboratory only after the final results were analyzed.

For all infection trials, mice were weighed daily and monitored for clinical signs of infection. Mice that had a >20% drop in weight were humanely euthanized. Mice infected via oral gavage were euthanized 5-7 days post-infection, and spleen, liver, mesenteric lymph nodes (MLN), and

cecum were collected. Mice infected IP were humanely euthanized 72 h post-infection, and spleen, liver, MLN, kidney, and blood were collected. Collected organs were placed in a 2 ml Eppendorf Safe-Lock tube containing 1 ml of phosphate-buffered saline and a 5-mm steel bead (Qiagen #69989), and homogenized using a mixer mill (Retsch; MM400) for 5 min at 30 Hz. To determine the number of CFU in the organ homogenates, serial dilutions were plated on LB agar supplemented with 50 μg mL^{-1} Kan or 20 μg mL^{-1} Cam. Organs collected from mice in the CI experiments were plated on both Kan and Cam agar. The same procedure was performed on initial challenge doses to ensure the proper inoculation size.

3.5.9 Ethics statement

All animals were cared for and used 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 20190071, which was approved by the University of Saskatchewan's Animal Research Ethics Board.

3.5.10 Macrophage Growth Conditions

THP-1 monocytes were maintained in RPMI complete (Gibco 11875085) supplemented with 10% heat-denatured FBS (fetal bovine serum; SAFC Biosciences #12103C). The monocytes were differentiated with PMA (phorbol-12-myristate-14-acetate ester) and seeded at 1.25x105 cells/0.5mL/well into 48-well plates 3 days prior to infection. Upon PMA treatment, THP-1 monocytes will differentiate into adherent macrophage-like cells and stop dividing. One day before *S.* Typhimurium infection, after the third day of treatment with PMA, media was replaced with fresh RPMI.

RAW 264.7 macrophages were grown in DMEM (Dulbecco's Modified Eagle's Medium) (MilliporeSigma #D5796) supplemented with 10% heat denatured FBS, 1mM sodium pyruvate and 50 μg mL^{-1} gentamicin. Prior to seeding cells into multi-well plates, the cells were washed with versene, centrifuged at 1200 rpm, and resuspended in DMEM with 10% FBS (without antibiotics). RAW 264.7 cells were seeded at 1.25 x 10^5 *S.* Typhimurium cells per 0.5mL per well in a 48-well plate. The cells were allowed to adhere for at least 3 hours before infection.

3.5.11 Macrophage survival assay

Approximately 1.25 x 10^5 macrophages were infected with *S.* Typhimurium at a multiplicity of infection (MOI) of ~100 for RAW 264.7 and ~10 for THP-1 macrophages. Infected macrophages

were centrifuged at 1000 rpm for 1 min and incubated at 37°C for 30 mins. Following *S.* Typhimurium internalization, macrophages were washed twice with warm PBS, and medium containing 20 µg mL-1 gentamicin was added to kill extracellular bacteria. Macrophages were lysed with 0.1% Triton X-100, at 30 mins and 18 hours post-infection to enumerate intracellular *S.* Typhimurium. To determine the number of intramacrophage bacteria, lysed cell mixtures were serial diluted and plated on LB agar or LB agar supplemented with 5 µg mL-1 Tet. Percent macrophage survival was calculated as follows: [(CFU of bacteria recovered at 18 h) / (CFU of bacteria recovered at 30 min) x 100]

3.5.12 Statistical analysis

Statistical analysis was performed using GraphPad Prism version 8.0. The normality of each data set was first assessed using the Sharpiro-Wilk normality test. For crystal violet assay the mean difference between groups was compared using an ordinary one-way ANOVA with post-hoc analysis via Holm-Sidak's multiple comparison test. For murine infections, if data sets were normally distributed, comparisons were performed using unpaired t-test with Welch's corrections. If any data sets were not normally distributed, comparisons were performed using the Mann-Whitney test. For CI experiments, if the data was not normally distributed the Wilcoxon signed-rank test was used to determine if the median CI was significantly different from 1.0. If the data was normally distributed one-sample t-test was used to determine if the mean was significantly different from 1.0. For macrophage survival assays, if the data were normally distributed, comparisons of the percent survival from multiple experiments were performed using unpaired t-test. If the data was not normally distributed, comparisons were performed using the Mann-Whitney test. In all cases, statistical significance was set at $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$, ns $p > 0.05$.

3.6 Supplementary information

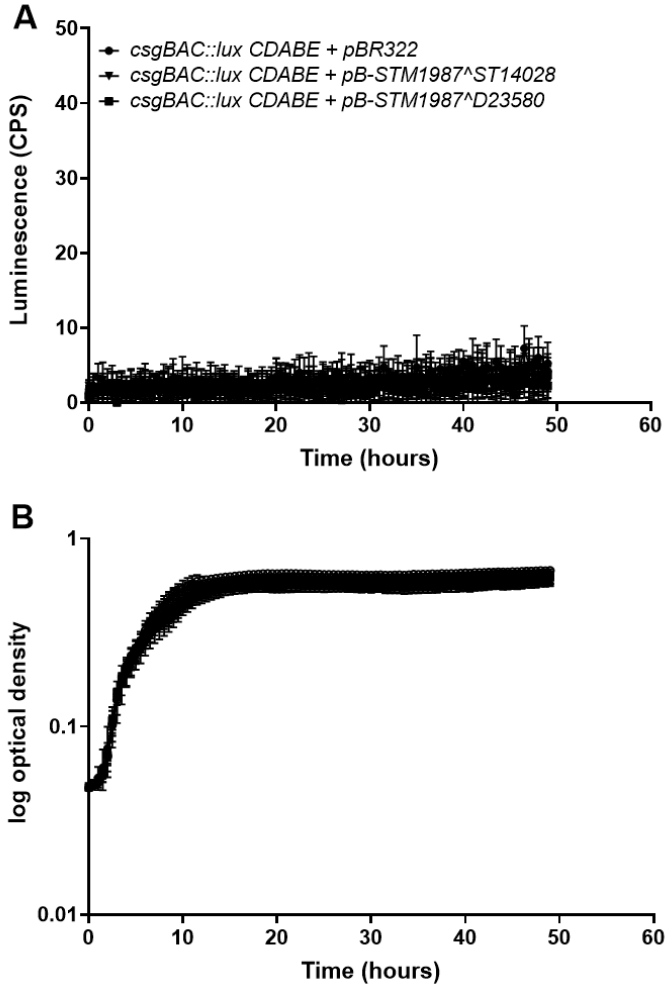

Figure S3.1: The curli biosynthesis operon is not expressed at 37°C. The *S.* Typhimurium 14028 Δ*stm1987* strain, containing a plasmid-based *csgBAC* promoter luciferase (*luxCDABE*) fusion designed to measure gene expression by light production, was transformed with pBR322, pBR322/STM1987^14028 or pBR322/STM1987^D23580. Strains were grown in LB broth for 48 hours at 37°C. A. Curve represent the raw, non-normalized gene expression values (light counts per second; CPS) in each culture as a function of time. B. Curves represent the optical density values (measured at 595 nm) of the cultures as a function of time.

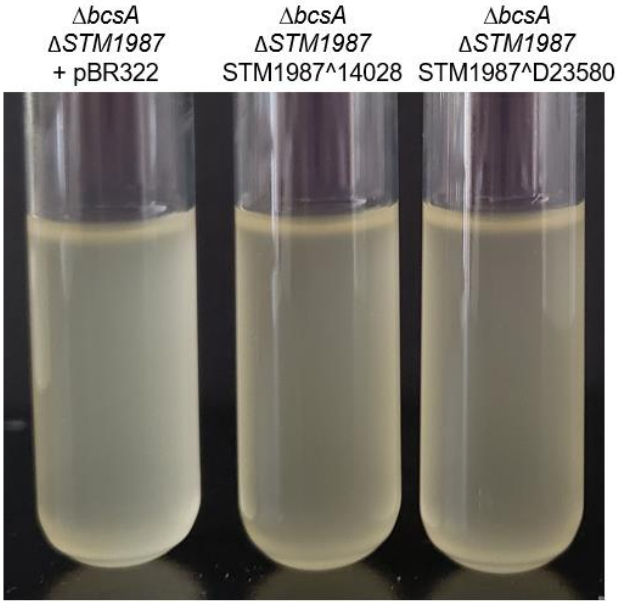

Figure S3.2: Phenotypic comparison of STM1987 alleles from *S.* Typhimurium 14028 and D23580. *S.* Typhimurium 14028 Δ*bcsA* Δ*stm1987* was transformed with pBR322 (control) or pBR322 harbouring the STM1987^14028 or STM1987^D23580 allele. Strains were grown in LB broth for 18 h at 37°C; and lack of cellulose production was determined by the absence of adherent ring of cells at the air-liquid interface.

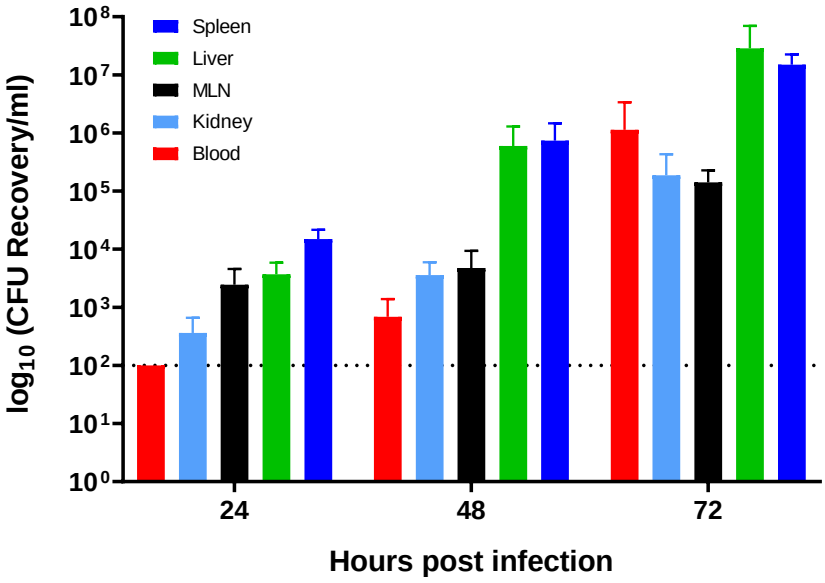

Figure S3.3: Colony forming units (CFU) of *Salmonella* recovered from mice at 24-72 hours post-infection. C57BL/6 mice infected intraperitoneal with *S.* Enteriditis D7795 or *S.* Typhimurium D23580 were euthanized 24, 48- or 72-hours post-infection. Blood, Kidney, MLN, Liver and Spleen were collected from euthanized mice, homogenized and plated on LB agar supplemented with 20 µg ml^{-1} CAM to enumerate the number of *Salmonella* recovered from infected mice 24, 48 or 72 hours post-infection.

Table S3.1: Strains and plasmids used in this study

Strains or plasmids	Genotype	Source or Reference
Strains		
S. Typhimurium 14028	Wild-type strain	ATCC
STM1987$_{(D23580)}$	*STM1987* from D23580	This study
Δ*STM1987*	Deletion of *STM1987* ORF	This study
P*csgD*$_{OFF}$ (-47 C>T)	P*csgD* SNP from D7795	[136]
Δ*csgD*	Deletion of *csgD* ORF	[176]
Δ*bcsA*	Deletion of *bcsA* ORF	[118]
S. Typhimurium D23580	Wild-type strain	G. Dougan [139]
STM1987$_{(14028)}$	*STM1987* from ST10428	This study
S. Enteritidis D7795	Wild-type strain	G. Dougan [12]
P*csgD*$_{ON}$ (-47 T>C)	P*csgD* from 14028	[136]
P*csgD*$_{OFF}$ (-47 T>T)	Native P*csgD*[a]	[136]
Plasmids		
pBR322/*STM1987*^14028	STM1987 from ST10428	This study
pBR322/*STM1987*^D23580	STM1987 from D23580	This study
pCS26	Bacterial luciferase	[177]
pCS26-*STM1987*	*STM1987* promoter	This study
pCS26-*adrA*	*adrA* promoter	[118]
pCS26-*csgB*	*csgBAC* promoter	[118]

Table S3.2. Oligonucleotides used in this study.

Primer	Sequence (5'-3')[a]	Purpose
STM14_2408-ko_sense	GTGCCGCACGAAACACTGTTAGACAATCAAGGCTGGTTTAAAAAGCTGGCGTGTAGGCTGTAGCTGCTTC	To amplify *cat* gene product from pKD3 to generate Δ*STM1987* strain by lambda-red recombination
STM14_2408-ko_antisense	GAATGGACTATTTCTTTTCCCGCTCCTGAGTCGCGTCGCTGGCGCAAATACCTCCTTAGTTCCTATTCCG	
STM1987detect1	TACCGTAAGCCATCAGGGGG	To confirm the deletion of *STM1987* from the genome of ST10428
STM1987detect2	TAAACCGGGAGGGCGTACAT	
STM14_2408for1	GATCCTCGAGAAATTCGCGGTGTTTCGCAC	Used to amplify *STM1987* promoter region from ST10428
STM14_2408rev2	GATCGGATCCCTAACAGTGTTTCGTGCGGC	
bcsA-ko_sense	CATGATGCGGGCGACAAAACGTCCGCCGGGAGCCTGCGATGAGCGCCCTTGTGTAGGCTGGAGCTGCTTC	To amplify *cat* gene product from pKD3 to generate Δ*bcsA* strain by lambda-red recombination
bcsA-ko_antisense	CTATTACCGCCGCACACATCCAGGACAATTTTCTTTTCATCGCATTATCACCTCCTTAGTTCCTATTCCG	
bcsA detect 1	TTCATCGCTGCGCATACCAA	To confirm the deletion of *bcsA* from the genome of ST10428
bcsA detect 2	CTTACGCATTGGCAGTCAGTT	
pZE05	CCAGCTGGCAATTCCGA	Used to verify fusion of *STM1987* promoter region to *luxCDABE*
pZE06	AATCATCACTTTCGGGAA	

STM1987forEco	GATCGAATTCAAACGGTGTTTCGCAC	To amplify *STM1987* with native promoters from *S.* Typhimurium strains for cloning into pBR322.
STM1987revAatII	GATCGACGTCGGACTATTTCTTTTCCCGCT	
STM1987revHind	GATCAAGCTTGGACTATTTCTTTTCCCGCT	Used with STM1987forEco primer to amplify STM1987 from *S.* Typhimurium strains for genome engineering
STM1987checkFOR	CAACGGTATGCATGAAGC	Used to confirm the presence or absence of the SNP at nucleotide 566 in *STM1987* in *S.* Typhimurium strains after genome engineering.
STM1987checkREV	TAATTATCGTGATCCAGCGG	

[a] Nucleotide sequences corresponding to restriction enzyme sites are underlined.

4.0 Interface

Invasive nontyphoidal *Salmonella* (iNTS) that cause bloodstream infections differ from standard gastroenteritis causing strains by >700 SNP. These SNPs contribute to the undergoing genomic degradation of iNTS, which are thought to be evolving towards host adaptation in a manner similar to human restricted TS serovars [12,115,139,178]. Current research is focused on correlating SNPs with the altered phenotypes and pathogenesis of iNTS serovars. In the previous chapter I described a SNP in diguanylate cyclase *stm1987* that led to increased *Salmonella* virulence and survival inside macrophages. iNTS and NTS serovars are major public health concerns, and due to the high fatality rate associated with diseases (primarily bloodstream infections) and increasing numbers of antibiotic resistance isolates [10,179] vaccines are urgently needed to reduce their prevalence.

NTS is endemic worldwide, while iNTS mainly reported in sub-Saharan Africa, is increasingly being reported in other parts of the world [23,180]. Compared to NTS serovars, iNTS serovars are more invasive and cause a systemic infection. However, both serovars share greater than 3000 genes, and similar antigens induce a protective immune response in humans [71,72,114]. Hence a single vaccine can be used to reduce the prevalence of bloodstream infections and gastroenteritis. Live attenuated vaccines are not recommended against NTS infections, due to the prevalence of iNTS in immunocompromised individuals, hence subunit vaccines are the focus of vaccine development. The next chapter is focused on the development of an extracellular polysaccharide-based vaccine against NTS serovars. The polysaccharides that have been identified in NTS are cellulose, colanic acid and O-Ag capsule. Although cellulose is likely produced during infection as an antivirulence factor [107], it is not an ideal vaccine candidate because, it is ubiquitous and may not induce a *Salmonella* specific immune response. In the next section I describe how random mutagenesis aimed at increasing the amount of O-Ag capsule produced by *S.* Typhimurium, led to increased colanic acid production. Immunization with colanic acid did not provide protection, however immunization with GMMAs purified from colanic acid overproducing bacteria induced a partially protective immune response against *S.* Typhimurium in a mouse model of infection.

5.0 A GMMA-EPS based vaccine for Non-typhoidal *Salmonella*

Akosiererem S. Sokaribo[1,2], Sumudu Perera[1,2], Zoe Sereggela[1,2], Ryan Krochak[2], Melissa Palmer[1,2], Lindsay Balezantis[1,2], Fangning Liu[2], Xiaohui Xing[3], Shirley Lam[1], Will Deck[1], Wade Abbott[3], Sam Attah-Poku[1], Shantanu Tamuly[4], Aaron P. White[1,2*]

Affiliations:

[1] Vaccine and Infectious Disease Organization-International Vaccine Centre, Saskatoon, Saskatchewan, Canada

[2] Department of Biochemistry, Microbiology and Immunology, University of Saskatchewan, Saskatoon, Saskatchewan, Canada

[3]Agriculture and Agri-Food Canada, Lethbridge, AB, Canada

[4]Department of Veterinary Biochemistry, College of Veterinary Science, Assam Agricultural University, Khanapara, Guwahati, Assam, India

To be submitted to MDPI vaccines

* Corresponding author e-mail: aaron.white@usask.ca

Author contributions

Akosiererem S. Sokaribo: Designed and performed experiments, analyzed data, and wrote the manuscript.

Sumudu Perera: Performed luciferase assays

Zoe Sereggela and Ryan Krochak: Assisted with exopolysaccharide purification

Melissa Palmer, Lindsay Balezantis and Fangning Liu: Assisted with animal trial experiments.

Xiaohui Xing and Wade Abbot: Performed monosaccharide analysis on purified exopolysaccharides.

Shirley Lam: Assisted in endotoxin removal from purified exopolysaccharide using Triton X-114.

Will Deck and Sam Atah-Poku: Purified exopolysaccharide using anion exchange and size exclusion chromatography.

Shantanu Tamuly: Assisted with purification of GMMAs and animal trial experiments

Aaron P. White: Disigned experiment, analyzed data, edited manuscript and obtained funding that supported this research

5.1 Abstract

Nontyphoidal *Salmonella* are a major cause of gastroenteritis worldwide, and bacteremia in sub-Saharan Africa with a high fatality rate. No vaccine is currently available for human use. Current vaccine development strategies are focused on extracellular polysaccharides present on the surface of nontyphoidal *Salmonella*. This study aimed to boost the amount of extracellular polysaccharide (EPS) purified from *S.* Typhimurium for immunization trials. Random mutagenesis with Tn*10* transposon increased the production of EPS colanic acid, by 10-fold compared to the wildtype production. Immunization with colanic acid or colanic acid conjugated to truncated glycoprotein D or inactivated diphtheria toxin did not induce a protective immune response in mice. However, immunization with Generalized Modules for Membrane Antigens (GMMA) isolated from colanic acid overproducing isolates induced a partially protective immune response against *Salmonella* in mice. Our results support the development of a GMMA-EPS based vaccine against nontyphoidal *Salmonella*.

5.2 Introduction

Salmonella enterica species are the major cause of foodborne diseases; typhoid fever and gastroenteritis [1]. Typhoid fever, common in developing countries is mainly caused by typhoidal *Salmonella* (TS) serovars: *S.* Typhi and *S.* Paratyphi [5]. Gastroenteritis occurs worldwide and is primarily caused by Non-typhoidal *Salmonella* (NTS) serovars: *S.* Typhimurium and *S.* Enteritidis [5]. The global incidence of NTS gastroenteritis is estimated at ~94 million cases annually with 155,000 deaths; ~80.3 million cases are contracted via food-borne transmissions [18]. NTS gastroenteritis is usually self-limiting in immunocompetent individuals and severe cases can be treated with antibiotics. However, there is an increase in multi drug-resistant isolates [2], and in sub-Saharan Africa, NTS serovars associated with gastroenteritis globally, are the leading cause of bloodstream infections that are distinct from typhoid fever [10,181]. A protective vaccine against NTS serovars, and implementation of effective food safety procedures, are needed to reduce the global incidence of gastroenteritis.

Extracellular polysaccharides (EPS) are carbohydrate-based polymers synthesized and secreted into the external environment by bacteria [182]. They can be present on the cell surface as slime with no visible attachment, as capsular polysaccharide covalently linked to the bacterial surface or

as lipopolysaccharides (LPS) anchored to the cell surface via lipid A [182]. EPS have been implicated in various biological processes such as, resistance to desiccation, protection against predation, biofilm formation and evasion of immune response [183]. Bacterial EPS are composed of regularly repeating units of one or more monosaccharides linked via glycosidic bonds. They can take the form of homo- or heteropolymers. Homopolymers are composed of identical monosaccharide, while heteropolymers are composed of two or more different monosaccharides [184]. EPS are incredibly diverse in terms of their structure and composition, because they can be composed of different monosaccharides connected by varying glycosidic linkages and modified with a diverse array of non-carbohydrate residues [184]. Based on serology, bacterial EPS can be divided into LPS O-antigen and capsular polysaccharide K antigen [185,186]. Approximately 170 different O-antigens and ~80 K-antigens have been identified in *E. coli*. Approximately 90 and 79 K-antigens have been identified in *Streptococcus pneumoniae* and *Klebsiella* spp respectively [187]. Due to their surface location, EPS stimulate immune responses to bacteria and are optimal targets for vaccine development. Several EPS-based vaccines have been developed and are currently licensed for use in humans.

The EPS that have been identified in *Salmonella* are cellulose, enterobacterial common antigen (ECA), colanic acid, Vi polysaccharide, LPS and O-Ag capsule. Cellulose composed of β (1 - 4) linked D-glucose units is one of the major components of biofilms, important for long-term survival and environmental persistence of *Salmonella* [118]. ECA is a glycolipid found in the outer leaflet of the outer membrane of all enterobacteriaceaes, and it is involved in *Salmonella* resistance to bile [188,189]. Colanic acid composed of repeating units of glucose, galactose, fucose and glucuronic acid decorated with acetate and pyruvate [190,191] is produced at temperatures below 30°C and is throught to be involved in the survival of adverse environmental conditions outside the host [87]. The Vi polysaccharide is a linear homopolymer of (1 – 4) α-D-galacturonic acid with N- and O-acetylation at its O2 and O3 positions. It is only present in *S*. Typhi, where it plays a critical role in resistance to phagocytosis and complement mediated killing. It induces a protective immune response against *S*. Typhi and is currently the basis of several licensed subunit vaccines against typhoid fever [192]. LPS consists of the lipid A region, core oligosaccharide and O-antigen (O-ag). As a major component of the outer membrane of Gram-negative bacteria, it plays a major role in bacterial pathogenesis, host adaptation, and stimulation of an immune response [193]. *Salmonella* LPS O-ag is comprised of tetrasaccharide repeating units of mannose, rhamnose,

galactose and tyvelose or abequose. O-Ag capsule is a group IV capsular polysaccharide comprised of >2300 repeating tetrasaccharide units similar to LPS O-ag with some structural modifications: the galactose and tyvelose or abequose residues are glucosylated [96,194]. O-Ag capsule has been implicated in the colonization and persistence of *Salmonella* on plant surfaces [195,196], biofilm formation on gallstones and cholesterol coated surfaces [90], resistance to desiccation [96], and resistance to killing by human immune serum in *vitro* [197]. Since LPS O-ag is the target of protective immunity against NTS [77], EPS-based vaccines are currently being developed against NTS serovars.

Outer membrane vesicles (OMVs) are spherical outer membrane blebs spontaneously released by Gram-negative bacteria [198,199]. OMVs are comprised of proteins, EPS and lipoproteins present on the outer membrane, and are released in all growth phases, and during infection [199]. OMVs are involved in bacteria-bacteria and bacteria-host interactions, and have been implicated in quorum sensing, nutrient acquisition, protein export, horizontal gene transfer, bacterial pathogenesis, and immune modulation [198,200]. OMVs are attractive vaccine candidates because they present antigens in their natural context to the immune system; however, the amount of spontaneously release OMVs are too small for vaccine development [199]. Genetic modifications that disrupt the integrity of the inner and outer membrane have been shown to greatly enhance the production of OMVs [201,202]. OMVs released from genetically modified bacteria are called Generalized Modules for Membrane Antigens (GMMAs) [203]. Deletion of *tolR* affects the stability of the linkage between the inner and outer membrane and results in increased GMMA production by *Salmonella* species [79,204]. GMMAs are targets for the development of subunit vaccines, because they lack the ability to cause disease, can deliver heterologous antigens [205,206], and are highly immunogenic [200,207]. Proteomic analysis has shown that GMMAs contain over 100 outer membrane and periplasmic proteins [208,209]. Due to the large number of antigens that GMMAs can present to the immune system, GMMAs are being exploited for the development of cross-protective vaccines, for diseases like gastroenteritis caused by several NTS serovars [210].

In this study, we describe conditions that improve EPS production in *S*. Typhimurium. We also show how random mutagenesis aimed at increasing the production of O-Ag capsule for vaccine development led to increased colanic acid production. Immunization with colanic acid did not

induce a protective immune response against *S.* Typhimurium. However, immunization with GMMAs purified from colanic acid overproducing *Salmonella* reduced bacterial colonization in mice.

5.3 Results

5.3.1 The yih operons are not involved in *Salmonella* virulence

LPS O-Ag is the target of protective immunity against NTS; however, the immune response is strain specific [71,72,76]. Since there are >1500 NTS serovars, a vaccine that can induce cross-protective immunity against the predominant NTS serovars (*S.* Typhimurium and *S.* Enteriditis) will reduce the prevalence of gastroenteritis. The O-Ag capsule is an attractive vaccine candidate because it is comprised of over 2300 repeat units with similar tetrasaccharide units as the LPS O-ag [96,194]. The Increased molecular weight of oligosaccharides has been shown to result in improved immune response [211], and glycoconjugates from high molecular weight polysaccharides are immunogenic in humans at lower doses [212]. Due to the large number of repeating units, we hypothesize that immune responses to conserved epitopes in O-Ag capsule could induce cross-protective immunity against the predominant NTS serovars that cause gastroenteritis.

The divergently transcribed operons *yihUTSRQPO* (*yihU*) and *yihVW* have been identified as the putative O-Ag capsule biosynthesis operons in *Salmonella* [96]. Using bioluminescence, White et al., [213] showed that the *yihU* operon is expressed during infection. Since virulence factors are often optimal vaccine candidates, we wanted to determine if the *yih* operons are involved in *Salmonella* virulence. We performed a competitive index experiment in C57BL/6 mice orally challenged with a 1:1 mixture of *S.* Typhimurium 14028 (wildtype) and *S.* Typhimurium 14028Δ*yih*. At 4-7 days post-infection, mice were euthanized and bacterial loads in the spleen, liver, mesenteric lymph nodes (MLN), and cecum were enumerated. An equal number of wildtype and *S.* Typhimurium 14028Δ*yih* were recovered from all organs tested (Fig 5.1), suggesting that the *yih* operons are not involved in *Salmonella* virulence.

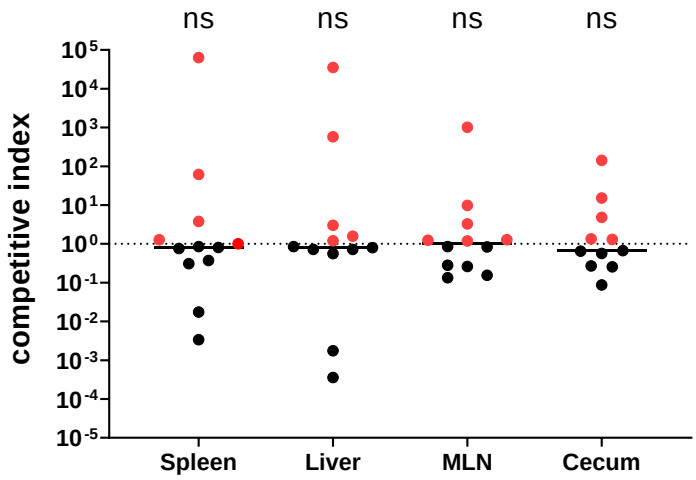

Figure 5.1: Evaluating the role of the *yih* operons in the virulence of *S.* Typhimurium. Competitive index experiment was performed with C57BL/6 mice orally infected with wildtype and *S.* Typhimurium 14028Δ*yih*. At 4-7 days post-infection, the CFU levels were enumerated from the liver, spleen, cecum, and MLNs. Each dot represents the CFU counts (per organ) from the designated organ from a single mouse. Competitive index values were calculated from each organ as follows: (CFU *yih* mutant/ wt)$_{output}$/(CFU *yih*/wt)$_{input}$. A CI value of 1, which represents a situation where both strains are equally virulent, is represented by the horizontal dotted line. Red circles represent CI values where the *S.* Typhimurium 14028 Δ*yih* strain won the competition. Statistical differences between groups of mice was noted as ns $p > 0.05$.

5.3.2 YihW represses expression of the *yih* operons

One of the problems of commercializing EPS-based vaccines is the difficulty to produce large quantities of EPS at an industrial scale. Therefore, our first objective was to boost O-Ag capsule production in *S.* Typhimurium by increasing the expression of the *yih* operons. It is often difficult and inefficient to purify EPS in the presence of cellulose, because it non-specifically binds the materials in the extracellular matrix together [118]. Therefore, a cellulose negative strain was used for *Salmonella* EPS purification [96]. Genes in the *yihU* operon have homologies to carbohydrate metabolism genes, while genes in the *yihVW* operon has homologies to regulatory genes (*yihV* have homology to kinases while *yihW* has homology to glycerol-3-P regulon repressor) [96]. To determine if genes in the *yihVW* operon have a regulatory effect on the expression of the *yih* operons, *yihU* and *yihVW*::*lux* promoter fusions were used to measure the promoter activity of both operons in *S.* Typhimurium Δ*bcsA* strain. Deletion of cellulose synthase (*bcsA*) had no effect on the expression of *yihU* (Fig 5.2A), and increased *yihVW* expression by ~70-fold (Fig 5.2B). Compared to *bcsA* deletion, plasmid-based overexpression of *yihVW* had no effect on *yihVW* expression and reduced *yihU* expression by ~20-fold, while deletion of *yihVW* or *yihW* alone increased *yihU* expression by ~100-fold and *yihVW* expression by ~10-fold (Fig 5.2A and B). Our

results suggest that YihW negatively regulates expression of the *yih* operons. Due to the increased expression of *yihVW* in a *bcsA* mutant, we speculate that cellulose positively regulates *yihU* expression via repression of the *yihVW* operon.

To determine if increased gene expression from the *yihU* operon would lead to increased production, O-Ag capsule was purified from *S.* Typhimurium Δ*bcsA* and *S.* Typhimurium Δ*bcsA* Δ*yihW* strains. Two times more crude EPS was purified from *S.* Typhimurium Δ*bcsA* Δ*yihW* compared to *S.* Typhimurium Δ*bcsA*. To isolate O-Ag capsule from crude EPS, anion and size exchange chromatography were performed, and O-Ag capsule containing factions were identified using EPS specific serum. Similar quantities of O-Ag capsule were purified from *S.* Typhimurium *ΔbcsA* and *S.* Typhimurium Δ*bcsA* Δ*yihW* after chromatography (Table 5.1), suggesting that the increased expression was not associated with increased production of O-Ag capsule.

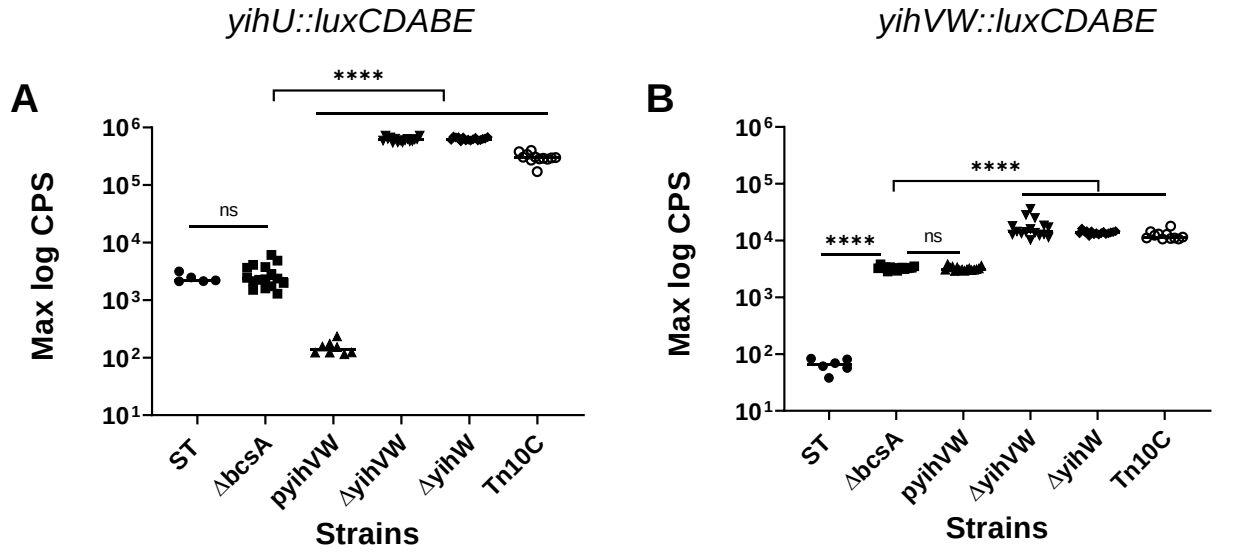

Figure 5.2: Expression of O-Ag capsule biosynthesis operon. The expression of A. *yihUTSRQPO* or B. *yihVW*; was determined in *S.* Typhimurium (ST, wildtype), *S.* Typhimurium Δ*bcsA* (ΔbcsA) *S.* Typhimurium Δ*bcsA* pBR322-yihVW (pyihVW), *S.* Typhimurium Δ*bcsA* Δ*yihVW* (ΔyihVW), *S.* Typhimurium Δ*bcsA* Δ*yihW* (ΔyihW) and *S.* Typhimurium Δ*bcsA* Δ*yihW* Tn10 (Tn10C), using luminescence. Cultures were grown in 1% tryptone at 28°C with agitation, and luminescence (In counts per second [cps]) were recorded every 30 mins for 48 hours. The log maximum CPS value recorded over 48 hours is shown. Statistical significance between groups was noted as: not significant (ns), $p > 0.05$, ****, $p < 0.0001$

5.3.3 Effect of precursor sugars on *yihUTSRQPO* promoter activity.

The lack of adequate amounts of precursor sugars could be one possible explanation for why 100-fold increased gene expression of the *yihU* operon in *S.* Typhimurium Δ*bcsA* Δ*yihW* strain did not lead to increased production of O-Ag capsule. Hence, we wanted to determine if increased amount of O-Ag capsule could be isolated from *S.* Typhimurium Δ*bcsA* Δ*yihW* grown in media supplemented with precursor sugars required for O-Ag capsule biosynthesis. *S.* Typhimurium O-Ag capsule is made up of tetrasaccharide repeating units of galactose, rhamnose, mannose and abequose, with abequose and galactose residues partially substituted with a glucose side chain [194]. Using a luciferase reporter assay we examined the effect of precursor sugars on *yihU* expression. Mannose, rhamnose and galactose increased *yihU* expression (by ~5-fold) in *S.* Typhimurium Δ*bcsA* (Fig 5.3A), while only mannose slightly increased *yihU* expression in *S.* Typhimurium Δ*bcsA* Δ*yihW* (Fig 5.3B). However increased gene expression in the presence of precursor sugars had no measurable effect on O-Ag capsule production (data not shown). This indicated that the lack of increased O-Ag capsule production by *S.* Typhimurium Δ*bcsA* Δ*yihW* was not due to the absence of adequate amount of precursor sugars.

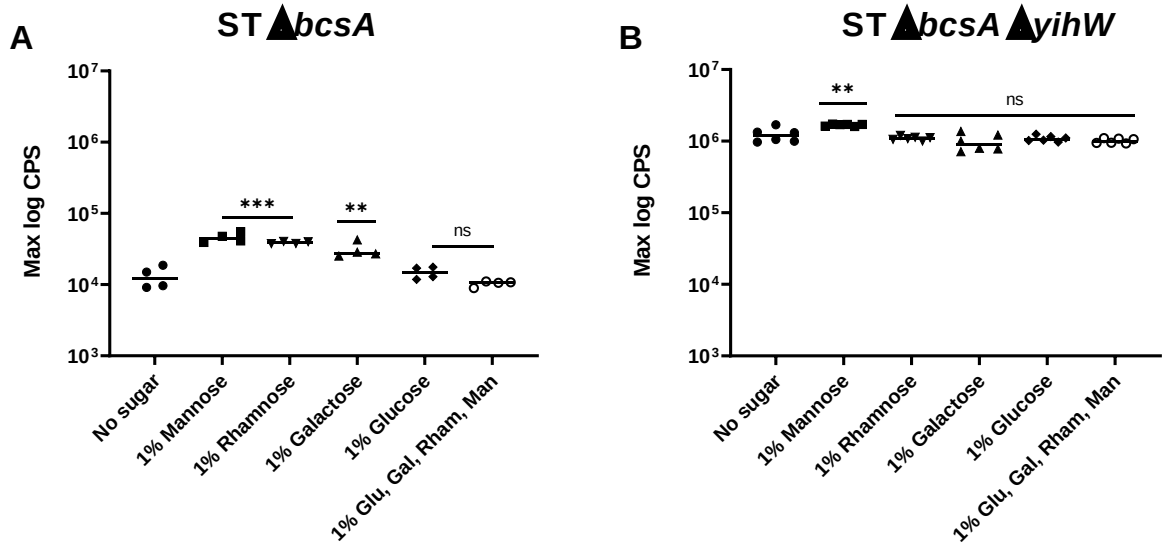

Figure 5.3: Effect of different precursor sugars on the expression of *yihUTSRQPO*. The *S.* Typhimurium Δ*bcsA* and *S.* Typhimurium Δ*bcsA* Δ*yihW* strains were grown in 1% tryptone media supplemented with or without different precursor sugars (glucose, galactose, rhamnose, mannose, or all four sugars), at 28°C. Luminescence (In counts per second [cps]) were recorded every 30 mins for 48 hours. The log maximum CPS value recorded over 48 hours is shown. Statistical differences were noted as *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, or ns $p > 0.05$.

5.3.4 Overproduction of EPS in transposon mutants.

Since increased expression of the *yih* operons and supplementation of growth media with precursor sugars did not boost the amount of O-Ag capsule purified from *S*. Typhimurium Δ*bcsA* Δ*yihW*, we hypothesized that other regulators besides YihW may be negatively regulating the biosynthesis of O-Ag capsule. To identify unknown factors that negatively regulate EPS biosynthesis in *Salmonella*, random mutagenesis was performed in *S*. Typhimurium Δ*bcsA* Δ*yihW* using Tn*10*dtet transposon. It was expected that random mutagenesis in *S*. Typhimurium Δ*bcsA* Δ*yihW* with ~100-fold increased gene expression of the O-Ag biosynthesis operon, *yihU*, would lead to the generation of transposon mutant strains that overproduces O-Ag capsule.

Bacterial colonies overproducing EPS often have mucoid and watery surfaces that can be visually detected, hence colony morphology was used to identify EPS overproducing strains. After random mutagenesis, seven potential high EPS producing isolates with mucoid morphologies were identified (named Tn*10*A-G). Nested PCR was used to identify the sites of Tn*10*dtet insertions (Table 5.1), and crude EPS was purified from each mutant. At least two times more crude EPS was isolated from Tn*10* C, D, E and G compared to *S*. Typhimurium Δ*bcsA* Δ*yihW*. After O-Ag capsule purification, ~10 times more material was obtained from Tn*10*C; hence, subsequent experiments were performed with this mutant. Using a luciferase assay we showed that the *yihU* and *yihVW* expressions were similar in Tn*10*C and *S*. Typhimurium Δ*bcsA* Δ*yihW*, suggesting that increased *yihU* expression in Tn*10*C was not associated with the increased amount of EPS produced.

Table 5.1. The amount of EPS purified from *Salmonella* wildtype and transposon mutants.

Strains	Identified site of Tn*10* insertion	Crude polysaccharide (mg)	Polysaccharide after chromatography (mg)	endotoxin removal with Triton X-114 (mg)
ST Δ*bcsA*	-	59.5	12.5	Few specks
ST Δ*bcsA* Δ*yihW*	-	130	18	Few specks
Tn*10*A	Tnp *IS200*	132	41.1	Few specks
Tn*10*B	*filD*	44	-	-
Tn*10*C	*srfA*	500	157.2	10
Tn*10*D	*STM14_2260*	209	37	< 2
Tn*10*E	*fhlA*	332	49.8	Few specks
Tn*10*F	*ompS*	67	-	-
Tn*10*G	*STM14_3662*	344	61	Few specks
Tn*10*C (MOPS)		2000	500	100

ST denotes *S.* Typhimurium. Tn*10*A-G are *S.* Typhimurium *ΔbcsA ΔyihW* mutants with Tn*10* transposon insertions. The amount of polysaccharide purified from 50 EPS agars are shown. Tn*10*C (MOPS) signifies strain Tn*10*C grown in EPS agar buffered with 40 mM MOPS. Few specks indicate that less than 0.5 mg of EPS was obtained.

5.3.5 Increased EPS production by *Salmonella* grown in buffered medium.

O-Ag capsule was purified from *S.* Typhimurium grown in EPS medium with a carbon to nitrogen ratio of 10:1. We reasoned that reduced pH due to increased glucose metabolism may reduce growth and limit the amount of EPS produced by S. Typhimurium Δ*bcsA* Δ*yihW* Tn*10*C. Therefore, we investigated the effect of buffering the pH on EPS production. Approximately four times more crude EPS was isolated from Tn*10*C grown in EPS medium buffered with MOPS compared to unbuffered medium (Table 5.1).

5.3.6 Removal of contaminating LPS from purified EPS.

LPS is a common contaminant of purified EPS from Gram-negative bacteria, and since it is toxic in high amounts, the presence of LPS is not desirable in vaccine formulations [214]. Triton X-114

or acid hydrolysis with acetic acid are routinely used to remove LPS from purified EPS [215,216]. To determine the most efficient method for LPS elimination, purified O-Ag capsule was treated with either Triton X-114 or acetic acid.

Triton X-114 is a non-ionic detergent that separates into a detergent-rich, and detergent-poor phase at room temperature [217]. When mixed with purified EPS, LPS preferentially goes into the detergent-rich phase, due to non-polar interactions between lipid A and Triton X-114 detergent. Several rounds of separation with Triton X-114 is usually performed in order to reduce the amount of LPS present in purified EPS [215]. We performed three rounds of LPS removal with Triton X-114; however, increasing amounts of materials were lost with each separation round. Only 50 mg of O-Ag capsule was isolated from 1000 mg of crude EPS.

Following acid hydrolysis with acetic acid, the endotoxic portion of LPS lipid A, is separated from the core oligosaccharide, and forms precipitates which can be separated by centrifugation. After lipid A removal, O-Ag capsule was isolated from crude EPS based on size using chromatography. Approximately 200 mg of O-Ag capsule was purified from 1000 mg of crude EPS.

After treatment with Triton X-114 or acid hydrolysis, limulus amebocyte lysate (LAL) assay was used to quantify the amount of LPS present in purified EPS. About 4.9×10^4 endotoxin units (EU)/ml was present after three rounds of LPS removal with Trition X-114, while ~1.4×10^4 EU/ml was present after LPS elimination with acetic acid. LPS at a concentration between 1×10^3 EU/ml and 4.55×10^6 EU/ml has been shown to be safe when administered to a 20g mouse [214,218]. Hence both methods reduced LPS to a safe level, however, less material is lost when acid hydrolysis is used for LPS elimination.

5.3.7 Over-production of colanic acid in *S.* Typhimurium

Although immune serum raised to whole *Salmonella* EPS [96] was used to guide the purification process, we performed monosaccharide composition analysis to characterize the final purified EPS. The amounts of fucose, galactose and glucose present in our crude and final purified EPS showed that colanic acid was purified from *S.* Typhimurium Δ*bcsA* Δ*yihW* Tn*10*C, and not O-Ag capsules (Table 5.2).

WcaJ is a glycosylase that is part of the *wca* gene cluster important for the biosynthesis and transport of colanic acid in *Salmonella* [219]. To verify the overproduction of colanic acid, we

generated a *S.* Typhimurium Δ*bcsA* Δ*yihW* Tn*10*C Δ*wcaJ* strain. Deletion of *wcaJ* resulted in the loss of the mucoid morphology associated with EPS overproduction (data not shown), indicating that colanic acid was being over-produced by *S.* Typhimurium Δ*bcsA* Δ*yihW* Tn*10*C.

Table 5.2. Monosaccharide composition (Mol%) of crude and final purified EPS from *Salmonella*

Monosaccharides	Crude EPS	Polysaccharide isolated from crude EPS	Colanic acid	O-Ag capsule
Rhamnose	3.6	1.5	NA	22
Fucose	32.3	31.9	27	NA
Mannose	5.7	8.4	NA	24
Galactose	35.2	34.6	28.8	28
Abequose /Tyvelose	-	-	NA	18
Glucose	22.4	22.4	17.9	9.1

Note: Experiments were conducted in triplicates. Sum of numbers presented in a column may not precisely be 100.0 due to rounding. NA not applicable, - not tested. Reference for O-Ag capsule composition and colanic acid composition [96,191].

5.3.8 Immune response induced by colanic acid

The objective of this study was to boost the amount of EPS purified from *S.* Typhimurium for immunization trials. Random mutagenesis with Tn*10* transposon aimed at increasing the amount of O-Ag capsule purified from *S.* Typhimurium led to the overproduction of colanic acid. Colanic acid has previously been associated with survival in the non-host environments [87]; however, since it is a conserved EPS, we wanted to determine if immunization with colanic acid could induce a protective immune response against *Salmonella*.

Groups of six BALB/c or C57BL/6 mice were immunized intramuscularly with purified colanic acid formulated with TriAdj. TriAdj is a novel combination adjuvant platform comprised of 1. polyI:C a TLR agonist, 2. an immunostimulatory host defense peptide (HDP) and 3. Polyphosphazene [220]. After primary immunization, two booster immunizations were administered at two- (BALB/c) or three-weeks intervals (C57BL/6). Immunization with colanic acid induced an anti-colanic acid IgG response in C57BL/6 (Fig 5.4A) and BALB/c (Fig 5.4B) mice.

To generate a specific and strong T-cell dependent response, EPS are often conjugated to carrier proteins [81,82]. Colanic acid was conjugated to truncated bovine herpesvirus 1 glycoprotein D (tgD) [221,222] or the inactive form of diphtheria toxin (CRM197) [223]. C57BL/6 mice were immunized with 50 µg of colanic acid conjugated to CRM197 (CA-CRM197), while BALB/C mice were immunized with 1 µg of colanic acid conjugated to tgD (CA-tgD). Immunization with colanic acid or CA-CRM197 induced similar levels of anti-colanic acid IgG in C57BL/6 mice (Fig 5.4A). Immunization with colanic acid or CA-tgD also induced similar levels of anti-colanic acid IgG in BABL/c mice (Fig 5.4B), however, mice were immunized with 50 times less CA-tgD.

Immunization with CA-CRM197 or CA-tgD induced robust anti-CRM197 IgG and anti-tgD IgG levels respectively (Fig 5.4C and D). These results indicate that immunization with colanic acid alone or colanic acid conjugated to a carrier protein induced an immune response in mice.

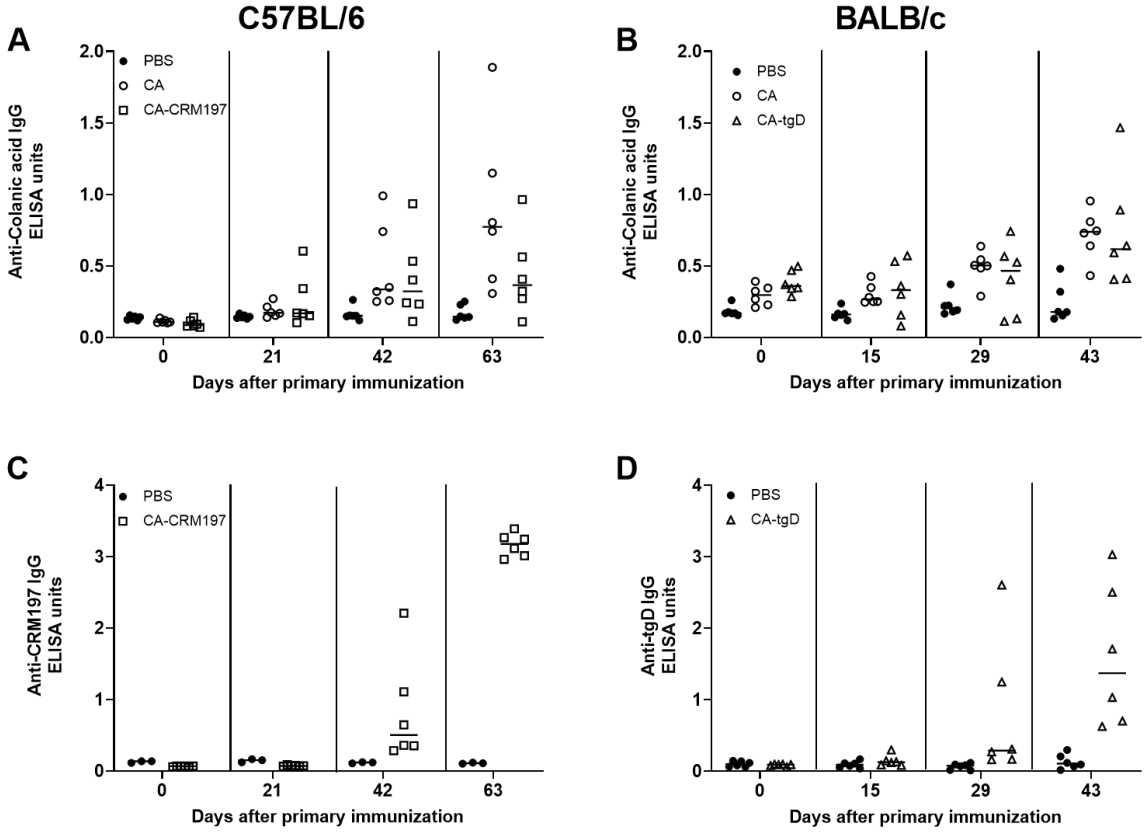

Figure 5.4: Immune response to colanic acid, truncated glycoprotein D and inactivated diphtheria toxin. ELISA was performed with sera collected from mice (A and C, C56BL/6, B and D BALB/c), immunized with phosphate buffered saline (PBS), colanic acid (CA), CA-CRM197 or CA-tgD. ELISA units = OD 405-490nm.

5.3.9 Immunization with colanic acid does not induce a protective immune response in mice

To determine if the immune response induced by colanic acid or colanic acid conjugated to a carrier protein is protective, mice were orally challenged with 10^7 CFU of *S.* Typhimurium. Four to seven days after challenge mice were euthanized and the liver, spleen, cecum, MLN, and blood were collected for bacteria enumeration. No significant difference in *Salmonella* CFU levels was observed in mice immunized with colanic acid, CA-CRM197, CA-tgD or PBS (control) (Fig 5.5A and 5.5B). These results indicated that the immune response induced by colanic acid alone or conjugated to CRM197 or tgD was not protective against *Salmonella* infections.

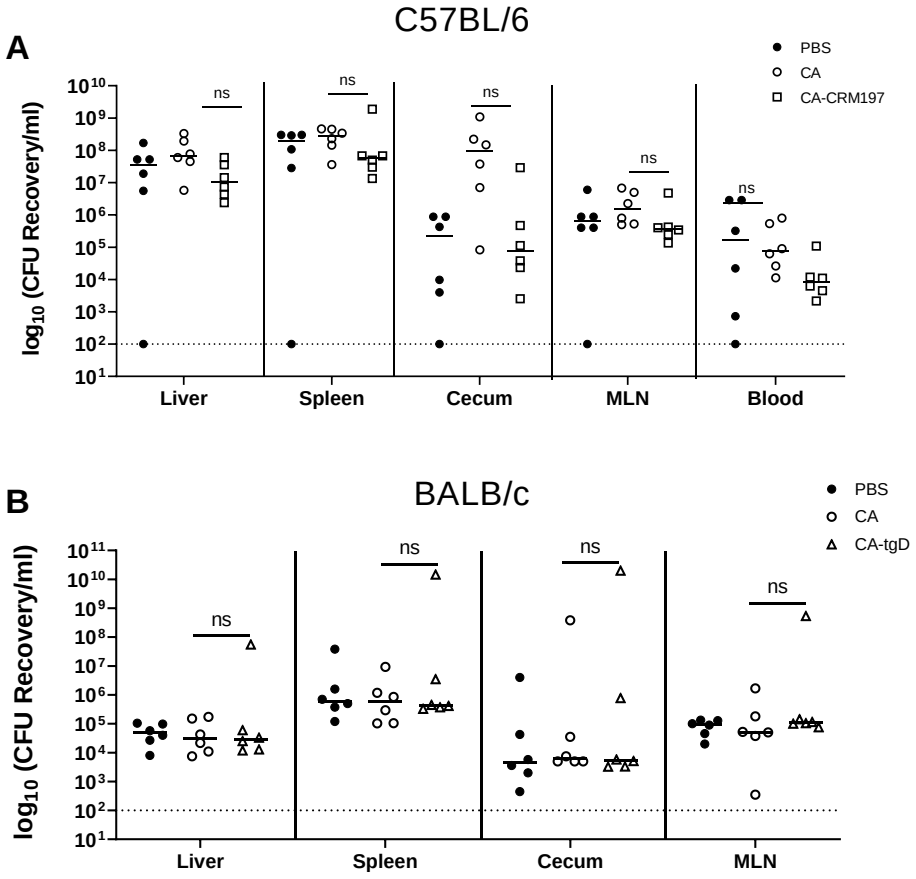

Figure 5.5: Bacteria counts from immunized mice. A. C57BL/6 mice were immunized with phosphate buffered saline (PBS), colanic acid (CA) or CA-CRM197. B. BALB/c mice were immunized with PBS, CA or CA-tgD. Immunized mice were orally challenged with *Salmonella* and 4-7 days post infection the liver, spleen, cecum, MLN and blood (C57BL/6) were harvested. Organs were homogenized before plating on LB agar supplemented with kanamycin. The $\log_{10}$ CFU of *Salmonella* recovered from each mouse is shown. The dashed line represents the limit of detection. For each group of mice, the black line represents the median values. Statistical significance: ns, $p > 0.05$,

5.3.10 Immunogenicity of GMMAs

GMMAs are targets for the development of subunit vaccines because, they are rich sources of outer membrane antigens that can induce a strong immune response. The antigen content of GMMAs can vary based on growth phase, growth condition, and genetic modifications present in the producing bacteria [199]. We wanted to determine if immunization with GMMAs purified from EPS over producing isolates would induce a protective immune response against *Salmonella*. A *tolR* mutation has previously been linked to increased GMMA production [201]; hence, we purified GMMAs from colanic acid overproducing Tn*10*C with *tolR* deletion (Tn10C GMMAs). As controls we also purified GMMAs from *S.* Typhimurium Δ*tolR* (wildtype GMMAs) and *S.* Typhimurium Δ*tolR* Δ*lon* (Lon GMMAs). Lon is a cytoplasmic serine protease responsible for the proteolytic cleavage of several proteins, and *S. enterica* strains with *lon* mutations have been shown to overproduce colanic acid [224].

Groups of six C57BL/6 mice were immunized intramuscularly with GMMAs purified from each strain. Two booster immunizations were given to each mouse at 3-week intervals. Serum from inmmunized mice were screened for the presence of GMMAs and colanic acid specific antibodies. Immunization with wildtype, Lon or Tn10C GMMAs induced a significant amount of anti-colanic acid IgG 42 days after primary immunization (Fig 5.6A). There were no statistically significant differences between the amount of anti-colanic IgG induced by wildtype, Lon and Tn*10*C GMMAs.

Immunization with wildtype, Lon or Tn10C GMMAs induced increasing amounts of anti-GMMAs IgG on days 21, 42 and 63 (Fig 5.6B, C, D). Our results indicate that immunization with GMMAs induced colanic acid and GMMA specific immune responses.

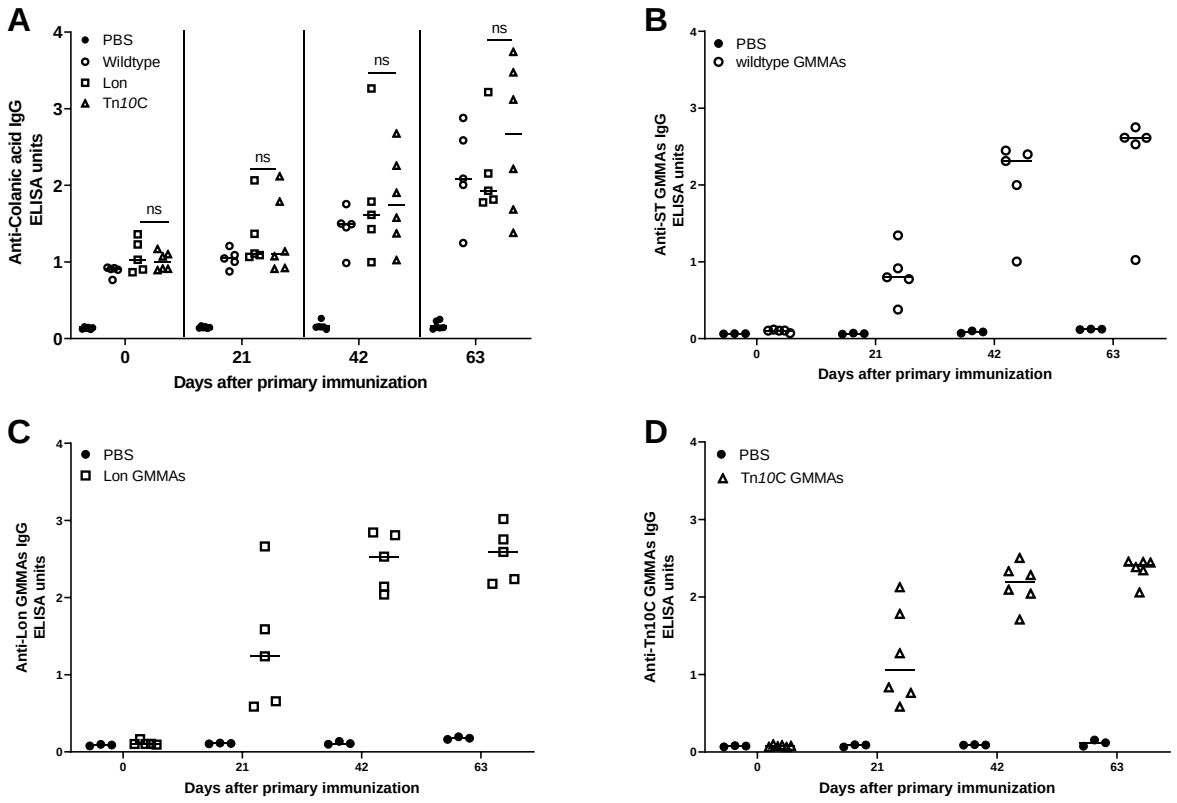

Figure 5.6: Immune response to GMMAs and colanic acid in immunized mice. Mice were immunized with GMMAs purified from *S.* Typhimurium 14028 Δ*tolR* (wildtype), *S.* Typhimurium 14028 Δ*tolR* Δ*lon* (Lon), Tn*10*C Δ*tolR* (Tn*10*C) or PBS. ELISA was performed with sera collected on days 0, 21, 42 and 63 and used to detect A. Anti-colanic acid IgG, B. Anti- S. Tm 14028 GMMAs IgG C. Anti-lon OMV IgG, D. Anti-Tn10C GMMAs IgG. Line represent median. Statistical significance: ns $p > 0.05$. ELISA units = OD 405-490nm.

5.3.11 Immunization with GMMAs provides partial protection.

To assess the level of protection induced by GMMAs, three weeks after final immunization mice were orally challenged with 10^7 CFU of *S.* Typhimurium. All mice were euthanized 4-7 days after challenge and protection was assessed by the CFU of *S.* Typhimurium recovered from the liver, spleen, cecum, MLN and blood.

3 of 5 mice immunized with wildtype and 4 of 5 mice immunized with Tn*10*C GMMAs showed ~5-log reduction of bacterial colonization in the liver and spleen, and ~3-log reduction in the cecum, MLN and blood, compared to control mice (Fig 5.7). Mice immunized with Lon GMMAs showed a ~1-log reduction in the liver, spleen, cecum, and MLN and a ~4-log reduction in the

blood compared to control mice (Fig 5.7). Our results indicate that immunization with GMMAs reduces bacterial colonization of the mice organs.

S. Typhimurium was not recovered from 3 of 5 mice immunized with wildtype GMMAs, 4 of 6 mice immunized with Tn10C GMMAs, 1 of 5 mice immunized with Lon GMMAs and 1 of 6 mice unimmunized control mice (Fig 5.7). Based on these results, we conclude that immunization with wildtype and Tn*10*C GMMAs induced a partially protective immune response against *S.* Typhimurium.

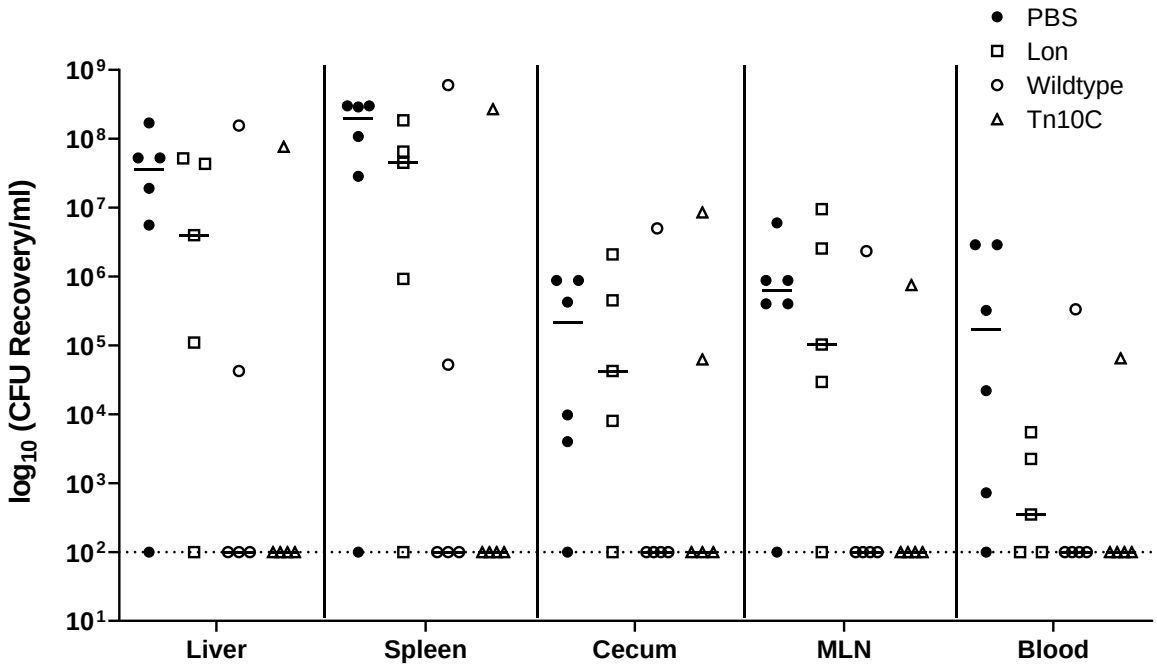

Figure 5.7: Colony forming units (CFU) of *S.* Typhimurium recovered from GMMAs immunized mice. C57BL/6 mice were immunized with PBS or GMMAs purified from *S.* Typhimurium Δ*tolR* (*S.* Tm 14028), *S.* Typhimurium Δ*tolR Δlon* (Lon) and *S.* Typhimurium Δ*tolR* Δ*bcsA* Δ*yihW* Tn*10*dtet (Tn*10*C). Immunized mice were orally challenged with 10^7 CFU of *S.* Typhimurium 14028, and 4-7 days post infected mice were euthanized, and liver, spleen, cecum, MLN, and blood were collected from bacterial enumeration. Organs were homogenized before plating on LB agar supplemented with kanamycin. The $\log_{10}$ CFU of *Salmonella* recovered from each mouse is shown. Dashed line represents the limit of detection. Black line represents median values. Statistical significance: Not significant (ns) $p > 0.05$.

5.4 Discussion

Immune responses directed against EPS form the basis for some of the most successful human vaccines. EPS-based vaccines have been developed and are currently licensed for use against various pathogens such as, *Haemophilius influenza* type b, *Neisseria meningitides*, *Streptococcus*

pneumonia and *Salmonella* Typhi [84,86,225]. Bacterial EPS are T-cell independent antigens and do not induce a robust immune response [81,82]. The lack of T- cell response to EPS vaccines can be overcome by conjugation to carrier proteins, which results in antibody production and immune memory [84,226]. EPS conjugated to carrier proteins are the basis for most EPS-based vaccines currently licenced for use in humans. There is currently no licensed vaccine against the NTS serovars that cause gastroenteritis and invasive bloodstream infections in sub-Saharan Africa. Due to the success of Vi EPS-based vaccines against typhoid fever [52,54,227], there is renewed interest in developing an EPS-based vaccine against NTS serovars that cause gastroenteritis and invasive bloodstream infections.

Colanic acid does not indue a protective immune response

Colanic acid is a common EPS mainly associated with bacterial survival in adverse environmental conditions [87]. However, it has been shown to contribute to *Salmonella* biofilm formation on mammalian cell lines and chicken intestinal epithelia [228], suggesting that colanic acid may be produced *in vivo* during infection. Hence, we wanted to determine if immunization with purified colanic acid could provide protection against a lethal *Salmonella* challenge in mice. The lack of protective immune response is supported by the susceptibility of *Salmonella* and *E. coli* overproducing colanic acid to the bactericidal activity of human serum and to phagocyte-mediated killing [229,230].

Immunization with GMMA provides partial protection

GMMAs are attractive vaccine candidates because they are comprised of multiple antigens that can stimulate an immune response [207]. Several studies have focused on developing GMMAs purified from bacteria with different genetic modifications, as vaccine candidates for NTS. GMMAs purified from *S*. Typhimurium with truncated LPS or deficient in flagellin production has been shown to induce cross reactive antibody responses and cross protection against *S*. Choleraesuis and *S*. Enteritidis [80,231]. Our results suggest that GMMAs purified from EPS overproducing strains can provide partial protection against *Salmonella* infection. Although Tn*10*C and Lon GMMAs were isolated from colanic acid overproducing *Salmonella*, immunization with Tn*10*C GMMAs reduced bacterial colonization to a greater extent compared to Lon GMMAs. This suggests the presence of different antigens on Tn10C and Lon-GMMAs. Several studies have shown that the composition of GMMAs can vary with regards to growth

phase, growth condition, and genetic mutation present in bacteria [232–234]. LPS-OAg or GMMAs have been shown to induce protective immune responses against NTS in mouse models of infection [71,80,231]. However, none of these antigens have been successfully developed as vaccine against NTS. We propose that engineered GMMAs with high EPS content can be developed as vaccine candidates. Compared to LPS-OAg, GMMAs induced a more diverse immune response and reduced bacterial colanization to a greater extent [78]. However, the LPS-OAg is the target of protective immune response against NTS [71, 72,76]. We speculate that engineered GMMAs with high concentrations of LPS-OAg will induce a diverse and protective immune response against NTS.

The diverse roles of the *yih* operons

The *S. enterica* O-Ag capsule was thought to be a potential vaccine candidate because it a has similar repeating unit as LPS O-Ag, which is the target of protective immunity against NTS [71,72,76]. Gibson et al., speculated that precursor sugars synthesized by the LPS O-Ag machinery, are modified, assembled, and translocated out of the cell as O-Ag capsule by gene products from the divergent operons *yihUTSRQPO* and *yihVW* [96]. The increased expression of *yihUTSRQPO* and *yihVW* operons in a Δ*yihW* mutant is consistent with YihW being a repressor of the *yih* operons. The repressive effect of YihW has also been described in *E. coli* where three *yihW* binding sites were identified in the *yih* promoter region [235,236]. Overexpression of genes often leads to the overproduction of specific proteins or EPS. However, 100-fold increased expression of the *yihUTSRQPO* operon in the Δ*yihW* mutant did not result in increased production of O-Ag capsule. The introduction of precursor sugars into growth media often leads to increased EPS biosynthesis [237,238]. However, O-Ag capsule biosynthesis was not increased in the presence of excess precursor sugars. In addition, random mutagenesis aimed at increasing the amount of O-Ag capsule produced by *S.* Typhimurium Δ*bcsA* Δ*yihW*, lead to increased colanic acid production. Based on these results, we speculate that the *yih* operons are not be involved in the biosynthesis of O-Ag capsule.

In *E. coli*, biochemical analysis with proteins purified encoded by the *yih* operons and mass spectrometric analysis of products revealed that they catalyze various stages of the sulphoglycolysis pathway, required for sulphoquinovose (SQ) catabolism [239,240]. SQ, a major reservoir of organosulphur, is degraded by bacteria as a source of carbon and sulphur. YihS

purified from *E. coli* was shown to be an isomerase that catalyzes the isomerization of SQ to 6-deoxy-6-sulphofructose (SF), which is phosphorylated by kinase YihV to 6-deoxy-6-sulphofructose-1-phosphate (SFP). Aldolase YihT catalyze the cleavage of SFP to dihydroxy-acetone phosphate (DHAP) and 3-sulpholactaldehyde (SLA). DHAP enters the glycolytic pathway, while reductase YihU converts SLA to 2, 3-dihydroxy-propane-1-sulphonate (DHPS), which is transported out of the cell [239] and degraded by other bacteria [241]. Besides SQ catabolism, genes in the *yih* operons have been shown to have enzymatic activities towards various sugars such as mannose, succinic semialdehyde and α-glycosyl fluoride [242–244]. Using transcriptional analysis Kaznadzey et al.,[236] showed that genes in the *yih* operons may be involved in an alternative pathway of lactose degradation in *E. coli* [236]. Analysis of *Salmonella* mutants indicated that genes in the *yih* operons are involved in persistence and serum resistance [197]. *S.* Typhimurium Δ*yihT* and Δ*yihO* mutants, have reduced competitive fitness on green tomato [196], and colonization of alfalfa sprout, respectively [195]. *S.* Typhimurium Δ*yihO* and Δ*yihQ* mutants have reduce ability to survive desiccation and are unable to form biofilms on cholesterol-coated surfaces [90]. Due to the high nucleotide (80%) and protein (80 – 92%) sequence identity with *E. coli yih*, we speculate that the *yih* operon is required for SQ catabolism in *Salmonella*. SQ is a major constituent of the human diet and about 10,000,000,000 tonnes (petagram) are produced by photosynthetic organisms annually [245]. It is likely a major source of carbon and sulphur and this can explain the conservation of the *yih* operons in most bacterial species [236]. This could also explain the expression of *yihU* in the murine intestinal tract during *S. enterica* serovar Typhimurium infections.

Due to the phenotypes associated with mutant strains of *S. enterica*, there is also a possiblilty that genes in the *yih* operons have dual functionality. In *Salmonella* the *yih* operon maybe involved in SQ catabolism and the synthesis of an unknown EPS, or modification of the antigenic content of the outer membrane. The serum used by Gibson et al., [96] for the identification of the *yih* operons, was generated against the whole EPS fraction and was found to be cross-reactive to an uncharacterized EPS [96]. This might explain the lack of capsular polysaccharide in *S.* Typhimurium Δ*yihO* observed by Marshall and Gunn using confocal microscopy [96,197]. A role for the *yih* operons in the modification of the antigenic content of the outer membrane, provides an explanation for the exclusive production of phase 1 flagellin FliC by *S.* Typhimurium Δ*yih* mutants. In addition, compared to wildtype, *S.* Typhimurium Δ*yih* mutants produce ~ 25 to 45%

more short LPS, with 1 to 8 LPS O-Ag repeating units [197]. More research is required to clarify the role of the *yih* operons in *Salmonella*.

The O-Ag capsule described by Gibson et al., [96] could be very long chain LPS O-Ag, and this provides an additional explanation for why increased expression of the *yihUTSRQPO* operon did not lead to increased biosynthesis. *S.* Typhimurium has a tri-modal distribution of LPS due to the varying length of the O-antigen repeat units. Short LPS O-Ag (S-OAg) is comprised of 1 to 15 repeat units, long LPS O-Ag (L-OAg) is composed of 16 to 35 repeating units and very long LPS O-Ag (VL-OAg) is comprised of more than 100 repeating units [246–248]. We speculate that the O-Ag capsule and VL-OAg could be the same EPS, because they both have similar banding patterns on SDS-PAGE [96,197], are composed of similar tetrasaccharide repeat units [194] and have been implicated in similar functions [197,247,249]. Although O-Ag capsule was found to be glycosylated at the tyvelose and galactose residues, high molecular weight LPS with varying glcosylation levels has previously been described in *Salmonella* [250]. Hence the O-Ag capsule may be VL-OAg glycosylated at the tyvelose and galactose sugars. More research is needed to determine the EPS present in *Salmonella*.

Although EPS has been the focus of subunit vaccine development against NTS, none have been tested in clinical trials. The immune response to LPS-OAg is strain specific, while colanic acid does not induce a protective immune response. Research on EPS based vaccines against NTS will have to focus on identifying novel EPS that can be developed as vaccines. Currently, GMMAs may be the best strategy for vaccine development, because they are highly immunogenic and deliver multiple antigens in their natural context, raising the possibility of inducing cross-protection between NTS serovars. With the rise in multidrug resistant isolates and the increasing incidence of invasive bloodstream infections, a vaccine is urgently needed to reduce the prevalence of NTS infections.

5.5 Materials and Methods

5.5.1 Bacterial strains, media, and growth conditions.

The bacterial strains used in this study are listed in Table S5.1. For standard growth, strains were inoculated from frozen stocks onto LB agar (lysogeny broth, 1% NaCl, 1.5% agar) supplemented with appropriate antibiotic (50 μg mL^{-1} kanamycin (Kan), 34 μg mL^{-1} chloramphenicol (Cam) or

5 µg mL^{-1} tetracycline (Tet)) and grown overnight at 37°C. Isolated colonies were used to inoculate 5 mL LB broth and the culture was incubated for 18 hours at 37°C with agitation at 200 rpm. For EPS purification, overnight cultures were grown for 5 days at 28 °C on agar supplemented with 1% glucose, 0.05% yeast extract, 10 mM Na_2HPO_4, 0.1% NH_4Cl, 0.3% KH_2PO_4, with or without 40 mM MOPS. For murine infection experiments, overnight cultures grown in LB broth were diluted to the desired CFU concentration in 100 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), pH 8, and used to infect mice.

For bioluminescence assays, overnight cultures of *S.* Typhimurium transformed with promoter luciferase fusions (*yihUTSRQPO*::*luxCDABE* or *yihVW*::*luxCDABE*), were diluted 1 in 600 in 1% tryptone broth supplemented with 50 µg ml^{-1} kanamycin (Kan) to a final volume of 150 µl in 96-well clear-bottom black plates (9520 costar Corning Inc) and overlaid with 50 µl of mineral oil. Cultures were assayed for luminescence (1s; in counts per second [cps]) and absorbance (590 nm, 0.1s) every 30 mins during growth at 28°C with agitation in a Victor X3 multilabel plate reader (Perkin-Elmer).

5.5.2 Generation of *S.* Typhimurium mutant strains

Lambda red recombinase knockout procedure [170] was used to generate *S.* Typhimurium Δ*lon*, Δ*tolR*, Δ*yihW* and Δ*yihVW* mutants. Primers containing 50-nucleotide sequences on either side of *lon*, *tolR*, *yihW* or *yihVW* (Table S5.2) were used to amplify the *cat* gene from pKD3 or *kan* gene from pKD13 using Phusion High-Fidelity DNA polymerase (New England Bio-Labs). The PCR products were purified and electroporated into *S.* Typhimurium 14028 cells containing pKD46. Mutants were selected by growth at 37°C on LB agar supplemented with 10 µg ml^{-1} Cam or 50 µg mL^{-1} Kan. Cam^R isolates were restreaked onto LB agar containing 34 µg ml^{-1} Cam. Mutations were transduced into *S.* Typhimurium strains with a clean background using P22 phage [171]. The *cat* or *kan* gene was resolved from the chromosome using pCP20 [170]. DNA sequencing of PCR products from the chromosomes of mutant *Salmonella* isolates was used to verify the loss of the *lon*, *tolR*, *yihW* or *yihVW* operons. Primers used in this study are listed in Table S5.2. The generation of *S.* Typhimurium Δ*bcsA* has been previously described [118].

5.5.3 Generation of transposon mutants.

To generate *S.* Typhimurium Δ*bcsA* Δ*yihW* transposon mutants [251,252], *S.* Typhimurium LT2 harbouring pNK972 was infected with P22 lysate of *S.* Typhimurium TT10423 containing

Tn10dtet on F'. The resulting transductants (~100, 000 colonies) were pooled and a P22 *S.* Typhimurium LT2 Tn10dtet transducing fragment library was generated, following the method outlined by Maloy [171]. The resulting P22 phage lysate was used to infect *S.* Typhimurium Δ*bcsA* Δ*yihW* and plated on 1% tryptone supplemented with 10 µg ml^{-1} Tet. The resulting transductants (10, 000 to 20, 000) were visually screened for the presence of a mucoid morphology which was indicative of high EPS production. To identify the site of transposon insertion nested PCR was performed on purified genomic DNA using primers TL or TR and ARB1 or ARB6. The product of the first reaction was further amplified using primers UniversalTn and ARB2. PCR products were purified and sequenced. To identify site of Tn*10* insertions DNA sequences were mapped to *S.* Typhimurium 14028 genome using Geneious.

5.5.4 Generation of plasmid vectors

To generate pBR322-*yihVW*, the DNA region containing *yihVW* was PCR amplified from *S.* Typhimurium 14028 genome using primers yihVWFOR, yihVWREV (Table S5.2) and Phusion polymerase (Fisher Scientific), with reaction conditions as recommended by the manufacturer. The resulting PCR product was digested with *AatII* and *PstI* and ligated into *AatII*/*PstI* digested pBR322 prior to electroporation into *S.* Typhimurium 14028. Positive clones were selected by growth on LB agar supplemented with 7 ug mL^{-1} tetracycline (Tet). The generation of pBR322-*yihVW* plasmid was confirmed by sequencing using primers yihVWseqF and yihVWseqR (Table S5.2). The generation of *yihUTSRQPO* and *yihVW* promoter region fused to *luxCDABE* operon has previously been described [96].

5.5.5 Purification of extracellular polysaccharide

The EPS purification protocol was adopted from Gibson *et al.*,[96] with some modifications. To separate EPS from other cellular materials, *S.* Typhimurium scraped off the agar surface was resuspended in 1% phenol, mixed vigorously by vortexing and centrifuged (16, 000 x g 4°C, 4h). Crude EPS was precipitated, by mixing supernatant with 4 volumes of ice-cold acetone while stirring continuously with a glass rod. The resulting precipitate was stored overnight (at -20°C to allow for further precipitation), collected by centrifugation (6, 000 x g 4°C, 15 mins) and air-dried. Crude EPS was solubilized in water, dialyzed in water for 48 h (MW cut-off 10 kDa; SnakeSkin dialysis tubing, Thermo Fisher Scientific) and lyophilized. The lyophilized polysaccharide was dissolved in buffer A (15 mM NaOAc, 0.05% Triton X – 100, pH 5.5) and 0.01% sodium azide.

The dissolved sample was heated twice at 37°C for 15 min prior to loading onto Q Sepharose FF xk50/11.5 and washing with 2 column volumes of buffer A. Materials were eluted with a stepwise gradient of buffer B (1.5 M NaOAc, 0.05% Triton X – 100, pH 5.5), which was increased sequentially from 17% (1.25 column volumes), to 50% (1.25 column volumes) to 100% (1.5 column volumes). Western blot analysis using EPS specific serum was used to identify colanic acid containing fractions.

For size exclusion chromatography colanic acid-containing fractions were pooled, concentrated (10 MWCO centrifuge filters, Amipore), and filtered through a 0.22 µm syringe tip filter, prior to loading on to the Superdex S300 prep grad xk26/95 column. The column was washed with 50 mM NH_4HCO_2 pH 7.72. Colanic acid containing fractions were identified using western blot and concentrated with a Millipore Amicon Ultra-15 Centrifugal Filter Device, dialyzed in water for 48 h (MW cutoff 10 kDa; SnakeSkin dialysis tubing, thermos scientific) and lyophilized.

5.5.6 Endotoxin removal from purified EPS

For endotoxin removal by acid hydrolysis using acetic acid, purified crude EPS was dissolved in 1% acetic acid and heated in an oil bath at 110°C for 2 hours [215]. The solution was centrifuged for 10 mins at 8,000 rpm. The pellet was discarded, and the supernatant was lyophilized before colanic acid isolation using anion exchange and size exclusion chromatography.

For endotoxin removal using Triton X-114, purified colanic acid was dissolved in pre-condensed Triton X-114, prepared by dissolving 20 ml of Triton X-114 and 16 mg of 2, 6-Di-tert-butyl-4-methylphenol at 4°C in 10 mM Tris-HCl (pH 7.4), 150 mM NaCl solution [216]. The mixture was incubated at 30°C until it separated into a detergent-depleted aqueous phase and a detergent-rich phase. The large aqueous phase was discarded and replaced with an equal volume of 10 Mm Tris-HCl (pH 7.4), 150 Mm NaCl. The solution was mixed and incubated at 30°C to enable phase separation. After three rounds of phase separation, the detergent-rich phase, with a Triton X-114 concentration of 11.0 % was collected and stored at room temperature [215].

For endotoxin removal using pre-condensed Triton X-114, purified EPS was dissolved in water to a final concentration of 0.5 mg/ml and mixed with 11% Triton X-114 to a final Triton X-114 concentration of 1% (w/v). The resulting cloudy solution was stirred for 30 min at 4°C until the solution became clear. The mixture was incubated at 37°C for 30 mins to induce separation into

two-phases, and subsequently centrifuged at 1200 x g for 30 mins at 25°C. The lower phase containing LPS was discarded and the upper phase containing EPS was mixed with 11% Triton X-114 to a final concentration of 2%. LPS extraction was repeated twice as described above. Centrifugation speed and time were increased by 1,000 x g and 30 mins for each round of purification to obtain the best separation.

To remove detergent from the EPS containing solution after LPS removal, the Triton X-114 treated sample was mixed with three volumes of methanol-chloroform (2 volumes of methanol and 1 volume of chloroform) and incubated at room temperature for 30 mins to enable phase separation. The Triton X-114 containing lower phase was eluted and the colanic acid containing upper phase was mixed again with the methanol-chloroform solution. This procedure was repeated two additional times. Residual methanol chloroform was removed from the purified O-Ag capsule using water aspiration. Samples were dialyzed for 48 h in ddH_2O at 4°C and lyophilized.

5.5.7 Conjugation of EPS to tgD and CRM197

20 mg of purified colanic acid dissolved in 0.15 M NaCl (2ml final volume) was allowed to react with 13 mg of sodium cyanoborohydride (CNBr, dissolved in 16 µl chloroform), the pH of the solution was maintained at 10.5 pH using NaOH (0.5 M). After 15 min at room temperature, adipic dihydrazide (ADH) was added to a final concentration of 12 mg/ml. HCl (0.5 M) was used to reduce the final pH to 8.5. The reaction mixture was incubated at room temperature overnight with stirring. The solution was dialyzed twice against 20 mM MES buffer (pH 6.0) using a 10kDa cut-off MWCO dialysis tubing for 3 hours at 4°C. Inactivated diphtheria toxin CRM197 was obtained from Fina Biosolutions LLC (Rockville, MD, USA). The purification of tgD has been previously described [253,254].

The resulting CNBr treated EPS (5 mg/ml, 3ml) was allowed to react with 1.5 ml tGD or CRM197 (2 mg/ml) in the presence of 12 mg EDC (dissolved in 20 mM MES buffer pH 6.0), at room temperature overnight. The resulting solution was dialyzed against 0.15 M NaCl using 10 kDa MWCO dialysis tubing for 3 h at 4°C. EPS conjugated to tgD or CRM197 was loaded on to a Sephacryl S300 xk26/94 column. The column was washed with 50 mM PBS, 15 mM NaCl pH 7.4 solution, and 10 ml fractions were collected. Fractions containing conjugated material were identified based on size after Western blot analysis with EPS specific serum.

5.5.8 GMMA production and purification

For GMMA production, bacteria grown overnight at 37°C were used to inoculate 1% tryptone broth (without antibiotics) to an optical density of 0.03 at 600 nm. Cultures were incubated at 30°, for 18 h with agitation. Culture supernatants were collected by centrifugation at 8000 x g for 10 min, filtrated with 0.45-µm filter, and concentrated by dialyzing (MW cutoff 10 kDa; SnakeSkin dialysis tubing, Thermo Fisher Scientific) against polyethylene glycol (PEG) 2000. GMMAs were pelleted by ultra-centrifugation at 186 000 x g for 2 h at 4°C. The resulting pellets were resuspended in endotoxin free water, filtered with 0.22-µm filter, lyophilized and resuspended in PBS.

5.5.9 Vaccine formulation

Vaccine antigens colanic acid (CA), CA-tgD and CA-CRM197 were formulated with triple combination adjuvant consisting of Poly (I:C) (PIC) (Invitrogen), host defence peptide (HDP) (genescript) and polyphosphazene (PCEP) (Idaho National laboratory) immediately prior to administration. Formulations were prepared by first mixing 10 µg of PIC and 20 µg HDP in PBS and incubating at room temperature (RT) for 15 mins. CA, CA-tgD or CA-CRM197 were added before the addition of 10 µg of PCEP making a final ratio of 1:2:1 of PIC: HDP: PCEP (TriAdj). Mixtures were incubated in the dark for 15 mins at RT prior to administration.

5.5.10 Murine immunization experiments

Six to eight-week-old female BALB/c mice were purchased from Charles River Laboratories (Kingston, ON), while C57BL/6 mice were purchased from Jackson Laboratory (Bar Harbor, ME). Mice were assigned to cage groups using a randomization table prepared on Microsoft Excel and individual mice were marked with ear notches.

Two groups of six BALB/c mice were immunized intramuscularly 3 times at 2 weeks interval with 50 µg colanic acid (CA), 1µg CA conjugated to tgD (CA-tgD), or phosphate-buffered saline (PBS). Serum was collected on days 0, 15, 29, 43 and 58. Three groups of six C57BL/6 mice were immunized intramuscularly 2 times at a 3 week interval with 50 µg CA, 50 µg CA conjugated inactivated diphtheria toxin (CA-CRM197), or PBS. Sera were collected on days 0, 21, 42 and 63.

For immunization with GMMAs, four groups of six C57BL/6 mice were immunized intramuscularly with PBS or 50 µg of GMMAs purified from *S.* Typhimurium Δ*tolR* (STm-

GMMA), *S.* Typhimurium Δ*lon* Δ*tolR* (lon-GMMA), or *S.* Typhimurium Δ*bcsA* Δ*yihW* Δ*tolR* Tn10C (Tn10C-GMMA). Secondary and tertiary immunizations were performed with 5 µg of GMMAs at three week intervals. Serum were collected on days 0, 21, 42 and 63 from each mouse.

5.5.11 Murine infection experiments

Six to eight-week-old female BABL/C or C57BL/6 mice purchased from Jackson Laboratory (Bar Harbor, ME) were assigned to cage groups using a randomization table prepared in Microsoft Excel, and individual mice were marked with ear notches.

For the competitive index (CI) experiment 2 groups of six C57BL/6 mice were challenged with a mixed inoculum consisting of a ~1:1 ratio of Kan^R and Cam^R *S.* Typhimurium 14028 strains containing sig70_16 *luxCDABE* construct [175]. Mice were challenged via oral gavage with a total CFU of ~10^7.

Immunized C57BL/6 or BABL/C mice were challenged via oral gavage with 10^7 CFU of Kan^R *S.* Typhimurium containing sig70_16 *luxCDABE* construct 21 days after final immunization.

Infected mice were weighed daily and monitored for clinical signs of infection. Mice that had a >20% drop in weight were humanely euthanized. All remaining mice were humanely euthanized 4-7 post-infection. Spleen, liver, MLN, and cecum were collected from each mouse. Blood was collected from C57BL/6 mice.

Collected organs were placed in a 2 ml Eppendorf Safe-Lock tube containing 1 ml of PBS and a 5-mm steel bead (Qiagen product 69989) and homogenized using a mixer mill (Retsch; MM400) for 5 min at 30 Hz. To determine the number of CFU in the initial challenges, serial dilutions of organ homogenates and blood were plated on LB agar supplemented with 50 µg ml^{-1} Kan. Organs collected from mice infected with a mixed inoculum were plated on both Kan and Cam agar. The CI values were calculated as follows: $(CFU\ Kan^R/CFU\ Cam^R)_{output}/(CFU\ Kan^R/CFU\ Cam^R)_{input}$.

5.5.12 Ethics statement

All animals were cared for and used 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 Protocols 20170066 or 20190071, which were approved by the University of Saskatchewan's Animal Research Ethics Board.

5.5.13 ELISA

Serum IgG levels specific for CA, tgD, CRM197 and GMMAs were measured by ELISA. Briefly, 96-well plates were coated with CA (2µg per well), tgD, CRM197 or GMMA (0.1µg per well) in coating buffer at 4°C overnight. Plates were blocked for 30 mins at RT with 5% skim milk dissolved in Tris-buffered saline (TBS) containing 0.05% tween 20 (TBST). Sera were serially diluted in TBST, starting at 1:100 in 5-fold dilutions. Alkaline phosphatase-conjugated goat-anti mouse IgG at a dilution of 1:1500 was used to detect bound IgG. The reaction was visualized with p-nitrophenyl phosphate, and absorbance was read at 405 nm with a reference wavelength of 409 nm.

5.5.14 Statistical analysis

Statistical analysis was performed using GraphPad Prism version 8.0. The normality of all data set was assessed using the Sharpiro-Wilk normality test. For luciferase assays, if the data were not normally distributed, comparisons were made using the Mann-Whitney test. If data were normally distributed, comparisons were made using unpaired t-test.

For murine infections if data sets were normally distributed, comparisons were performed using unpaired t-test with Welch's corrections. If any data sets were not normally distributed, comparisons were performed using the Mann-Whitney test.

For CI experiments, if the data was normally distributed one sample t-test was used to determine of the mean was significantly different from one. If the data was not normally distributed the Wilcoxon signed-rank test was used to determine if the median CI was significantly different from one. In all cases statistical significance was set at $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$, ns $p > 0.05$.

5.6 Supplementary information

Table S5.1. Strains and plasmids used in this study

Strains or plasmids	Genotype	Source or Reference
Strains		
S. Typhimurium LT2		
S. Typhimurium LT2 TT10423	*proAB47* / F' *pro*(+) *lac*(+) *zzf*-1831::*Tn10*(del) 16 (del) 17	[251]
S. Typhimurium 14028	Wild-type strain	ATCC
Δ*bcsA*	Deletion of *bcsA* ORF	[118]
Δ*yihVW*	Deletion of *yihVW* ORF	This study
Δ*yihW*	Deletion of *yihW* ORF	This study
Δ*yih*	Deletion of *yih* ORF	This study
S. Typhimurium 14028 ΔbcsA ΔyihW	Deletion of *bcsA* and *yihW* ORF	This study
Δ*wcaJ*	Deletion *wcaJ* ORF	This study
Tn*10A*	Tn*10* dtet insertion in *Tnp IS200*	This study
Tn*10B*	Tn*10* dtet insertion in *filD*	This study
Tn*10C*	Tn*10* dtet insertion in *srfA*	This study
Tn*10D*	Tn*10* dtet insertion in *STM14_2260*	This study
Tn*10E*	Tn*10* dtet insertion in *fhlA*	This study
Tn*10F*	Tn*10* dtet insertion in *OmpS*	This study
Tn*10G*	Tn*10* dtet insertion in *STM14_3662*	This study
Tn*10C* Δ*tolR*	Tn*10* dtet insertion in *srfA* and deletion of *tolR* ORF	This study
Δ*tolR*	Deletion of *tolR* ORF	This study
Δ*lon* Δ*tolR*	Deletion of *lon* and *tolR* ORF	This study
Plasmids		
PNK972	pBR322 derived plasmid with Tn*10* transposase gene	[252]

pBR322/*yihVW*	*yihVW* from *S*. Typhimurium 14028	This study
pCS26	Bacterial luciferase	[177]
pCS26-*yihUTSRQPO*	*yihUTSRQPO* promoter	This study
pCS26-*yihVW*	*yihVW* promoter	This study

Table S5.2. Oligonucleotides used in this study.

Primer	Sequence (5'-3')	Purpose
yihVWkoFOR	TTCGTGAAATTAAAATGAGCACATCGAAAATGCTTGAGGAATGACCATGGGTGTAGGCTGGAGCTGCTTC	To amplify cat gene product from pDK3 to generate Δ*yihVW* strain by lambda-red recombination
yihVWkoREV	TTGGCCGGATAAAGCGCTGACGCGACCCTCCGGCGCAAGGGCGCTTGTCACCTCCTTAGTTCCTATTCCG	
yihWkoFOR	TAATATGAGCAGTAGGAAGCTTTTAGAGGAATGCTCATGAGTGTAGGCTGGAGCTGCTTC	Used with yihVWkoREV to amplify cat gene from pDK3 to generate Δ*yihW* strain by lambda-red recombination
yihVWdetect1	GCACATCGAAAATGCTTGAGGA	To confirm the deletion of *yihVW* and *yihW* from *S.* Typhimurium 14028
yihVWdetect2	ATATCGCCTGCATCACAGCG	
yihVWFOR	CGCGCTGCAGCTGTTTGTGATCGTATTTGTAATTTAT	Used to amplify *yihVW* from *S.* Typhimurium 14028 for cloning into pBR322
yihVWREV	GATCGACGTCGCATCACAGCGCCGTTTTATTG	
yihVWseqF	GATCTTGCCGGGAAGCTAGAGTAAG	To confirm the cloning of *yihVW* into pBR322
yihVWseqR	GATCTTCTTGAAGACGAAAGGGCCT	
TL	TCCATTGCTGTTGACAAAGGGAAT	

TR	ACCTTTGGTCACCAACGCTTTTCC	Used with arb01 or arb06 for nested PCR (first reaction) to identify the site to Tn*10* dtet insertion
ARB1	GGCCACGCGTCGANNNNNNNNGATAT	Used with TL or TR for nested PCR (first reaction) to identify the site of Tn*10* dtet insertion
ARB6	GGCCACGCGTCGANNNNNNNNACGCC	
UniversalTn	GACAAGATGTGTATCCACCTTAAC	Used for nested PCR (second reaction) to identify the site to Tn*10* dtet insertion
ARB2	GGCCACGCGTCGACTAGTAC	
lonF	CTATACTATCTGATTACCTGGCGGACACT AAACTAAGAGAGAGCTCTATGATTCCGG GGATCCGTCGACC	To amplify *kan* gene product from pkd13 to generate Δ*lon* strain by lambda-red recombination
lonR	TTATTAGCGCTATTTGCGCGAGGTCACTA TTTTGCGGTTACAACCTGCATTGTAGGCT GGAGCTGCTTCG	
lonPF	AACACGCCGTTGAATGTGTG	To confirm the deletion of *lon* from *S.* Typhimurium 14028
lonPR	TTATATCAGGCCTGCCACGC	
wcaJ-ko-F	ATCTCCCCTTACCGCCTGCGGGTAAGGG GCCAATCACAGGAACAACGATGATTCCG GGGATCCGTCGACC	To amplify *kan* gene product from pkd13 to generate Δ*wcaJ* strain by lambda-red recombination
wcaJ-ko-R	GTAAAATAGCCTTGTGGGTCAGGTTCTTA ATACGCCGCTTTATTAACAAATGTAGGCT GGAGCTGCTTCG	
wcaJ-verF	CCAGAACCTGTTCACAAGGC	To confirm the deletion of *wcaJ* from *S.* Typhimurium 14028
wcaJ-verR	GCCTGAATGTGGAATCACGC	
TolR-ko_For	TTCTGCACCGCCAGGCGTTTACCGTAAGC GAAAGCAACAAGGGGTAAGCCGTGTAG GCTGGAGCTGCTTC	To amplify cat gene product from pDK3 to generate Δ*tolR*

TolR-ko_Rev	AAACTGTTCGCCTGTTACTCGCCGTCTTT CAAGCCAACGGGACGCAGACTCCTCCTT AGTTCCTATTCCG	strain by lambda-red recombination
TolR-F	CTGCTCGACGTACTGTTG	To confirm the deletion of *tolR* from *S.* Typhimurium 14028
TolR-R	ATCACCTGTTCAGACGGCAG	

6.0 Interface

In the previous section, I described the development of an EPS based vaccine against NTS. In addition to vaccines, mitigation strategies that reduce transmission will further reduce the global burden of NTS infections. NTS serovars are the leading cause of foodborne outbreaks, and approximately 90% of infections are acquired through the consumption of contaminated food [1,18]. Biofilm formation has been linked to *Salmonella* persistence on food produce and in food processing plants [255]. The eradication of biofilms will reduce transmission and the incidence of NTS food outbreaks. Transcription factor CsgD is the main regulator of biofilm formation, and *Salmonella* strains with mutations in *csgD* are unable to form biofilms [87,92,136]. In the next chapter I will discuss the effects of different environmental factors, such as osmolarity, nutrient availability, and temperature on *csgD* expression (biofilm formation). Understanding the regulation of *csgD* expression is important because, CsgD is an ideal target for the development of antimicrobials that can inhibit *Salmonella* biofilm formation.

7.0 Metabolic Activation of CsgD in the Regulation of *Salmonella* Biofilms

Akosiererem S. Sokaribo[1,2], Elizabeth G. Hansen[1], Madeline McCarthy[1,2], Taseen S. Desin[2, 3], Landon L. Waldner[1], Keith D. MacKenzie[4, 5], George Mutwiri Jr.[1], Nancy J. Herman[1], Dakoda J. Herman[1], Yejun Wang[6] and Aaron P. White[1,2,*]

[1] Vaccine and Infectious Disease Organization-International Vaccine Centre, University of Saskatchewan, Saskatoon S7N 5E3,
[2] Department of Biochemistry, Microbiology and Immunology, University of Saskatchewan, Saskatoon S7N5E5, Canada.
[3] Basic Sciences Department, King Saud bin Abdulaziz University for Health Sciences, Riyadh 11481, Saudi Arabia
[4] Institute for Microbial Systems and Society, Faculty of Science, University of Regina, Regina S4S 0A2, Canada
[5] Department of Biology, University of Regina, Regina S4S 0A2, Canada
[6] Department of Cell Biology and Genetics, School of Basic Medicine, Shenzhen University Health Science, Shenzhen 518060

***** Correspondence: aaron.white@usask.ca; Tel.: +01-306-966-7485
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Author Contributions

Akosiererem S. Sokaribo: Designed and performed experiments, analyzed data, wrote, reviewed, and edited manuscript.

Elizabeth G. Hansen: Performed luciferase assays and prepared amino acids stock solutions.

Madeline McCarthy: Took pictures of rdar colonies

Taseen S. Desin: Transformed *Salmonella* with plasmids.

Landon L. Waldner and Yejun Wang: Designed and performed experiments and analyzed data

Keith D. MacKenzie, George Mutwiri Jr, Dakoda J. Herman: Generated reporter plasmids

Nancy J. Herman: Generated *Salmonella* mutants.

Aaron P. White: Designed experiments, analyzed data, wrote, reviewed, and edited manuscript.

7.1 Abstract

Among human food-borne pathogens, gastroenteritis-causing *Salmonella* strains have the most real-world impact. Like all pathogens, their success relies on efficient transmission. Biofilm formation, a specialized physiology characterized by multicellular aggregation and persistence, is proposed to play an important role in the *Salmonella* transmission cycle. In this manuscript, we used luciferase reporters to examine the expression of *csgD*, which encodes the master biofilm regulator. We observed that the CsgD-regulated biofilm system responds differently to regulatory inputs once it is activated. Notably, the CsgD system became unresponsive to repression by Cpx and H-NS in high osmolarity conditions and less responsive to the addition of amino acids. Temperature mediated regulation of *csgD* on agar was altered by intracellular levels of RpoS and cyclic-di-GMP. In contrast, the addition of glucose repressed CsgD biofilms seemingly independent of other signals. Understanding the fine-tuned regulation of *csgD* can help us to piece together how regulation occurs in natural environments, knowing that all *Salmonella* strains face strong selection pressures both within and outside their hosts. Ultimately, we can use this information to better control *Salmonella* and develop strategies to break the transmission cycle.

Keywords: biofilm; *Salmonella*; CsgD; curli; cellulose; CpxR

7.2 Introduction

Salmonella enterica strains that cause gastroenteritis and typhoid fever were recently ranked first and second in terms of global disease impact (i.e., disability adjusted life years) among 22 of the most common food borne pathogens [1]. *S. enterica* strains are distributed within >2000 serovars, with yearly estimates of approximately 94 million cases of gastroenteritis [18] and 21 million cases of typhoid fever worldwide [109]. The serovars associated with typhoid fever (i.e., Typhi, Paratyphi and few others [178]) consist of human-restricted strains and are collectively referred to as typhoidal *Salmonella* (TS). The serovars associated with gastroenteritis (i.e., Typhimurium, Enteritidis and >1600 others) consist of host-generalist strains and are collectively referred to as nontyphoidal *Salmonella* (NTS) [5]. NTS outbreaks are relatively common occurrences and are often linked to the consumption of contaminated food produce, such as poultry [19,256], fruits, vegetables [20,87] and processed foods [21]. In general, NTS strains

have a remarkable ability to persist and survive in harsh conditions, including extremes of drying and nutrient limitation [118,257,258].

The majority of NTS strains can form biofilms, a specialized physiology that is characterized by multicellular aggregation, long-term survival, and resistance. Biofilm formation has been linked to *Salmonella* persistence on food surfaces, plants, and other produce, and is thought to provide protection during food processing [255,259,260]. Aside from the food-borne aspects, biofilm formation is hypothesized to be an integral part of the life cycle of gastroenteritis-causing *Salmonella* strains, by ensuring long-term survival of cells as they cycle between hosts and the environment [255,261]. We have speculated that biofilms are connected to the host generalist lifestyle since the environment (soil and water) would be a common collecting point for multiple host species. In contrast, there is widespread loss of biofilm formation in TS strains and other more invasive strains, such as the specialized NTS strains associated with human bloodstream infections in sub-Saharan Africa [136,150], although TS produce alternative biofilms on gallstones inside human carriers [262]. There are multiple selection pressures acting on biofilm formation in diverse *Salmonella* strains. In short, biofilms are thought to represent the most dominant form of bacterial life on the planet and understanding the regulation of this specialized physiology is important.

Biofilm-forming strains of *S. enterica* can be identified by the production of distinct rdar (red, dry and rough) morphotype colonies when grown on agar-containing media supplemented with the dye Congo red. Cells within the colony are held together by curli fimbriae for short-range interactions and cellulose for long-range interactions [91,92,118]. In addition, other polymers are part of the extracellular biofilm matrix, including polysaccharides (i.e., O-Ag capsule, colanic acid and cellulose) proteins (i.e., BapA, curli, and flagella), lipopolysaccharides and DNA [87,263]. Curli, cellulose and the biofilm matrix impart survival and persistence properties on cells within the biofilm [118,255,258]. It is not known if the survival traits are specific to the polymers themselves or are emergent properties associated with cells entering a unique physiological state [263–265]. Perhaps the microenvironments generated within a biofilm are responsible for the adaptations, heterogeneity and cellular differentiation observed during biofilm formation [93,266].

In *Salmonella*, regulation of curli, cellulose and other polymers is coordinated through CsgD, the main transcriptional controller of biofilms. The activation of CsgD in vitro has been well-defined, with growth conditions of low osmolarity, lower temperatures and limiting nutrients necessary to activate *csgD* transcription [89]. Expression of *csgD* is repressed tightly at early stages of growth but is induced up to 370-fold when cells enter the stationary phase of growth [88]. The same general principles apply in *E. coli*, which shares the CsgD, curli, and cellulose biofilm components [267]. In the stationary phase of growth, cell density in the culture is high, nutrients become limiting and cells express the alternative sigma factor RpoS [268]. RpoS controls the general stress response [99] and selectively transcribes *csgD* [269,270]. The effects of osmolarity are mediated through the EnvZ/OmpR and CpxA/CpxR two-component signal transduction systems [102]. In low osmolarity, low levels of phosphorylated OmpR bind to a high-affinity binding site −50.5 bp upstream of the *csgD* transcription start sites, which activates *csgD* transcription [103]. In high osmolarity, transcription is repressed through binding of phosphorylated CpxR to multiple sites on the *csgD* promoter [102], as well as phosphorylated OmpR binding to a low-affinity site in the *csgD* promoter [271]. We realized that the complex regulatory network behind *csgD* activation [87] was even more dynamic when it was discovered that CsgD was produced in a bistable manner [213,272,273]. Biofilm cells are maintained in a CsgD-ON state due to a predicted feed-forward loop consisting of RpoS, CsgD and IraP, a protein that stabilizes RpoS [176,270]. The remaining single cells are in a CsgD-OFF state and express several important virulence factors [255]. The connection between persistence and virulence during biofilm formation brings into question the hierarchical regulation of this process, as well as determining how individual cells become activated and remain in their CsgD-ON or -OFF states.

The regulation of *Salmonella* biofilms is also strongly influenced by the intracellular levels of the second messenger, cyclic-di-GMP (c-di-GMP). It is synthesized from two guanosine 5′-triphosphate molecules by diguanylate cyclases (DGCs) and degraded by specific phosphodiesterases (PDEs). In general, high levels of c-di-GMP are associated with biofilm formation, sessility and persistence, and low levels of c-di-GMP are associated with motility and virulence [274]. The change in c-di-GMP levels in *S. enterica* is controlled by the enzymatic activity of 17 different DGCs and PDEs. For biofilms, the cellulose synthase enzyme, BcsA, is

allosterically activated by c-di-GMP that is produced by AdrA, a DGC that is transcriptionally activated by CsgD. Expression of CsgD itself is influenced by c-di-GMP synthesis and breakdown by a network of DGC and PDE enzymes [105]. The importance of c-di-GMP regulation is underscored by the observation that *S. enterica* isolates that are defective in the production of DGCs are both avirulent and unable to form biofilms [127].

In this manuscript, we analyzed the regulation of *csgD* transcription and the activity of CsgD through activation of curli biosynthesis (*csgBAC*) and cellulose production (*adrA*). We examined the response to different environmental signals (i.e., temperature, osmolarity, nutrients) and discovered that there is a hierarchy of regulation. These environmental signals were selected because their effects on *csgD* expression before induction have been well established and we hypothesize that these conditions would be encountered during food processing and in both host and non-host environments. We established that the CsgD system responds differently or not at all to known regulatory inputs once it has been activated. This is similar to some dedicated, point of no return processes, such as sporulation in *Bacillus subtilis* [108]; however, we show that the CsgD system can be reversed by other signals, such as glucose. The implications for the *Salmonella* lifecycle are discussed.

7.3 Results

7.3.1 Osmolarity Has No Effect Once *csgD* Transcription Is Activated

Osmolarity is a key regulatory factor for *Salmonella* biofilm formation *in vitro* [89,275]. In the presence of high concentrations of NaCl, *csgD* transcription is abolished [95]. In *E. coli*, this repression is mediated through the CpxA/R two-component system [103]. We performed transcription experiments with *S. enterica* serovar Typhimurium ATCC 14028 (i.e., *S.* Typhimurium 14028). Consistent with *E. coli*, expression of *csgD* was highest in low osmolarity media (i.e., no salt) and reduced sequentially in media supplemented with increasing concentrations of NaCl (Figure 7.1A). Reduced *csgD* expression in media supplemented with 75 mM or more salt correlated with basal expression of *csgBAC* (curli biosynthesis) and *adrA* (cellulose biosynthesis) (Figure 7.1B, C). To gauge the activity of the CpxA/R system, and its potential role in repression, we monitored expression of *cpxP*, a known regulatory target of

CpxR [276]. Expression of *cpxP* was highest in media supplemented with 150 mM NaCl (Figure 7.1D), which was inversely correlated with *csgD* expression levels. This was consistent with CpxR-mediated repression of *csgD* transcription. Regulation of *csgD* expression via the CpxA/R system is thought to be a dynamic process involving surface-sensing and feedback during curli production [102,277]. Therefore, we performed experiments where salt was added to growing cultures after 18 h of growth, rather than being premixed into the media before growth. At 18 h of growth, *csgD* expression level is rapidly increasing and *csgBAC* and *adrA* expression are just beginning to increase [265]. Under these conditions, *csgD* expression did not change when increasing concentrations of salt were added during growth; the expression curves were nearly superimposable regardless of the amount of salt added (Figure 7.1E). Expression of *csgBAC* was also not inhibited by the addition of salt and was actually increased at high salt concentrations (Figure 7.1F). For *adrA*, mild repression was observed, but expression was well above background levels, even in the presence of 150 mM salt (Figure 7.1G). *cpxP* expression, on the other hand, was similar to the premixed experiments, with highest expression in the 150 mM salt media and lowest expression in non-supplemented media (Figure 7.1H). These results indicated that the Cpx system was activated by the addition of salt during growth but was no longer causing repression of *csgD* transcription and the downstream genes involved in curli and cellulose production.

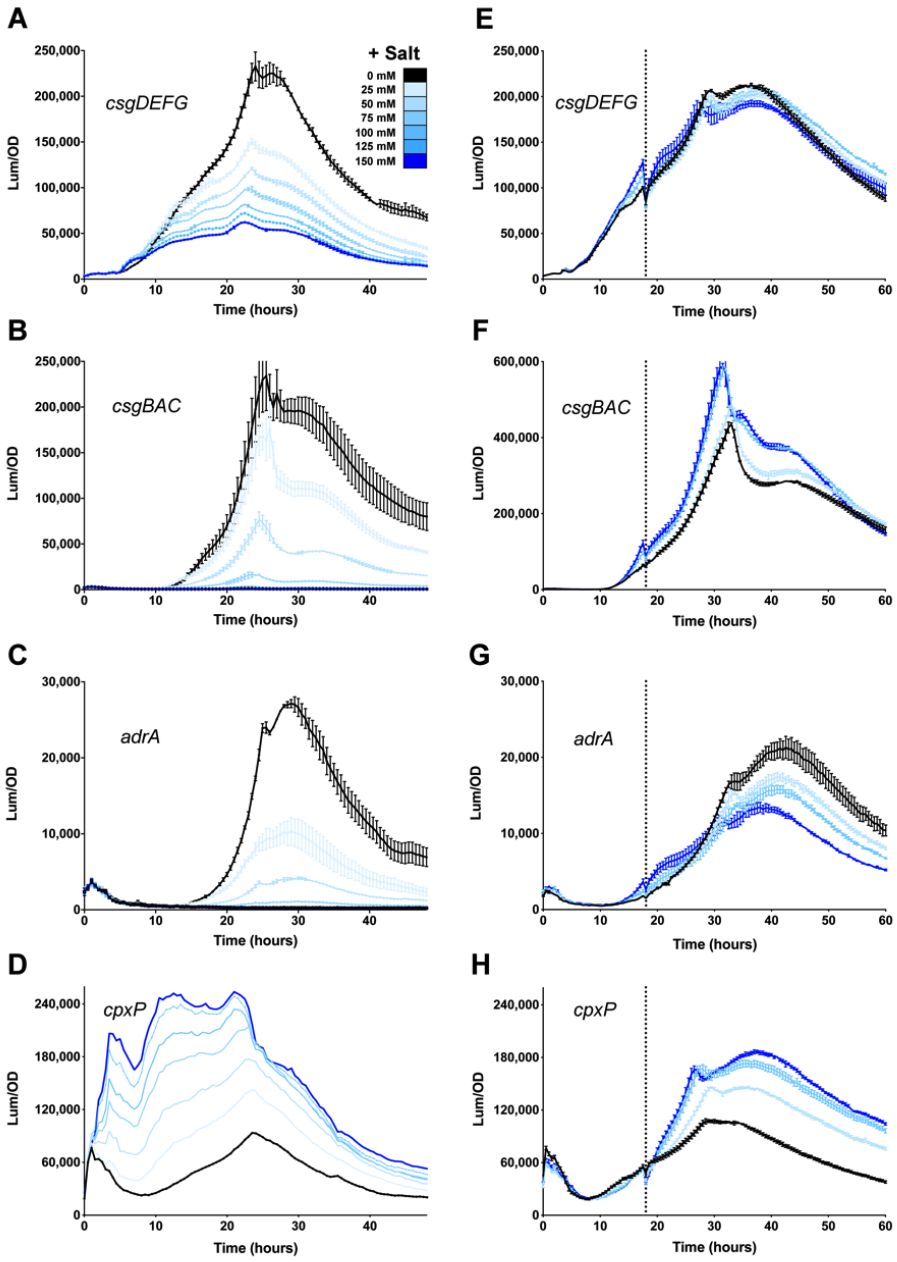

Figure 7.1: Response of the *Salmonella csgD* regulatory network to changes in osmolarity. *csgDEFG* (A and E), *csgBAC* (B and F), *adrA* (C and G), and *cpxP* (D and H) expression was measured in *S.* Typhimurium 14028 during growth at 28°C in media premixed with 25, 50, 75, 100, 125 or 150 mM salt (A–D) or with 50, 100, or 150 mM salt added during growth (E–H; vertical line shows the time of addition at 18 h). For each graph, luminescence (light counts per second) divided by the optical density at 600 nm (Lum/OD) was plotted as a function of time with each curve representing a single growth condition. The mean and standard deviations are plotted from experiments performed in triplicate (A–C, E–H) or from a single representative experiment (D).

7.3.2 Repressive Effect of CpxR on *csgD* Transcription Is Alleviated During Growth

To examine the effects of Cpx-mediated repression of *csgD* transcription in more detail, we monitored gene expression in a Δ*cpxR* mutant background. The Cpx system can be activated by high concentrations of metals and a variety of other signals, with each thought to represent a form of periplasmic stress [277,278]. Growth of *S*. Typhimurium 14028 in media supplemented with 1 mM copper chloride resulted in activation of the Cpx system, as measured by an increase in *cpxP* expression (Figure 7.2A, + inducer). As expected, *cpxP* expression was off in the Δ*cpxR* strain background (Figure 7.2A, red line). In the presence of copper chloride, *csgD* expression reached high levels, similarly to when in the presence of non-supplemented media (Figure 7.2B). There was also a slight increase in the Δ*cpxR* strain, which was consistent with CpxR being a repressor of *csgD* transcription. This effect was more pronounced for *csgBAC*, as expression was approximately four times higher in the Δ*cpxR* strain (Figure 7.2C). We performed the same experiment with the addition of copper chloride after 18 h of growth. The Cpx system was activated normally, as shown by elevated *cpxP* expression levels in the presence of the inducer (Figure 7.2D). However, expression of *csgDEFG* and *csgBAC* was unchanged in the Δ*cpxR* mutant strain, showing no evidence of CpxR-mediated repression (Figure 7.2 E, F). This indicated that once the *csgD* network was activated, the system was unresponsive to CpxR.

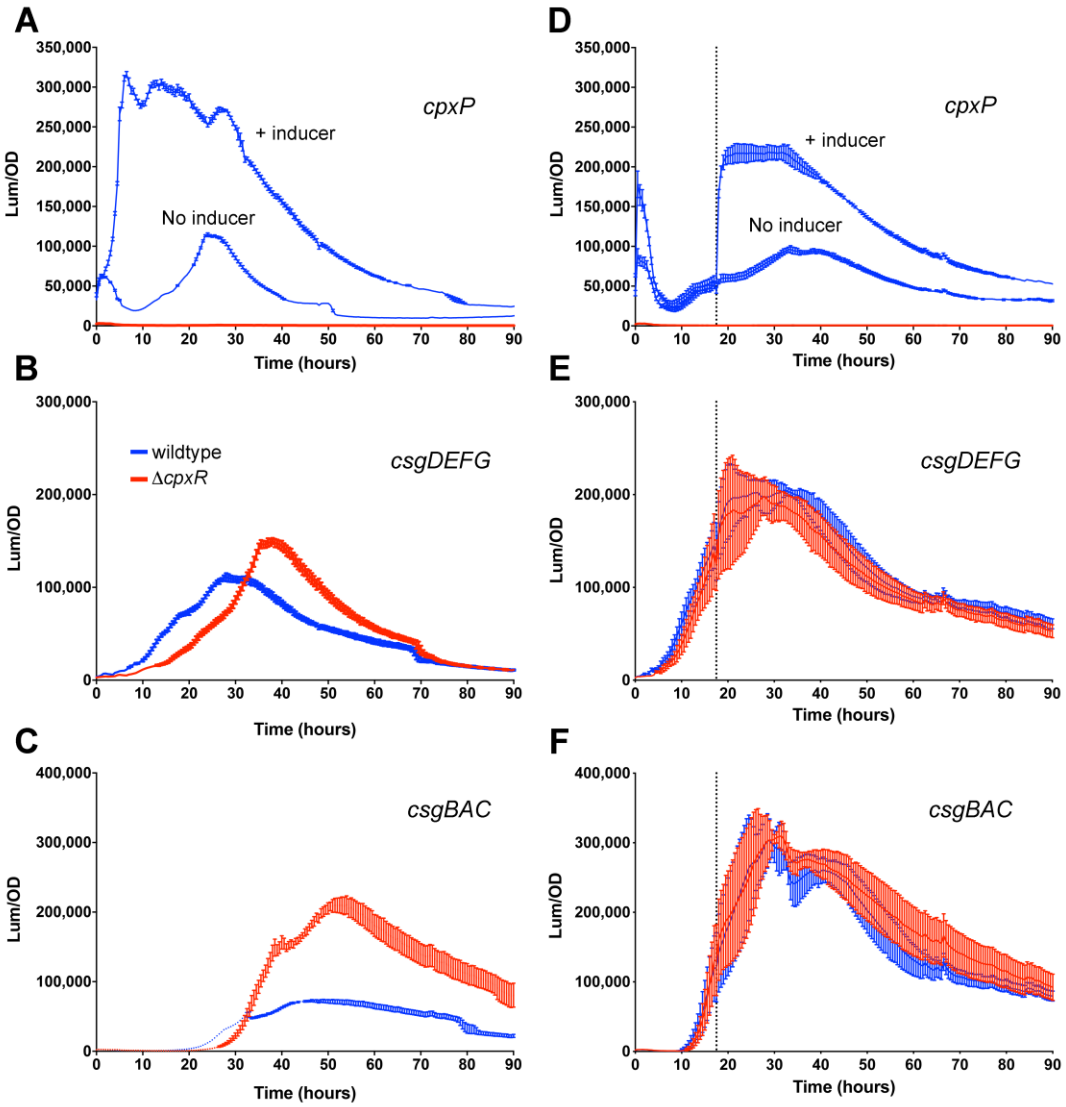

Figure 7.2*:* The Cpx system has no repressive effect on *csgD* transcription once the biofilm network is activated. Expression of *cpxP* (A and D), *csgDEFG* (B and E), and *csgBAC* (C and F) operons was measured during growth of *S.* Typhimurium 14028 wild-type (blue) or Δ*cpxR* strains (red) at 28°C in media supplemented with 1.0 mM CuCl2 (+ inducer) added at the beginning of growth (A–C) or added after 18 h of growth (D–F; the vertical, dotted line represents the time of addition). For each graph, luminescence divided by the optical density at 600 nm (Lum/OD) was plotted as a function of time and each curve represents a single growth condition. The mean and standard deviations are plotted from three biological replicate experiments measured in triplicate.

We also measured biofilm gene expression after the addition of sucrose (Figure 3). In *E. coli*, sucrose has been shown to repress *csgD* transcription, due to the activity of H-NS [102]. Sucrose is also a cleaner measure of osmolarity because unlike salt, it does not result in a change of ionic strength. In general, the *csgDEFG*, *csgBAC* and *adrA* expression profiles were consistent with

what was measured in response to salt addition. When sucrose was added to the media before growth, significant repression was observed for all three promoters (Figure 7.3 A, B, C). However, when sucrose was added to growing cultures at 18 h, there was no repression measured (Figure 7.3 E, F, G). The addition of sucrose had minimal effect on *cpxP* expression (Figure 7.3 D, H), and, therefore, did not appear to engage the CpxR/A system, similar to what was observed in *E. coli* [102]. These results indicated that the *S.* Typhimurium *csgD* biofilm network is not influenced by changes in osmolarity after it has been activated. Moreover, this appears to be a general effect that is not restricted to repression by the Cpx system.

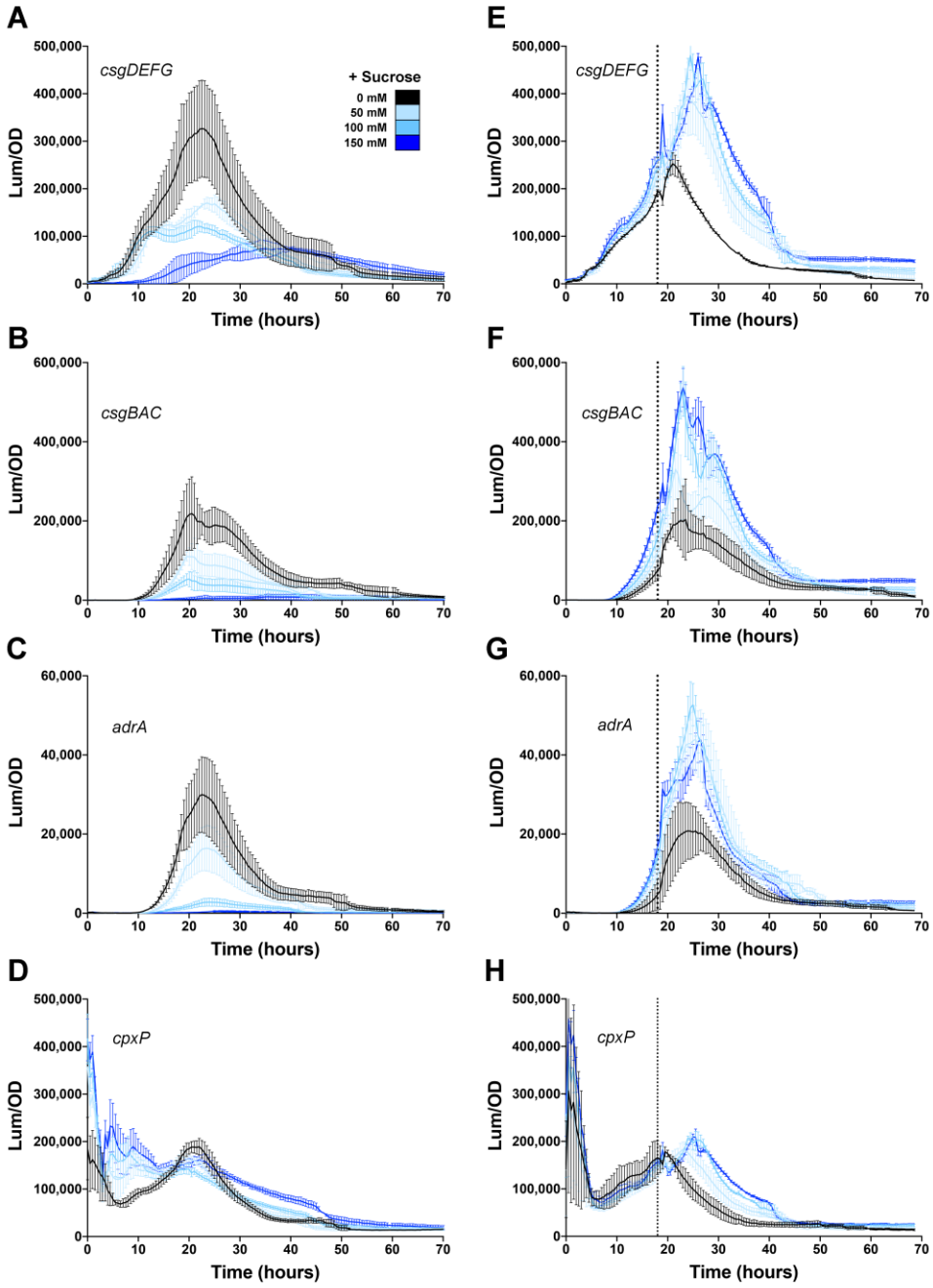

Figure 7.3: Effect of sucrose addition on the *Salmonella csgD* regulatory network. Expression of *csgDEFG* (A,E), *csgBAC* (B,F), *adrA* (C,G), and *cpxP* (D,H) operons was measured during growth of *S.* Typhimurium 14028 at 28°C in media premixed with 50, 100 or 150 mM sucrose (A–D) or with sucrose added during growth (E–H; vertical line represents the time of addition at 18 h). For each graph, luminescence (light counts per second) divided by the optical density at 600 nm (Lum/OD) is plotted as a function of time and each curve represents a single growth condition. The mean and standard deviations are plotted from three biological replicate experiments measured in triplicate.

7.3.3 Temperature and Glucose Repress *csgD* Expression

The idea that the biofilm system can become unresponsive to known regulatory inputs once it is activated fits with one of the hallmarks of a bistable gene expression system, in that a proportion of cells can remain activated even when the inducer is absent [279]. There are other bacterial physiologies, such as sporulation, where the cellular differentiation process is irreversible [108]. The *csgD* biofilm network has been shown to have bistable expression [176,272]. We wondered if the response we had observed with osmolarity was representative of a non-reversible system. In most *Salmonella* and *E. coli* strains, *csgD* expression and biofilm formation is activated at temperatures below 30°C and repressed at higher temperatures [95]. There are strains that produce biofilms at higher temperatures (i.e., 37°C), but these typically possess single nucleotide polymorphisms in the *csgD* promoter region that allows for dysregulated expression [95,171,280,281]. We tested whether increased temperature could shut off activated biofilm gene expression by first growing cells at 28°C for 18 h and then shifting the temperature to 30, 32, 35, or 37°C. At 30°C or 32°C, there was a measurable drop in *csgDEFG*, *csgBAC* and *adrA* expression, but it was still above background levels (Figure 7.4A, B, C). However, a temperature shift above 32°C reduced gene expression to baseline levels (Figure 7.4A, B, C). This showed that high temperature was able to override the activation of *csgD* and biofilm related genes. Glucose is another powerful repressor of *csgD* expression and biofilm formation *in vitro* [265,282]. For *S.* Typhimurium, the addition of glucose to growing cultures at 18 h rapidly abolished *csgDEFG* (Figure 7.4D), *csgBAC* (Figure 7.4E) and *adrA* (Figure 7.4F) expression, even at the lowest added concentration of 25 mM. We tested lower concentrations of glucose and found that in each case, *csgD* transcription was immediately repressed but was restored at later timepoints, presumably when all glucose was metabolized (data not shown). This showed that glucose was a powerful repressive signal. Together, these experiments showed that activation of the *S.* Typhimurium *csgD* biofilm network is a reversible process and suggested the existence of a regulatory hierarchy.

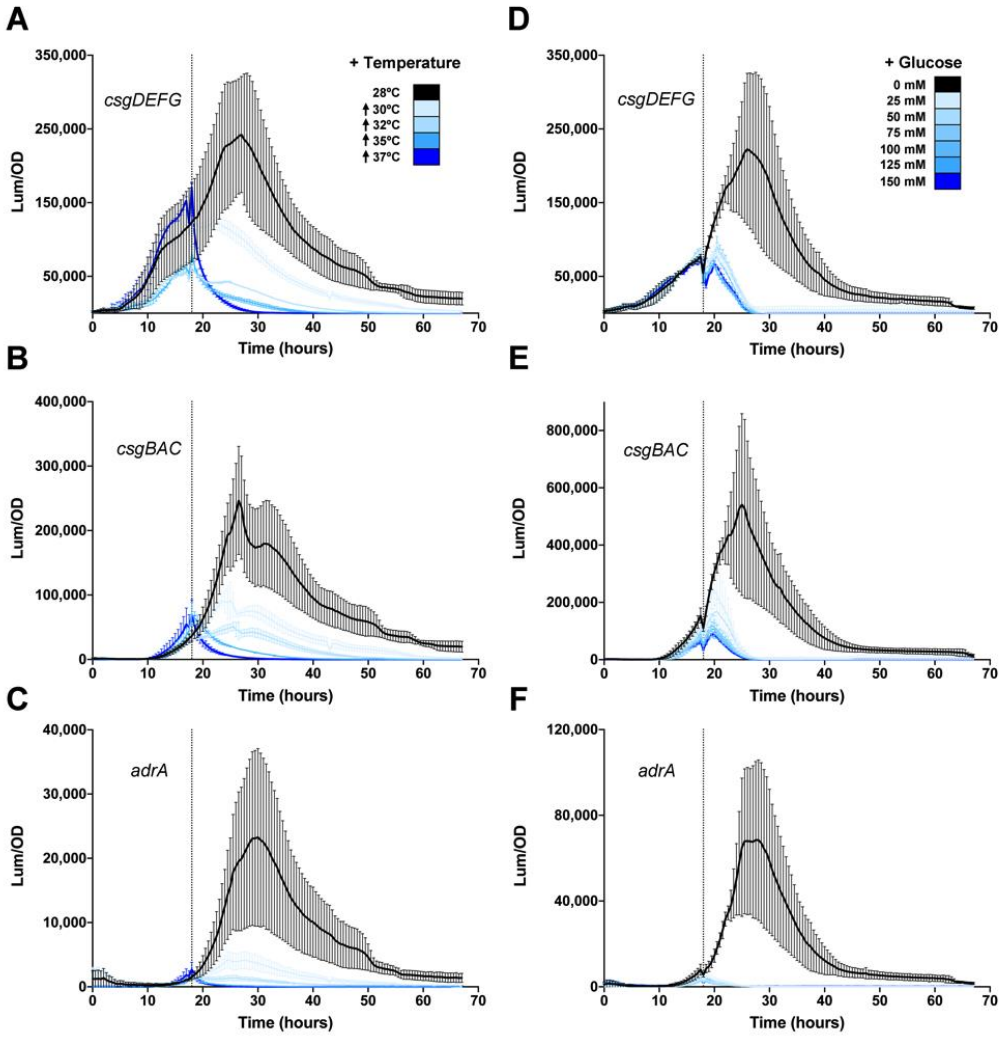

Figure 7.4: The *csgD* biofilm network in *Salmonella* is repressed by the addition of glucose or an increase in growth temperature. Expression of *csgDEFG* (A, D), *csgBAC* (B, E), and *adrA* (C, F) was measured during growth of *S*. Typhimurium 14028 at 28°C for 18 h prior to temperature shift (A–C) or the addition of 25, 50, 75, 100, 125, or 150 mM glucose (D–F). The vertical dotted line represents the time of temperature shift or glucose addition. For each graph, luminescence divided by the optical density at 600 nm (Lum/OD) is plotted as a function of time and each curve represents a single growth condition. The mean and standard deviations are plotted from three biological replicate experiments measured in triplicate.

7.3.4 Effect of Casamino Acids on Biofilm Formation

Expression of *csgD* is known to be activated once cells reach a critical density and nutrients start to run out [88]. Since 1% tryptone is primarily an amino acid-based media [283], we speculated that the addition of amino acids would reduce or delay expression of *csgD* and other biofilm genes. Casamino acids (CAA) are a complex mixture of amino acids and small peptides that are

used for nutritional investigations of bacterial growth. The addition of CAA to the medium prior to *S*. Typhimurium growth reduced *csgDEFG* expression approximately 15-fold in the presence of 0.5, 1.0 or 2.0% CAA (Figure 7.5A). *csgBAC* and *adrA* expression dropped to near baseline levels when CAA was added at the beginning of growth (Figure 7.5B, C). The addition of CAA to growing *S.* Typhimurium 14028 cultures also reduced the expression of all three promoters, but in a more dose dependent manner. Expression of *csgDEFG* was reduced to ~75%, 50% and 25% of initial levels after the addition of 0.5% CAA, 1.0% CAA and 2% CAA, respectively (Figure 7.5D). Expression of *csgBAC* was reduced after the addition of 0.5% or 1.0% CAA, but the promoter was still considered active, whereas expression returned to baseline after the addition of 2.0% CAA (Figure 7.5E). *adrA* expression returned to near baseline levels, even with the addition of 0.5% CAA (Figure 7.5F). These experiments demonstrated that there is a metabolic feedback into *csgD* expression and that the system responds differently once it has been activated.

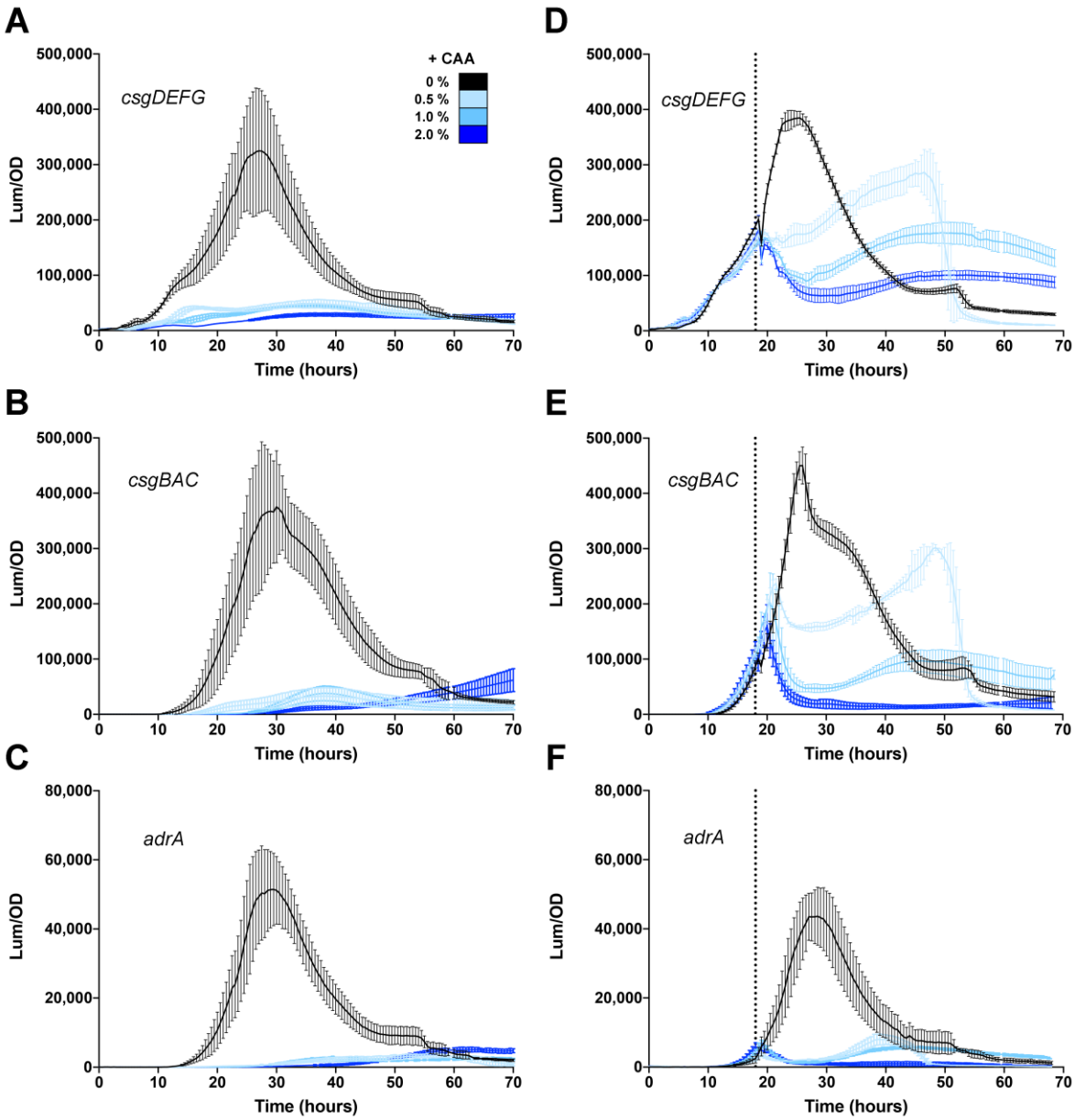

Figure 7.5: The *csgD* biofilm regulatory network in *Salmonella* is repressed by the addition of amino acids. Expression of *csgDEFG* (A, D), *csgBAC* (B, E), and *adrA* (C, F) was measured during growth of *S.* Typhimurium 14028 at 28°C in media premixed with 0.5%, 1.0% or 2.0% casamino acids (A–C) or in media where casamino acids were added during growth (D, E, F; the dotted line represents the time of addition at 18 h). For each graph, luminescence (light counts per second) divided by the optical density at 600 nm (Lum/OD) is plotted as a function of time and each curve represents a single growth condition. The mean and standard deviations are plotted from three biological replicate experiments measured in triplicate.

7.3.5 Differing Effects of Individual Amino Acids on *csgD* Gene Expression

We wanted to test how individual amino acids contributed to the repression of biofilm gene expression caused by CAA. We measured *csgBAC* expression i.e., curli production) as a proxy for biofilm formation and as readout for CsgD activity. Only Asn, Pro and Arg had a direct repressive effect on *csgBAC* expression when added individually (Figure 7.6A; blue bars). The expression curves were lower for the entirety of growth (Figure 7.6B). Six amino acids had no significant effect (Figure 7.6A, grey bars; examples in 7.6C) and seven amino acids caused an increase in expression (Figure 7.6A, pink bars). The addition of Gly and Thr yielded an approximately three-fold boost to *csgBAC* expression (Figure 7.6D), which was unexpected. These results indicated that the repression caused by CAA must have been due to the cumulative effect of multiple amino acids. When individual amino acids were added to *S.* Typhimurium cultures after 18 h of growth, the effects on *csgBAC* expression were not predictable based on their previous groupings (Figure 7.6E; see color distribution). No amino acids caused a decrease in expression, and some that were repressive when added before growth (i.e., Arg, Pro), now caused a significant boost in expression (Figure 7.6E, F). Eight amino acids had no significant difference from the water control (Figure 7.6G, Lys, Ser). Val, Ala, Gln and Thr led to increased *csgBAC* expression when added before or during growth, suggesting that these amino acids have a positive effect on curli fimbriae synthesis. Glycine, on the other hand, had no significant effect when added during growth (Figure 7.6H). Overall, we could not explain the differing effects of individual amino acids when added during growth. However, the results were consistent with our previous observation that the *S.* Typhimurium biofilm network responds differently to regulatory inputs after the *csgD* network has been activated.

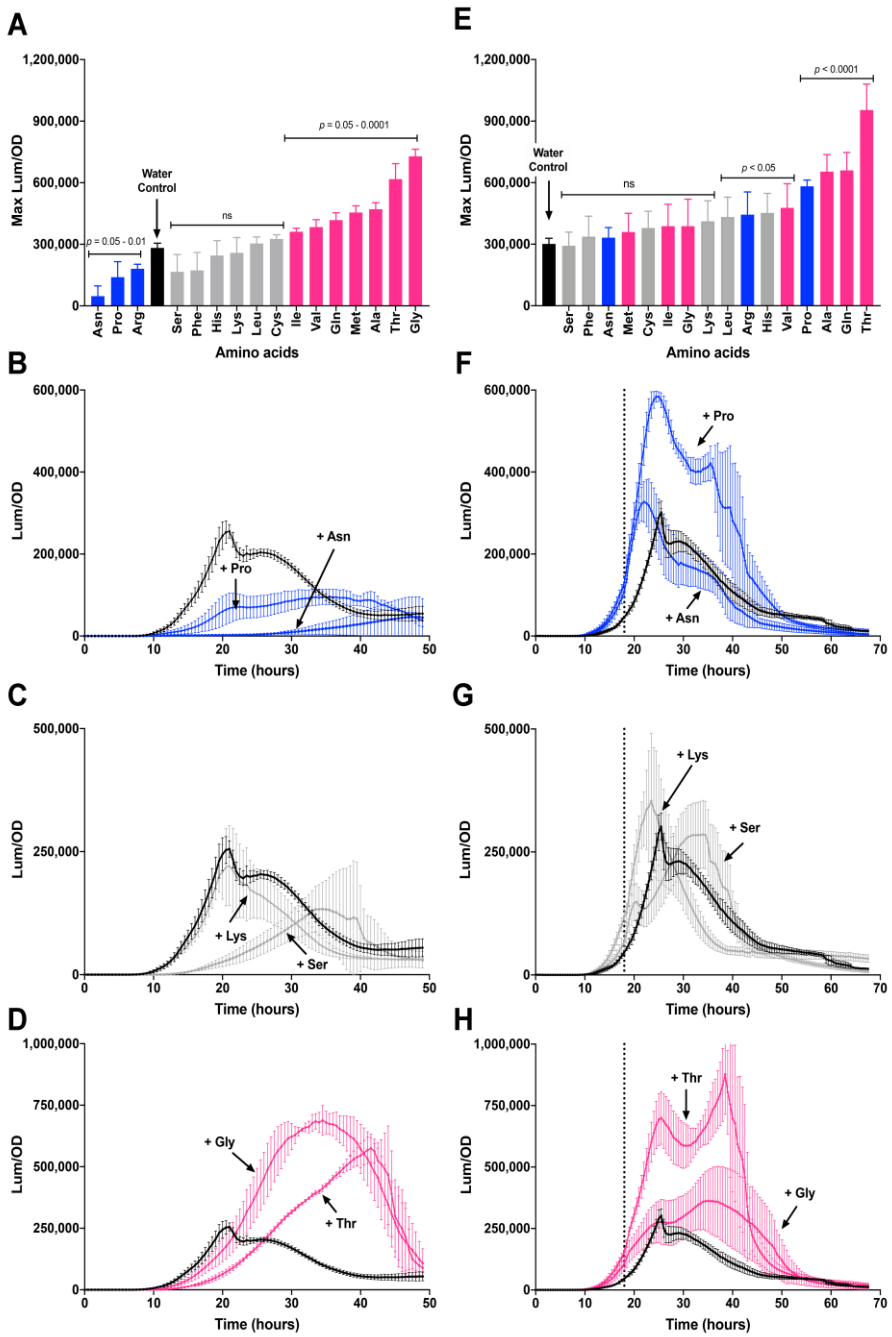

Figure 7.6: Individual amino acids have differing effects on the *csgD* biofilm regulatory network in *S.* Typhimurium 14028. Maximum expression of the *csgBAC* operon (curli production) was recorded during growth of *S.* Typhimurium 14028 at 28°C in media premixed with 15 mM of individual amino acids (A) or in media where the amino acids were added after 18 h of growth. The maximum Lum/OD values after addition of each amino acid were statistically compared to a water control and amino acids were determined to have a repressive (blue), neutral (grey) or stimulatory effect (purple) on *csgB* expression (A). This color scheme was used to represent the same amino acids when they were added after 18 h of growth (E). Lum/OD values were plotted as a function of time corresponding to selected amino acids premixed into the media (B–D) or added at 18 h of growth (F–H; the dotted line represents the time of addition). For each curve, the mean and standard deviations are plotted from three biological replicate experiments measured in triplicate.

7.3.6 Regulation of Rdar Morphotype on Agar-Containing Media

In the bistable expression of CsgD, the proportion of cells in the "ON" state is thought to be maintained by a feed-forward loop consisting of RpoS, the stationary phase sigma factor that controls *csgD* transcription, IraP, a protein that stabilizes RpoS, and CsgD itself [176,270]. In addition, *csgD* expression and CsgD activity can be influenced by the bacterial secondary messenger, cyclic-di-GMP (c-di-GMP) [105]. We wanted to investigate how these additional regulatory components influenced metabolic control of the *S.* Typhimurium biofilm regulatory network. Strains were grown at 28°C or 37°C on 1% tryptone agar, with different components added to the media. To modulate intracellular c-di-GMP levels, strains were transformed with plasmids over-expressing *stm1987*, encoding a DGC enzyme that generates c-di-GMP, or *yhjH*, encoding a PDE enzyme that breaks down c-di-GMP. To analyze the proposed feed-forward loop, we utilized a plasmid over-expressing RpoS and measured gene expression in Δ*rpoS* and Δ*iraP* strains. Each strain was transformed with a luciferase reporter plasmid so that we could visualize *csgBAC* expression. The vector-only *S.* Typhimurium 14028 control strain displayed robust light production at 28°C, with faint *csgBAC* signals also observed in the presence of 25mM salt (Figure 7, vector). Overexpression of *rpoS* appeared to elevate *csgBAC* expression under most conditions, including in the presence of salt and at 37°C (Figure 7, *rpoS*). The importance of RpoS was emphasized in that the Δ*rpoS* strain had no visible *csgBAC* expression under all tested conditions (data not shown), unless it was co-transformed with pACYC/*rpoS* (Figure 7; 28°C Δ*rpoS*). A strong stimulatory effect was also caused by the over-expression of *stm1987*, which allowed for robust *csgBAC* expression and biofilm colony morphology under most conditions (Figure 7, *stm1987*). The strain transformed with pBR322/*stm1987* was the only one to have detectable *csgBAC* expression at 37°C in the presence of 25mM salt (Figure 7). This indicated that elevated levels of c-di-GMP may be enough to overcome temperature-based repression of *csgBAC*. Emphasizing the importance of c-di-GMP, the expression of *yhjH* was sufficient to abolish *csgBAC* expression at 28°C (Figure 7, *yhjH*), as well as in all other tested conditions (data not shown). In contrast, deletion of *iraP* appeared to have little effect on *csgBAC* expression, with only a mild reduction observed at 28°C (Figure 7, Δ*iraP*). Finally, the presence of glucose in the media abolished *csgBAC* expression in all strain and plasmid combinations (Figure 7, 0.2% Glc). This experiment indicated that increased levels of RpoS and

c-di-GMP could partially overcome some *csgBAC* repression, and that glucose was perhaps the most powerful metabolic signal feeding into the *S.* Typhimurium *csgD* regulatory network.

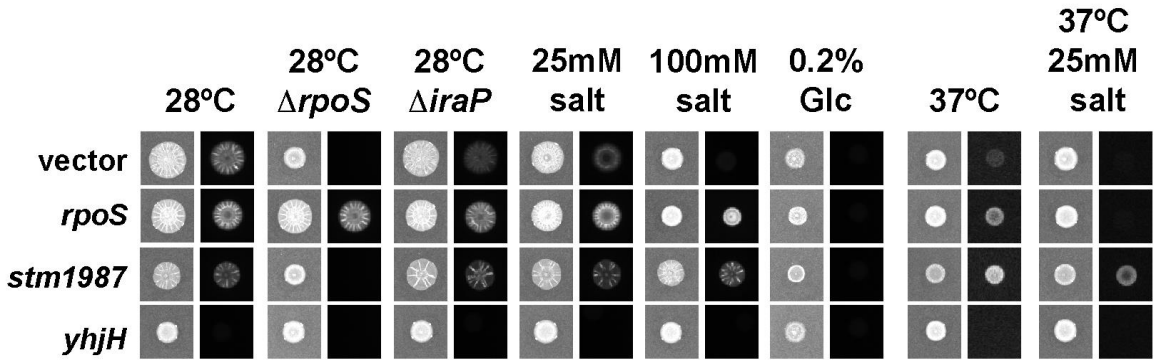

Figure 7.7: Visualization of *S*. Typhimurium curli expression in response to changing growth conditions. *S*. Typhimurium 14028 wild-type, Δ*rpoS* or Δ*iraP* reporter strains containing a *csgBAC* promoter–luciferase fusion were transformed with pBR322 (vector), pACYC/*rpoS* (rpoS), pBR322/*stm1987* (*stm1987*) or pBR322/*yhjH* (yhjH) plasmids. Cells were inoculated onto T agar or T agar supplemented with 0.2% glucose, 25 mM or 100 mM NaCl and grown at 28°C or 37ºC. Colony morphology (left column) and luminescence (right column) was recorded after 48 h growth. Control strains containing pACYC were also tested, but the *csgBA*C expression profiles were similar to strains transformed with pBR322; therefore, only the pBR322 pictures are shown.

7.4 Discussion

Biofilm formation is subject to tight and complex regulation through transcription factor CsgD. In *S*. Typhimurium, the intergenic region between divergent *csgDEFG* and *csgBAC* operons is among the longest non-coding region with 582 bp, which allows for a highly sophisticated signaling network. CsgD expression is regulated at the transcriptional, post transcriptional, translational and post translation level, in response to a variety of external and internal signals [87]. In this study we show that once activated, the CsgD biofilm network responds differently to metabolic inputs.

The ability of *S. enterica* strains to form biofilms is thought to be critical for the success of *Salmonella* as pathogens, particularly for gastroenteritis-causing strains [255]. With bistability of CsgD synthesis resulting in distinct cell types—multicellular aggregates associated with persistence (CsgD-ON), and single cells associated with virulence (CsgD-OFF) [176]—there is a need to have a flexible and dynamic response. We speculated that this phenotypic heterogeneity was a form of bet-hedging. A bet-hedging strategy ensures that at least one group of cells will be

more adapted for a specific set of conditions that is encountered [284]. For some bacterial processes, such as sporulation, the advantage of the sporulating cell is obvious; however, for the non-sporulating cells, the advantage lies in being capable of more rapid growth when an influx of new nutrients occurs [285]. For *Salmonella*, there is a lot of energy devoted to generating the polymers associated with biofilm aggregates [265,286]; in the virulent, single cell group, synthesis of the type three secretion apparatus also requires a significant outlay of energy [287]. This type of population split makes the most sense in response to the unpredictability of transmission [288] or perhaps for modulating host–pathogen interactions, as observed for *Vibrio cholerae* [289]. We analyzed regulation before *csgD* activation, which has been tested before in *S.* Typhimurium and *E. coli* and generally had the expected results, and compared this to regulation after *csgD* activation, which to our knowledge has not been tested before. We observed that *csgD* transcription and activation of downstream biofilm components was no longer repressed by increased osmolarity, and that the response to nutrient addition was also different, either as individual amino acids or a set of pooled amino acids. In contrast, the addition of glucose and temperatures above 32°C rapidly repressed *csgD*, *csgB* and *adrA* expression even after induction. We approached these experiments from the point of view of biofilm formation as a developmental process [290,291], and our results show that CsgD biofilm formation is reversible, but can also be viewed as irreversible, depending on the signal. Our results, therefore, suggest the existence of a regulatory hierarchy among external signals that regulate biofilm formation.

For osmolarity, it has been well established that the optimal conditions for *csgD* expression and rdar biofilm formation in vitro include low osmolarity [95,119,275]. Key transcription factors have been identified (i.e., OmpR, CpxR, H-NS, MlrA and others) and binding within the *csgD* promoter region has been characterized [89,102–104,292]. Yet, there are still some intriguing aspects; for example, *S. enterica* biofilm cells produce high levels of osmoprotectants even when growing in low osmolarity conditions [265]. To explain the accumulation of osmoprotectants, we hypothesized that there could be high osmolarity microenvironments created within biofilms due to nutrient and ion trapping by the extracellular matrix [263,293]. The presence of hyperosmolar environments was recently observed with *E. coli* biofilms [294]. Our experiments show that once CsgD has activated downstream target genes (i.e., *csgBAC* (curli) and *adrA* (cellulose),

transcription of all units becomes unresponsive to increases in osmolarity. This was specific to the CpxR/A two-component system in high salt conditions and by activating CpxR in ways that are not expected to significantly cause a change in osmolarity (i.e., metal stress) [278]. There has been some recent controversy about the role of CpxR in surface sensing or adhesion [295], but it is a well-established regulator of *csgD* [296]. The osmolarity effect was also general, as similar gene expression patterns were observed after the addition of sucrose, which was shown to repress *csgD* transcription in *E. coli* by acting through H-NS [102]. In our experiments, the CpxR/A system was not activated by the addition of sucrose, therefore, we assume that the same H-NS-mediated signaling occurs in *Salmonella*. To explain the results with *csgD*, it is possible that the presence of osmoprotectants produced early on during biofilm formation could mute the signaling effects associated with high external osmolarity [297]. Although we have shown that several osmoprotectant-associated genes are produced in time with *csgBAC* and *adrA* [265], we do not know the detailed time course for the appearance of the molecules themselves. The biological relevance for a lack of response to increased osmolarity is not clear, however, a recent paper described a real-world scenario where such a characteristic could be favored. Grinberg et al. 2019 [298] demonstrated that bacterial aggregates have enhanced survival on the surfaces of leaves in microdroplets that are not visible to the naked eye. As liquid evaporates from the leaf surfaces, solutes become concentrated and the microdroplets become hyperosmolar solutions. One could envision *S. enterica* biofilm aggregates surviving well in this scenario due to their stress-resistance adaptations and the altered *csgD* regulatory program identified here. We hypothesize that these microdroplets represent an environment where biofilms, and presumably biofilm-forming strains, would be favored over individual cells that do not aggregate together or strains that do not form biofilms.

Nutrient limitation was one of the first activating signals identified for *csgD* transcription [88,275]. In 1% tryptone or lysogeny broth, which are predominantly comprised of amino acids [283], *csgD* transcription occurs when cell density increases, and cells start to run out of nutrients [87,286]. While this was initially attributed to phosphate and nitrogen depletion [88], we tested if supplementation with additional amino acids would delay or prevent activation of *csgD* transcription. When amino acids were added together (i.e., casamino acids), the transcription of *csgD* and downstream biofilm genes was delayed for almost the entire 70-h

growth period, well after high cell densities were reached. When CAA were added during growth, *csgD* expression was shifted down in a dose dependent manner. This showed that after induction, *csgD* expression was still responsive to negative regulation by CAA. The dose response could represent a subpopulation of *S.* Typhimurium cells that retain metabolic flexibility [299] and are able to shift their metabolism away from biofilm formation. Based on the results with CAA, we predicted that individual amino acids might also have a repressive effect on biofilm formation. We measured the expression of the curli biosynthesis operon (i.e., *csgBAC*), a direct target of CsgD. Only Asn, Pro and Arg reduced *csgB* expression when added before growth, while Ile, Val, Gln, Met, Ala, Thr and Gly all increased expression. This indicated that the repression observed with CAA was the cumulative effect of the individual amino acids, as recently observed [300]. When added during growth, Leu, Arg, His, Val, Pro, Ala, Gln and Thr increased *csgB* expression, and no single amino acid decreased expression. This again showed that the CsgD biofilm network responds differently once it is activated. The production of sugars from gluconeogenesis is important for biofilm formation, as *S.* Typhimurium strains with mutations of *pckA* and *ppsA* are unable to form biofilms [265]. PckA and PpsA are important gluconeogenic enzymes required for the synthesis of phosphoenolpyruvate (PEP). Pck catalyzes the conversion of oxaloacetate to PEP [301], while Pps catalyzes the conversion of pyruvate to PEP. Ala, Gly and Thr are gluconeogenic amino acids that enter the gluconeogenic pathway through pyruvate [302]. In support of this, Ala and Thr increased *csgB* expression when introduced before and during growth. Gly also increased *csgB* expression when added before and during growth, but the change was not statistically significant. CsgD was shown to directly stimulate Gly biosynthesis during *E. coli* biofilm formation [303], presumably to ensure there is enough Gly supply to produce large quantities of the major curli subunit, CsgA (i.e., 16% Gly residues). Increased *csgB* expression in the presence of Ala, Gly and Thr is consistent with their conversion to pyruvate contributing to gluconeogenesis. For the aromatic amino acids, due to solubility and concentration problems, we only tested Phe, which had no significant effect on *csgB* expression. This was unfortunate since *S. enterica* strains defective in aromatic amino acid biosynthesis are unable to form biofilms [304], and tryptophan has been shown to have an important role in *S.* Typhimurium biofilms [305]. Tryptophan was also not present in CAA, as it is destroyed during the acid hydrolysis

process [306]. More research is needed to understand the impact of individual amino acids on *csgD* expression.

Glucose was the most powerful external signal tested in our experiments. Under all growth conditions, the presence of exogenous glucose completely repressed the transcription of *csgD*, *csgB* and *adrA*. Expression of *csgD* was repressed in the presence of glucose even when *rpoS* was over expressed from a plasmid or when levels of c-di-GMP were enhanced due to STM1987 activity. In the initial paper on carbon source foraging [307], the presence of glucose had a streamlining effect on the metabolism of *E. coli* when compared with growth on lower-quality carbon sources. This study was a genome-wide illustration of carbon catabolite repression [308], where growth on optimal carbon sources occurs first and genes for the metabolism of other carbon sources are repressed, usually acting through cyclic AMP (cAMP) and cAMP receptor proteins (CRP). Glucose had a repressive effect on biofilm formation in both *S.* Typhimurium and *E. coli* [118,282,300,309], however how the regulation is mediated is reported to be the opposite. High levels of cAMP repress *csgD* transcription in *S.* Typhimurium [300] but stimulate *csgD* transcription in *E. coli* [309]. It was also initially reported in *S.* Typhimurium that cAMP/CRP had no effect on *csgD* transcription [88]. It is hard to believe that the conserved divergent *csgDEFG* and *csgBAC* operons [310], biofilm networks and large intergenic region are capable of having opposite regulation in *S.* Typhimurium and *E. coli*. However, as pointed out by Hufnagel et al.[309], *E. coli* and *S.* Typhimurium have different evolutionary histories, hence could have differing regulatory responses to glucose. Another important aspect of cAMP/CRP regulation and glucose metabolism pertains to the quality of nitrogen source available [311], making this complex regulatory network in need of further study. It should be noted that the repressive effect of glucose did not change according to whether *csgD* transcription was activated or not, which was in contrast to the other nutritional signals that we tested.

The effects of temperature and c-di-GMP on *csgD* transcription were also evaluated. Temperature was one of the first conditions identified to regulate biofilm formation [95,171]. Activation at temperatures below 30°C is known to represent RpoS-dependent transcription of *csgD*. *S.* enterica strains with *csgD* promoter mutations can alleviate temperature-based repression by shifting transcription to be RpoD-dependent [95,275]. This may be a way for

natural *rpoS* mutant strains to retain the ability to form biofilms, as there are always a few isolates within natural collections that display temperature-independent biofilm formation [91,118,171]. Temperature was able to shut off the biofilm network even after *csgD* was activated, proving that it is also a strong regulatory signal. *S.* Typhimurium biofilm colonies were only formed at 37°C if c-di-GMP levels were enhanced by *stm1987* overexpression, with partial restoration if *rpoS* was overexpressed. Although these conditions are somewhat artificial, the c-di-GMP regulatory principles could be an important observation. We recently discovered that curli can be synthesized by *S.* Typhimurium during murine infections, with *csgD* transcription activated at 37°C in vivo [166]. It is also of note that iron limitation [95] and exposure to bile [312] can alleviate temperature-based repression of *csgD* transcription. Finally, expression of the c-di-GMP-degrading enzyme, YhjH (or STM3611), was enough to repress *csgD* expression in all tested conditions, which is similar to previous observations [125,313].

7.4.1 Conclusions

We have started to dissect the external signal hierarchy that regulates *csgD* transcription and CsgD-mediated biofilm formation in *S. enterica*. Most significantly, we identified differences in the regulatory responses based on whether or not *csgD* was activated before being exposed to a signal. These findings are summarized in Figure 7.8A, B. We hypothesize that the differences upon activation are related to the bistable expression of CsgD [176,272], similar to dedicated processes in other bacterial species. Even seemingly well-understood processes, such as diauxie—the switching of *E. coli* growth between two carbon sources—is subject to heterogeneity, as one sub-population of cells ceases growth once glucose has been exhausted, while the other subpopulation begins to grow on the second carbon source without delay [299]. The diauxic behavior was originally interpreted as the whole population of cells stopping growth during a transition period before starting growth on the second carbon source [314]. We hypothesize that many of the *csgD* regulatory elements that we have examined here are consistent between *S.* Typhimurium and *E. coli* [273], with some notable differences. With respect to phenotypic heterogeneity, we may only fully understand biofilm regulation once we are able to examine the fate of individual cells [266].

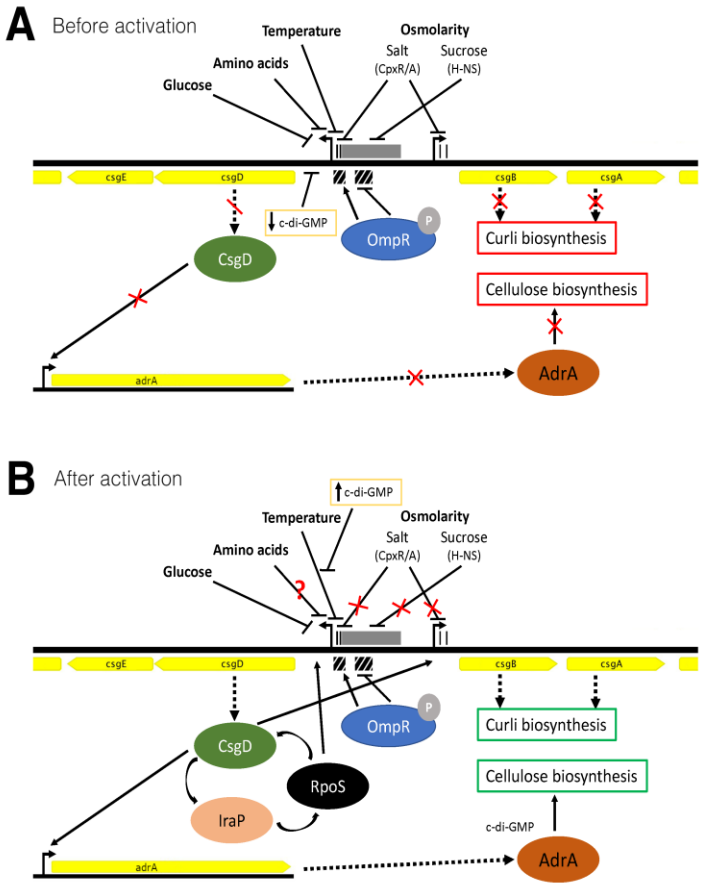

Figure 7.8: Graphical illustration of the CsgD regulatory principles identified in this manuscript. The divergent *csg* operons are shown (without c*sgFG* and *csgC*) with the intergenic region highlighted by transcription factor binding sites that have been experimentally verified in *Salmonella* (CpxR—black bars; H-NS—grey box; OmpR—hatched boxes). Phosphorylated OmpR binds the proximal, high affinity site under conditions of low osmolarity, which activates *csgD* transcription, and binds the distal, low affinity sites under conditions of high osmolarity, which represses *csgD* transcription[271]. The different regulatory elements that we have tested are shown: glucose; amino acids; growth temperature; and osmolarity, with sodium chloride, which is known to act via the CpxR/A system [103], and sucrose, which is known to act via H-NS [102]. The a*drA* gene encodes a diguanylate cyclase, which produces cyclic-di-GMP and allosterically activates cellulose production. (A) Glucose (> 25 mM), amino acids (> 0.5% casamino acids), temperature (> 32°C), salt and sucrose (> 25 mM) caused a reduction in *csgD* transcription and blocked transcription of *csgBAC* and *adrA*, preventing curli and cellulose biosynthesis. The effect of reduced c-di-GMP was tested by overexpression of the YhjH phosphodiesterase. The addition of individual amino acids was variable, with three leading to reduced *csgD* transcription (Asn, Pro, Arg), and seven leading to increased *csgD* transcription (Ile, Val, Gln, Met, Ala, Thr, Gly). (B) When the same regulatory components were tested after 18 h of growth, the effects were different. We assume that by this time point, the CsgD-IraP-RpoS feedforward loop [270] is activated, although deletion of *iraP* in our experiments had little effect. The addition of salt and sucrose had no effect on *csgD* transcription, and casamino acids were not as repressive. The effect of increased c-di-GMP was tested by overexpression of the diguanylate cyclase *STM1987*, which was able to relieve temperature-based repression of *csgD* transcription. The response to individual amino acids was again variable, however, none caused a reduction in *csgD* transcription and eight were stimulatory (Leu, Arg, His, Val, Pro, Ala, Gln, Thr). The question mark signifies that we do not fully understand the regulatory effects of individual amino acids.

7.5 Materials and Methods

7.5.1 Bacterial Strains, Media, and Growth Conditions

The bacterial strains used in this study are listed in Table 7.1. For standard growth, strains were inoculated from frozen stocks onto LB agar (lysogeny broth, 1% NaCl, 1.5% agar) supplemented with appropriate antibiotic (50 μg mL−1 kanamycin (Kan), or 5 μg mL−1 tetracycline (Tet)) and grown overnight at 37°C. Isolated colonies were used to inoculate 5 mL LB broth and the culture was incubated for 18 h at 37°C with agitation at 200 RPM. For analysis of colony morphology and gene expression, 4 μl of overnight culture was spotted on 1% tryptone agar supplemented with 0.2% freshly made glucose, 25 mM salt or 100 mM salt (agar supplemented with glucose were used within 24 h). Plates were incubated at 28°C or 37°C for two days. Visible and luminescence images were captured with a spectrum CT in vivo imaging system (PerkinElmer, Waltham, MA, USA).

Table 7.1. Strains and plasmids used in this study.

Strains or Plasmids	Genotype	Reference
Strains		
Salmonella enterica subsp. *enterica* serovar Typhimurium		
14028	Wild-type strain	ATCC
14028 Δ*cpxR*	Deletion of *cpxR*	This study
14028 Δ*iraP*	Deletion of *iraP*	This study
14028 Δ*rpoS*	Deletion of *rpoS*	[118]
Plasmids		
pCS26, pU220	Bacterial luciferase	[177]
pCS26-*stm1987*::luxCDABE	*stm1987* promoter	This study
pU220-*cpxP*::*luxCDABE*	*cpxP* promoter	This study
pU220-c*sgD*::*luxCDABE*	*csgDEFG* promoter	[118]

pCS26-*csgB*::*luxCDABE*	*csgBAC* promoter	[118]
pCS26-*adrA*::*luxCDABE*	*adrA* promoter	[118]
pBR322		
pBR322/*stm1987*	*stm1987*^14028	This study
pBR322/*yhjH*	*yhjH*^14028	This study
pACYC184		
pACYC/*rpoS*	*rpoS*^14028	[172]

7.5.2 Generation of *S*. Typhimurium 14028 Mutant Strains

Lambda red recombination [170] was used to generate Δ*cpxR* and Δ*iraP S.* Typhimurium mutant strains. Primers containing 50-nuclelotide sequences on either side of *cpxR* or *iraP* (Table 7.2) were used to amplify the *cat* gene from pKD3 using Phusion high-fidelity DNA polymerase (New England Bio-Labs, Ipswich, MA, USA). The PCR products were solution purified and electroporated into *S*. Typhimurium 14028 cells containing pKD46. Mutants were first selected by growth at 37°C on LB agar supplemented with 10 μg ml−1 chloramphenicol (Cam) before streaking onto LB agar containing 34 μg ml−1 Cam. PCR primers upstream and downstream of *cpxR* or *iraP* (Table 7.2) were used to amplify sequence from the genome of mutant S. Typhimurium 14028 strains and verify loss of the corresponding open reading frames. The ΔcpxR or Δ*iraP* mutations were moved into a clean *S.* Typhimurium strain background with P22 phage [171]. The *cat* gene was resolved from the chromosome using pCP20 [170].

Table 7.2. Oligonucleotides used in this study.

Primer	Sequence (5′–3′) [a]	Purpose
cpxRko sense	AAGATGCGCGCGGTTAAACTTCCTATC ATGAAGCGGAAACCATCAGATAGGTG TAGGCTGGAGCTGCTTC	To amplify *cat* gene product from pKD3 to generate Δ*cpxR* strain by lambda red recombination
cpxRko antisense	CCTGTTAGTTGATGATGACCGAGAGCT GACTTCCCTGTTAAAAGAGCTCCCCTC CTTAGTTCCTATTCCG	
cpxR ver F	CCAGCATTAGCACCAGCGCC	To confirm the deletion of *cpxR* from *S.* Typhimurium 14028
cpxR ver R	TCTGCCTCGGAGGTACGTAAACA	
cpxR1	GCCCTCGAGGTAACTTTGCGCATCGCT TG	To amplify the *cpxR* and *cpxP* promoter regions from *S.* Typhimurium 14028
cpxR2	GCCGGATCCTTCATTGTTTACGTACCT CCG	
iraPko sense	GGCAGTGGTTCTTCATAGTGATAACGT CACCCTGGAACTAATAAGGAAATGTG TAGGCTGTAGCTGCTTC	To amplify *cat* gene product from pKD3 to generate Δ*iraP* strain by lambda red recombination
iraPko antisense	TGTTATTTCATAAAAGTAACGTTATAA CAACTGTGTTGTTTTAAATACGACCTC CTTAGTTCCTATTCCG	
iraPko-detect1	CAAAAAGCGAAAGGCCAATA	To confirm the deletion of *iraP* from *S.* Typhimurium 14028
iraPko-detect2	TAGCACCATCCTTTTGTCAG	
STM14_2408for1	GATCCTCGAGAAATTCGCGGTGTTTCG CAC	To amplify the *stm1987* promoter region from *S.* Typhimurium 14028
STM14_2408rev2	GATCGGATCCCTAACAGTGTTTCGTGC GGC	

STM1987forEco	GATCGAATTCAAACGGTGTTTCGCAC	To amplify *stm1987* with native promoter region from *S.* Typhimurium 14028
STM1987revAatII	GATCGACGTCGGACTATTTCTTTTCCCGCT	
yhjHforEco	GATCGAATTCTTGACAAGTTTCGGGGGCTG	To amplify *yhjH* with native promoter region from *S.* Typhimurium 14028
yhjHrevAatll	GATCGACGTCGTATTACGGGAACAGTCTGG	
pZE05	CCAGCTGGCAATTCCGA	Used to verify promoter fusions to *luxCDABE*
pZE06	AATCATCACTTTCGGGAA	

[a] Nucleotide sequences corresponding to restriction enzyme sites are underlined.

7.5.3 Generation of Bacterial Luciferase Reporters and Other Plasmid Vectors

Luciferase fusion reporter plasmids containing the promoters of *csgDEFG, csgBAC* and *adrA* have been previously described [118]. The *cpxP* reporter plasmid was generated to monitor the levels of CpxA/CpxR activation within the cell. The intergenic region containing the *cpxR* and *cpxP* promoter sequences was PCR amplified from *S.* Typhimurium 14028 using primers cpxR1 and cpxR2 (Table 2) and Phusion high-fidelity DNA polymerase (New England BioLabs, Ipswich, MA, USA). The resulting PCR product was purified, sequentially digested with *Xho*I and *Bam*HI, and ligated (in the *cpxP* direction) using T4 DNA ligase (New England BioLabs, Ipswich, MA, USA) into pU220 digested with *Xho*I and *Bam*HI. The *stm1987* luciferase reporter plasmid was generated similarly using primers STM14_2408for1 and STM14_2408rev2 (Table 2), with cloning into pCS26. PCR screening with primers pZE05 and pZE06 was used to verify the successful fusion of promoter regions to *luxCDABE.* For plasmid-based overexpression of cyclic-di-GMP related enzymes, fragments containing *stm1987* and *yhjH* genes with their native promoters were PCR amplified from *S.* Typhimurium 14028 gDNA using Phusion high-fidelity DNA polymerase and appropriate primers (Table 2). Resulting PCR products were purified, digested with *Eco*RI and *Aat*II, and ligated using T4 DNA ligase into *Eco*RI/*Aat*II-digested pBR322. The pACYC-*rpoS* plasmid vector has previously been described[172] [48]. Reporter plasmids and overexpression plasmids were co-transformed into *S.* Typhimurium strains

by electroporation and selected by growth at 37°C on LB agar supplemented with 50 μg mL−1 Kan (pCS26) and 10 μg mL−1 Tet (pBR322 or pACYC).

7.5.4 Luciferase Reporter Assays

96-well bioluminescence assays were performed with *S.* Typhimurium luciferase reporter strains. Overnight cultures were diluted 1 in 600 into individual wells of black, clear bottom 96-well plates (9520 Costar; Corning Life Sciences, Tewksbury, MA, USA) containing 150 μL of 1% tryptone broth supplemented with 50 μg mL-1 of kanamycin (Kan). When noted, media was supplemented before growth with NaCl (25,150 mM), sucrose (50,150 mM), CuCl2 (1 mM), casamino acids (12%) or individual amino acids (15 mM) to the final concentrations as indicated. For the addition of media supplements during growth, cells were inoculated into 135 μL of media and grown for 18 h at 28°C before supplements were added as 15 μL aliquots to the appropriate wells. This included glucose ranging from 25–150 mM. To minimize evaporation of the media during the assays, cultures were overlaid with 50 μL of mineral oil per well. Cultures were assayed for absorbance (600 nm, 0.1 s) and luminescence (1s; in counts per second (CPS)) every 30 min during growth at 28°C with agitation in a Victor X3 multilabel plate reader (Perkin-Elmer, Waltham, MA, USA).

8.0 Interface

In the previous chapter I showed that transcription factor *csgD* responds differently to some regulatory factors once activated. *S.* Typhimurium grown in liquid culture under biofilm-inducing conditions, such as low temperature (28°C) and osmolarity, differentiates into two subpopulations of cells: multicellular aggregates and planktonic cells. This phenomenon is attributed to the bistable synthesis of CsgD. Multicellular aggregates are in a CsgD-ON state (produce high levels of CsgD), while planktonic cells are primarily in a CsgD-OFF statae (produce low levels of CsgD) [176]. Comparative transcriptome sequencing (RNA-seq) identified 1856 genes differentially expressed between both cell types. Multicellular aggregates had increased expression of genes involved in *Salmonella* biofilm formation, while planktonic cells had increased expression of virulence genes. Of 1856 differentially expressed genes, 798 were function unknown genes.

Function unknown (FUN) genes are increasingly being identified during genetic screens. Some FUN genes have no homologs in functionally annotated gene databases; hence it is difficult to design experiments that can aid in the identification of their function. There is currently no framework to identify the role of FUN genes. In the next chapter, using FUN genes differentially expressed between *S.* Typhimurium multicellular aggregates and planktonic cells, I highlight the difficulties and propose a framework that can be used to identify the roles of FUN genes.

9.0 The Challenges of identifying the roles of function unknown genes

Akosiererem S. Sokaribo[1,2], Yejun Wang[3], Ryan Krochak[1], Zoe Sereggela[1,2], Beatrice Chung[1,2], Shirley Lam[1], Wolfgang Koster[1], Jeffrey M. Chen[1], and Aaron P. White[1,2*]

Affiliations:
[1] Vaccine and Infectious Disease Organization-International Vaccine Centre, Saskatoon, Saskatchewan, Canada
[2] Department of Biochemistry Microbiology and Immunology, University of Saskatchewan, Saskatoon, Saskatchewan, Canada
[3] Department of Cell Biology and Genetics, School of Basic Medicine, Shenzhen University Health Science, Shenzhen, China

* Corresponding author

E-mail: aaron.white@usask.ca

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Authors contribution

Akosiererem S. Sokaribo: Designed and perform experiments, analyzed data and wrote manuscript.

Yejun Wang: Performed bioinformatic analysis

Ryan Krochak and Zoe Sereggela: Assisted with RNA purifications

Beatrice Chung and Jeffrey M. Chen: Grew RAW macrophages used for survival assays

Shirley Lam and Wolfgang Koster: Grew Caco2 intestinal cells used for invasion assays

Aaron P. White: Designed experiments, analyzed data, edited manuscript, and obtained funding for this research

9.1 Abstract

Slow progress in the identification of the roles of function unknown (FUN) genes is a major challenge associated with next generation sequencing. With advances in technologies and low sequencing cost, hundreds of functions unknown (FUN) genes are increasingly being identified. FUN genes are present in most sequenced bacterial genomes, and are identified during high throughput screens, and RNA-sequencing experiments. Since most FUN genes have no homologs to functional annotated genes present in databases, it is often difficult to identify conditions in which FUN genes mutant strains exhibit altered phenotypes compared to wildtype. Using FUN genes differentially expressed between *S.* Typhimurium multicellular aggregates and planktonic cells, we highlight the challenges of studying FUN genes and propose a framework that can be used to identify the roles of FUN genes. The fact that FUN genes are expressed in both the host and non-host environments, suggest that they play important roles in bacterial survival. A framework that aids in the identification of the roles of FUN genes will improve our understanding of bacterial pathogenesis, and identify novel antimicrobial targets.

9.2 Introduction

Salmonella species are important foodborne pathogens that cause two main types of disease in humans: typhoid fever and gastroenteritis [1]. Typhoid fever is caused by typhoidal *Salmonella* (TS) serovars Typhi and Paratyphi, while gastroenteritis is predominantly caused by non typhoidal *Salmonella* (NTS) serovars: Typhimurium and Enteritidis [5]. Most NTS serovars are considered host generalists and can cause disease in a wide variety of hosts, while TS serovars are host restricted [5]. Typhoid fever is endemic in regions that lack clean water and adequate sanitation, which facilitates the spread of TS via the fecal-oral route [5]. While gastroenteritis occurs worldwide, and outbreaks are often linked to the consumption of contaminated produce such as poultry, vegetables, and processed foods [19–21]. Biofilm formation has been associated with the broad host specificity and the persistence of NTS serovars on food produce [87].

Biofilms, which are formed when cells aggregate and become embedded in a self produced extracellular matrix, are thought to be the predominant life form of bacteria. Biofilm cells are difficult to eradicate because they have enhanced resistance to antimicrobials, disinfectants, and desiccation [87]. Biofilm formation has been linked to *Salmonella* persistence in the environment and could play an integral role in transmission. The red, dry and rough (rdar) morphotype is the

most studied form of *Salmonella* biofilms [95]. When present in the media, the Congo red dye accumulates within the rdar colonies due to the presence of curli fimbriae which facilitates short range interactions, and cellulose which facilitates long range interactions between cells embedded in the extracellular matrix [118]. In general, most NTS serovars are rdar positive, while TS serovars are rdar negative due to mutations in transcription factor CsgD [91,136].

Transcription factor CsgD is the main regulator of *Salmonella* biofilm formation. It positively regulates the expression of polymers in the extracellular biofilm matrix such as curli, cellulose, BapA, colanic acid and lipopolysaccharides [87]. The bistable synthesis of CsgD in *S.* Typhimurium leads to the formation of two distinct cell populations in liquid culture: multicellular aggregates and planktonic cells [176,272]. Using transcriptome comparisons MacKenzie et al.,[176] showed that 1856 genes (which represents 35% of all *S.* Typhimurium genes) are differentially expressed between both cell types [176]. Multicellular aggregates had higher expression of genes involved in biofilm formation, stress response, amino acid, and carbohydrate metabolism, while planktonic cells had higher expression of genes involved in virulence including *Salmonella* pathogenicity islands 1 and 2 genes [176,305]. Compared to planktonic cells, multicellular aggregates displayed enhanced resistance to desiccation and antibiotics. Planktonic cells had increased synthesis of T3SS, which correlated with enhanced invasion of human intestinal cell lines and significantly increased virulence in mice, compared to multicellular aggregates [176]. CsgD-based differentiation is hypothesized to be a form of bet-hedging that promotes *S.* Typhimurium transmission, with multicellular aggregates adapted for environmental persistence and planktonic cells adapted for virulence. Approximately 43% (798) of the 1856 genes differentially expressed between planktonic cells and multicellular aggregates were genes of unknown functions [176]. Due to the perceived adaptation of both cell types, the presence of function unknown (FUN) genes differentially expressed between multicellular aggregates and planktonic cells presents an opportunity for the identification of novel genes in *Salmonella* persistence and virulence.

Next generation sequencing has broadened our understanding of structural and functional genomics and provided new insight into pathogen evolution. Over the past decade, sequencing technologies have advanced tremendously, and reduced cost has led to the sequencing of the genomes of many organisms. Most sequenced genomes contain genes of unknown function. In

addition, an increasing number of FUN genes are identified during high throughput screening and RNA-seq experiments. Unfortunately, the slow pace in the identification of the roles of FUN genes is a major challenge in this era of genome sequencing. Since some FUN genes may be vital for pathogen survival and pathogenesis, identifying the roles of FUN genes will not only improve our understanding of host-pathogen interactions, but can lead to the identification novel targets for the development of antimicrobials against pathogenic organisms.

Using FUN genes differentially expressed between *S.* Typhimurium multicellular aggregates and planktonic cells, we highlight the challenges associated with identifying experimental conditions were FUN gene mutants have altered phenotypes. We also propose a framework that can be used to identify the roles of FUN genes. Identifying novel genes involved in persistence and virulence may hold the key for understanding bacterial transmission and pathogenesis, which can lead to the development of antimicrobials and strategies to mitigate *Salmonella* infections.

9.3 Results and Discussion

9.3.1 Selection of FUN genes with potential roles in *Salmonella* virulence and persistence

We hypothesize that a proportion of the FUN genes differentially expressed between multicellular aggregates and planktonic cells have roles in *Salmonella* virulence and/or persistence. Our first objective was to reduce 798 genes to a list of genes that have the best potential to be involved in *Salmonella* virulence and/or persistence.

The transcriptome of multicellular aggregates and planktonic cells were compared at four different time points, which corresponds to the induction of *csgD* expression (preaggregation stage, 8 hours), increasing *csgD* expression level (appearance of multicellular aggregates, 13 hours), peak *csgD* expression level (18 hours) and reduction of c*sgD* expression to near baseline levels (cells within the culture appear fully differentiated, 32 hours) [176]. FUN genes expressed at 8 hours were eliminated from our analysis, because at this time cells appeared homogenous, and multicellular aggregates were not visible in growth culture. Of 309 FUN genes highly expressed in multicellular aggregates, 111 genes were expressed in more than one time point (13, 18 and/or 32 hours), while 137 of the 492 FUN genes expressed in planktonic cells were expressed in more than one time point. Using a combination of transcription start and termination sites, intergenic distances and co-expression information obtained from RNA

sequencing, Wang et al.,[315] was able to distribute a large group of *S*. Typhimurium genes into operons [315]. In theory, proteins encoded in an operon often have related or coordinated functions or participate in the same or related biological processes. Analysis of operon knockout strains can aid in the identification of the roles of FUN genes, therefore we decided to focus our analysis on operons entirely comprised of FUN genes. The 111 differentially expressed FUN genes in multicellular aggregates mapped to 84 operons, while 137 FUN genes expressed in planktonic cells mapped to 97 operons (Figure 9.1). By comparing the genomes of the identified operons to other bacterial genomes using Kyoto encyclopedia of genes and genomes (KEGG) and gene ontology (GO), 23 FUN operons with potential roles in *Salmonella* virulence and persistence were selected for further analysis (Figure Table 9.1).

Operon knockout strains were generated using lambda red recombinase [170]. No difference in growth rate was observed between any of the FUN operon knockout strains and wildtype, in LB broth and M9 minimal media supplemented with glucose or succinate (Fig 9.2). This suggested that deletion of FUN operons had no effect on *Salmonella* growth under the conditions tested.

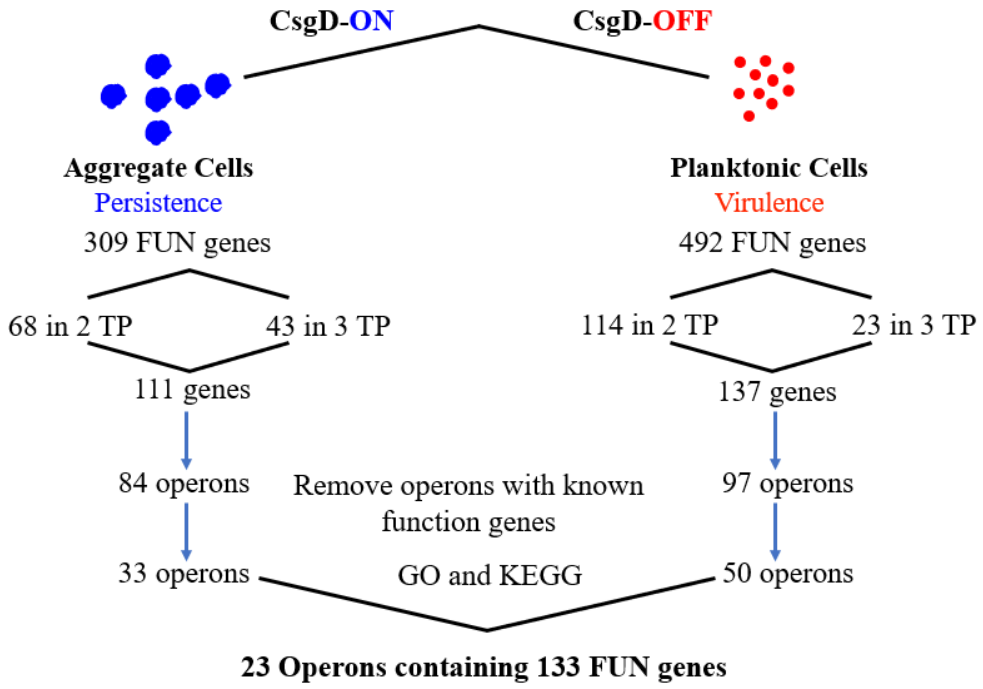

Figure 9.1: Selection of FUN genes with potential roles in *Salmonella* virulence and persistence. 309 and 492 FUN genes were expressed in multicellular aggregates and planktonic cells, respectively. Genes expressed in more than one time point (111 genes for multicellular aggregates and 137 genes for planktonic cells) were mapped to operons. Using KEGG and GO databases, 23 operons with potential roles in *Salmonella* virulence and persistence were selected for further analysis.

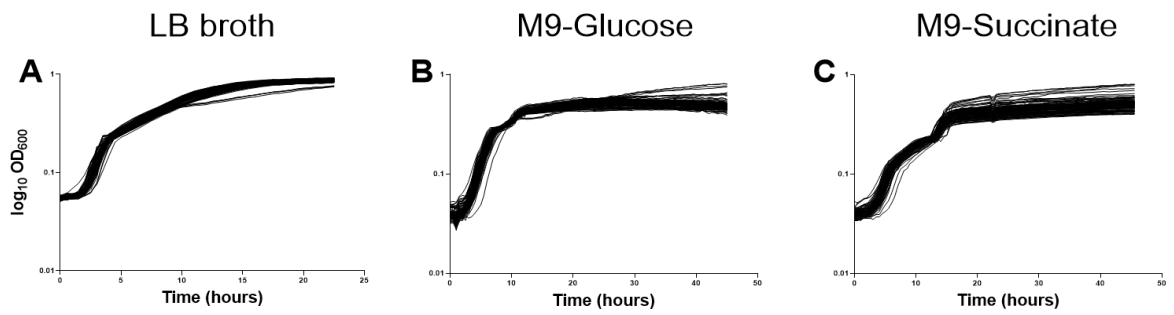

Figure 9.2: Growth of *S.* Typhimurium mutant strains with deletion of selected FUN operons. The optical density of each growing culture was measured at a wavelength of 590 nm (OD_{590}). Strains were grown for 24 h at 37°C in LB (A), or for 48 h at 28°C in M9 minimal media supplemented with 0.4% glucose (B) or 0.4% succinate (C). OD measurements were recorded using a Perkin Elmer Victor X^2 multilabel plate reader.

9.3.2 Invasion of a human intestinal cell line and survival inside murine macrophages by FUN operon knockout strains

Genes encoded by SPI-1 and SPI-2 were highly expressed in planktonic cells [25], hence we wanted to determine if the selected FUN operons have roles in *S.* Typhimurium virulence. The type three secretion systems (T3SS) encoded by SPI-1 and SPI-2 are the most important *Salmonella* virulence factors. SPI-1 T3SS delivers effectors required for *Salmonella* invasion of intestinal epithelial cells, while SPI-2 T3SS delivers effectors required for survival inside phagocytic cells such as macrophages [25]. *S.* Typhimurium SPI-1 or SPI-2 mutants are attenuated for virulence in a mouse model of infection. To determine if selected FUN operons have roles in virulence, the ability of FUN operon knockout strains to invade intestinal epithelial cells and survive inside murine macrophages was tested.

The invasion of intestinal epithelial cells was assayed using Caco-2 invasion assay. Caco-2 cells are a human colon carcinoma cell line with features similar to differentiated intestinal epithelial cells, often used to assay *Salmonella* invasion [316]. Of 20 FUN operon mutants analyzed, only one strain displayed altered rate of invasion compared to wildtype (Fig 9.3A). FUN 4768 had a slightly higher rate of invasion compared to wildtype (Fig 9.3A). Our result suggests that genes in the FUN 4768 operon may negatively affect *Salmonella* invasion of epithelial cells.

To determine if FUN operon knockout strains have roles in intra-macrophage survival, murine RAW 264.7 macrophages were infected with FUN operon knockout strains and the CFUs

recovered from macrophages 21 hours post infection were enumerated. FUN operons 2475 and 4889 had ~10-fold reduced level of survival compared to wildtype (Fig 9.3B), suggesting that these operons may be involved in *Salmonella* intra-macrophage survival.

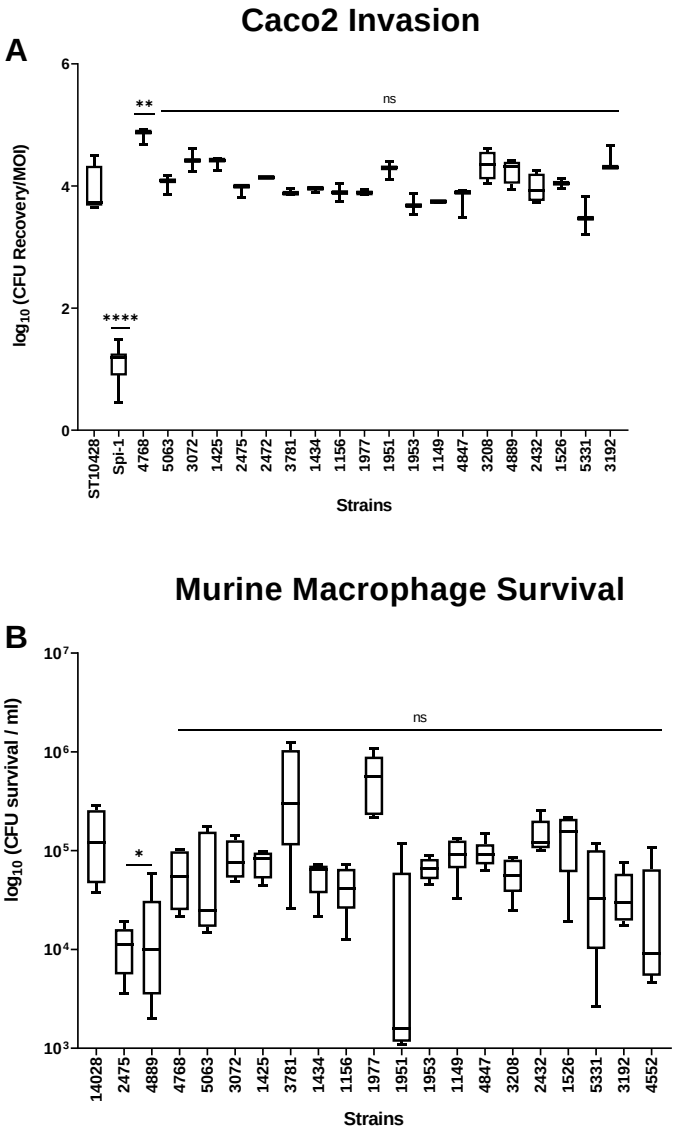

Figure 9.3: *S.* Typhimurium invasion of Caco-2 cell lines and survival inside murine macrophages. A. Polarized Caco-2 cell lines were infected with *S.* Typhimurium wildtype or FUN operon mutants at a multiplicity of infection (MOI) of 10. One-hour post-infection cells were lysed and CFU recovered from lysed cells were used to determine invasion rate. B. Murine RAW 264.7 macrophages were infected with *S.* Typhimurium wildtype or FUN operon mutants at a MOI of 100. CFU recovered 21 hours post-infection was used to determine the rate of survival. Statistical significance between each mutant and wildtype was noted as *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, or ns $p > 0.05$.

9.3.3 Biofilm formation assay using rdar morphotype and crystal violet staining

Genes involved in biofilm formation were highly expressed in multicellular aggregates, hence we wanted to determine if selected FUN operons were involved in *Salmonella* biofilm formation. Biofilm-forming *Salmonella* strains can be identified by the production of distinct rdar (red, dry, and rough) morphology, when grown on agar supplemented with Congo-red dye [95]. No difference in rdar morphotype was observed between wildtype and any of the FUN operon knockout strains (data not shown).

To quantify the amount of biofilm produced, crystal violet staining was performed, on FUN operon knockout strains grown under biofilm-inducing conditions in 96 well plates. As expected, *S.* Typhimurium strains defective in curli (Δc*sgD*, Δ*csgA*) and cellulose (Δ*bcsA*) biosynthesis produced reduced amount of biofilm compared to wildtype (Fig 9.4). No difference in biofilm formation was observed between wildtype and most FUN operon mutant strains. However FUN 3208, 1953, 1951, 1149 and 5063 produced statistically significant higher amounts of biofilms compared to wildtype (Fig 9.4), suggesting that genes in these operons may negatively regulate *Salmonella* biofilm formation.

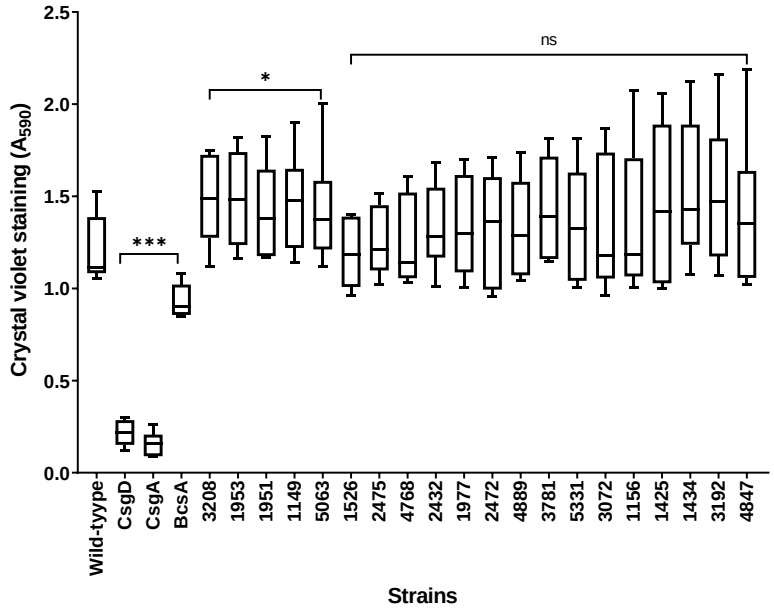

Figure 9.4: Quantification of biofilms produced by *S.* Typhimurium using crystal violet staining. *S.* Typhimurium mutants and wildtype were grown in tryptone at 28°C for 48 hours. The amount of biofilm attached to 96 well plates were stained with crystal violet and quantified with spectrophotometer with optical density at 590 nm. Assay was performed twice in three replicates. Statistical significance between each mutant and wildtype was noted as *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, or ns $p > 0.05$.

9.3.4 Expression of FUN operons

To analyze expression, the promoter region of each FUN operon was cloned into a luciferase reporter plasmid. Of the 23 promoters analyzed, only 7 had expression levels above baseline in tryptone broth at 28°C (Fig 9.5A), while 5 had expression levels above baseline in M9-glucose media at 28°C (Fig 9.5B). The promoter activity in tryptone broth at 37°C was low for most FUN operons, only FUN 1526 and 1148 had expression levels above baseline (Fig 9.5C).

The promoter activity of FUN 1148, 1149 and 1526 were ~100-fold above baseline in tryptone and M9-glucose broth at 28°C. The promoter activity of FUN 4889 was ~10-fold above baseline in tryptone broth at 28°C (Fig 9.5A and B). Since genes differentially expressed between multicellular aggregates and planktonic cells were isolated from *S.* Typhimurium grown in tryptone at 28°C, FUN operons were expected to have high promoter activities in tryptone. The intergenic regions upstream of the first gene in each operon were cloned into luciferase plasmids as the promoter region. It is conceivable that wrongly defined promoter regions may be why most FUN operons had low expression levels in tryptone broth.

Among the promoters examined, FUN 1148, 1149, and 1156 had the highest promoter activities under the conditions tested. Genes in these operons are homologous to Gifsy-2 prophage genes. Due to their high expression levels, we speculate that genes in these operons may be involved in assembly and production of prophages during *Salmonella* growth [317]. Although these operons were found not to play a role in virulence (in terms of *Salmonella* invasion and intramacrophage survival), it has previously been shown that curing of bacteria of the Gisfy-2 prophage significantly reduced *Salmonella* ability to establish a systemic infection in mice [318].

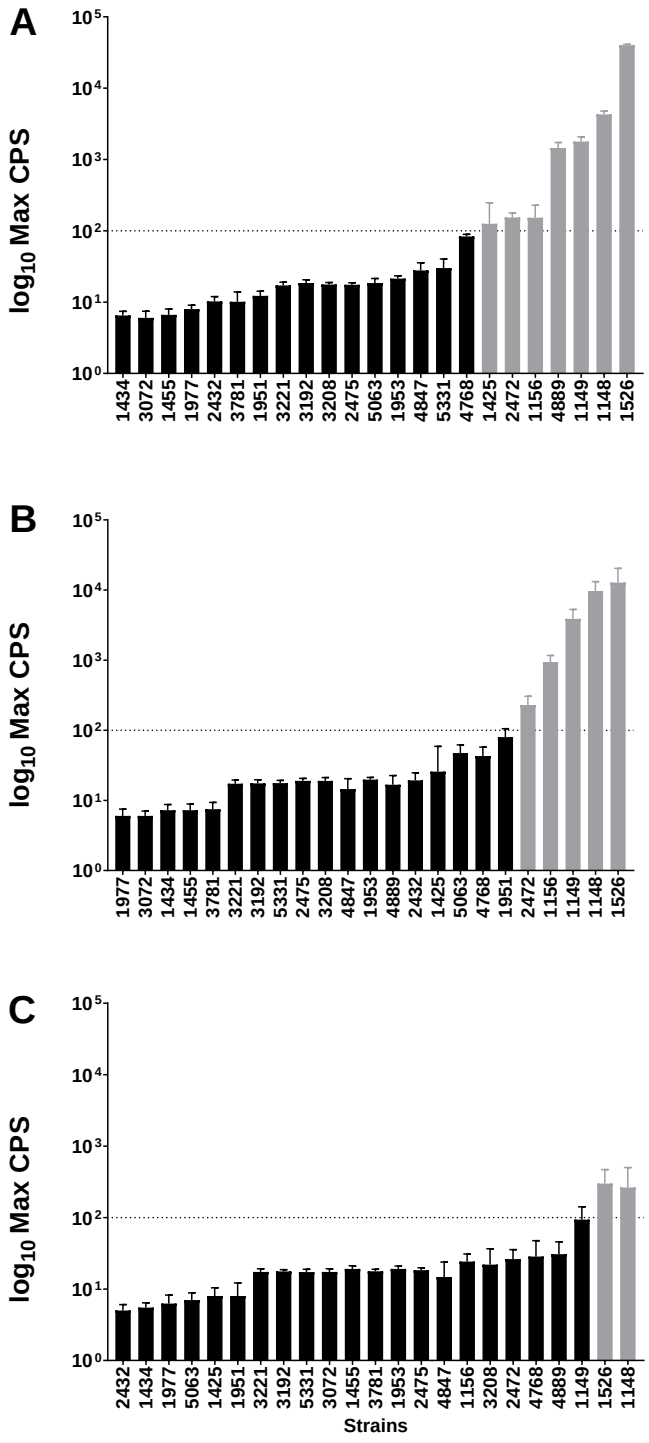

Figure 9.5: Expression of FUN operons. *S.* Typhimurium was transformed with FUN operon promoter-luciferase (*luxCDABE*) fusion, designed to measure gene expression by light production. For each reporter, maximum log luminescence value (counts per second, CPS) in A. tryptone at 28°C. B. M9 minimal media supplemented at glucose at 28°C. C. tryptone at 37°C, are shown. Error bars represent the mean of at least three biological replicates. The horizontal dotted line represents baseline expression level.

9.3.5 FUN operons conserved in *Salmonella* species

Genome degradations and gene acquisitions are the hallmarks of bacteria evolution. *Salmonella* is made up of two species, *bongori* and *enterica*. *S. enterica* is divided into six subspecies: *enterica, arizonae, diarizonae, salamae, houtenae* and *indica* [3]. *Salmonella enterica* subspecies are composed of host generalist and host restricted serovars. Genome sequencing has shown that the host range of different *Salmonella* subspecies can be correlated to the loss and acquisition of specific genes [139]. Therefore, genes not required for survival can be lost over time and the assumption is that highly conserved genes across *Salmonella* subspecies probably play important roles in the *Salmonella* life cycle.

Since the majority of our FUN operons had very low promoter activities, and had no roles in virulence or persistence under the conditions tested, we wanted to determine if the selected FUN operons were conserved across *Salmonella enterica* subspecies (*enterica, arizonae, diarizonae, salamae, houtenae* and *indica*). Using the National Center for Biotechnology Information (NCBI) nucleotide database, we showed that FUN operon 3781 was conserved in 5 of 6 *S. enterica* subspecies, 5331, 3062, 4768 and 1526 were conserved in 4, while 1951 and 1977 were conserved in 3 *S. enterica* subspecies (Table 9.1). Since the identified operons likely play important roles in the *Salmonella* life cycle, further analysis will be focused on the conserved FUN operons (3781, 5331, 3062, 1526, 1951, and 1977).

9.3.6 Identification of repressors that negatively regulate the promoter activity of FUN operons

Some transcription factors are known to regulate the expression of multiple genes under specific conditions. The identification of transcription factors that regulate gene expression can give insights into the roles of FUN genes. To identify genes that negatively regulate the expression of FUN operons, we performed random mutagenesis using Tn*10* transposon in *S.* Typhimurium transformed with FUN operon promoter::lux fusion plasmids. It was expected that mutation of a transcriptional repressor would lead to increased promoter activity of FUN operons and increase luminescence. After random mutagenesis, transposon mutants with increased luminescence were identified using the IVIS lumina II imager (Fig 9.6). Identified mutants were isolated and increased luminescence was verified using luciferase reporter assays. The promoter activity of FUN 5331 was increased by a 1000-fold, while FUN 1977 promoter activity was increased by a

100-fold compared to wildtype (Fig 9.7A and B). Insertional inactivation of STM14_5430 was found to increase the expression of FUN 5331, whereas inactivation of *leuO* by transposon was found to increase expression of FUN 1977. These results indicate that the promoter activity of 5331-5341 operon may be regulated by STM14_5340 which is part of the operon, suggesting that STM14_5340 may be an autoregulator that negatively regulates its own expression. STM14_5340 has homology to the bifunctional antitoxin/transcriptional repressor *relB*. LeuO is a LysR-type transcriptional regulator that regulates the expression of several genes involved in stress response, virulence, and multidrug resistance [319].

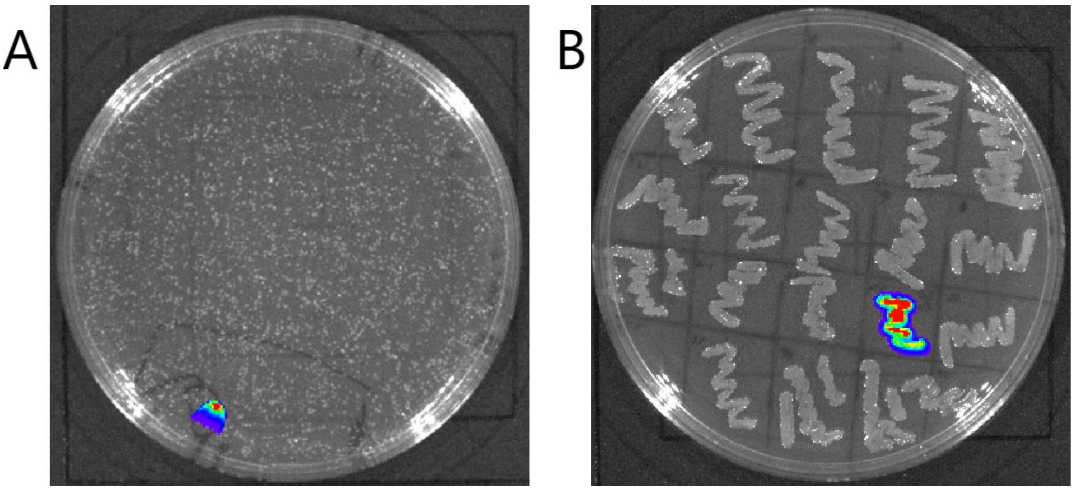

Figure 9.6: Identification of negative regulators of FUN operon promoters. *S*. Typhimurium transposon mutant with increased luminescence were identified using IVIS lumina II imager. Colonies in luminescent area were isolated and luminescent colony was identified.

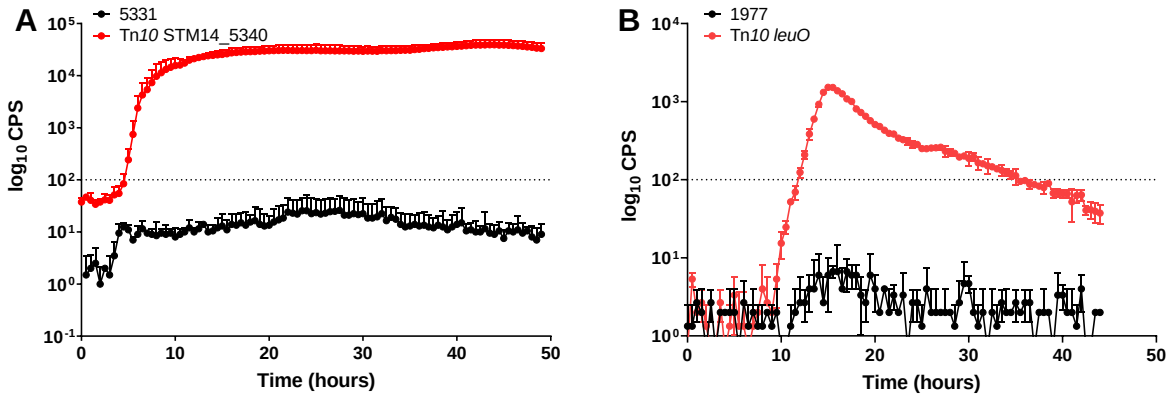

Figure 9.7: Expression of Fun operons in transposon mutants. Luciferase assays was performed with *S*. Typhimurium transposon mutants containing A. FUN 5331 promoter::*luxCDABE* plasmid and B. FUN 1977 promoter::*luxCDABE* plasmid. Luminescence (CPS, counts per second) was measured every 30 mins during growth at 28°C.

9.3.7 Transcriptome analysis of FUN operons

The analysis of mutant strains is indispensable for the identification of the roles of FUN genes. However, mutants must be analyzed under experimental conditions where they exhibit altered phenotypes compared to wildtype for the role of FUN genes to be identified. We hypothesize that genes differentially expressed between FUN operon mutant strains and wildtype can be used to identify pathways with altered expression. Based on the biological pathways altered in FUN operon mutants, experiments can be designed to analyze the roles of FUN genes. Comparative transcriptome sequencing (RNA-seq) was used to identify genes differentially expressed between FUN operon mutants (5331, 1977, 3781, and 4889) and wildtype. Using KEGG, the pathways with altered expression in FUN operon mutants were identified.

FUN 4889

Genes in FUN 4889 (STM14_4889 – 4894) operon are homologous to putative Na^+/galactoside symporter (STM14_4889), putative dehydrogenase (STM14_4890), putative ADP-ribosylglycohydrolase (STM14_4891), GntR family transcriptional regulator (STM14_4892) and putative periplasmic (STM14_4893) and cytoplasmic (STM14_4894) proteins. Using SalmoNet [320], STM14_4889 was predicted to interact with PurK and ThiC, and purified STM14_4890 has been shown to have an aminoimidazole riboside (AIRs) kinase activity [321]. AIR is the last metabolite common to the purine and thiamine biosynthesis pathway. Purine and thiamine are essential metabolic substrates required for numerous aspects of bacterial survival [321]. Using transcriptome analysis, genes with altered expression in FUN 4889 were grouped into the following categories: ribosome, carbon metabolism, glucose metabolism, oxidative phosphorylation, methane metabolism, tricarboxylic acid (TCA) cycle, lysine degradation and arginine and proline metabolism (Fig 9.8A). The involvement of genes in FUN 4889 in the thiamine and purine biosynthetic pathways supports the various categories of genes with altered expression identified by transcriptome analysis. This may also provide an explanation for the reduced survival of the FUN 4889 mutant strain inside macrophages (Fig 9.3B).

FUN 3781

The four gene operon containing FUN 3781 was conserved in 5 of 6 *S. enetrica* subspecies and is part of the *Salmonella* pathogenicity island-13 (SPI-13). Deletion of SPI-13 has been shown to attenuate *Salmonella* virulence in streptomycin pre-treated mice [322]; therefore, we wanted to determine if FUN 3781 is involved in virulence. FUN 3781 was recovered in higher proportions

than the wildtype strain in all organs analyzed, however, statistical significance was only observed in the liver (Fig 9.10A). This result suggests that genes in FUN 3781 may negatively regulate *Salmonella* colonization during infection. SPI-13 has also been shown to contribute to the survival of *Salmonella* in murine macrophages [323], however, deletion of FUN 3781 had no effect on intramacrophage survival (Fig 9.3B). It is possible that genes in FUN 3781 negatively regulate the expression of SPI-13 genes and this can explain the negative effect of FUN3781 on *Salmonella* colonization.

Transcriptome analysis showed that genes with altered expression in FUN 3781 were grouped into several categories, however genes involved in microbial metabolism in diverse environments were the most identified gene (Fig 9.8B). In agreement, genes in FUN 3781 has previously been shown to be involved in the metabolism of tyramine, a metabolic by product in the gastrointestinal tract and other host tissues [324]. We predict that FUN 3781 may be involved in *Salmonella* metabolism and further analysis will focus on the roles of FUN 3781 and SPI-13 in *Salmonella* virulence.

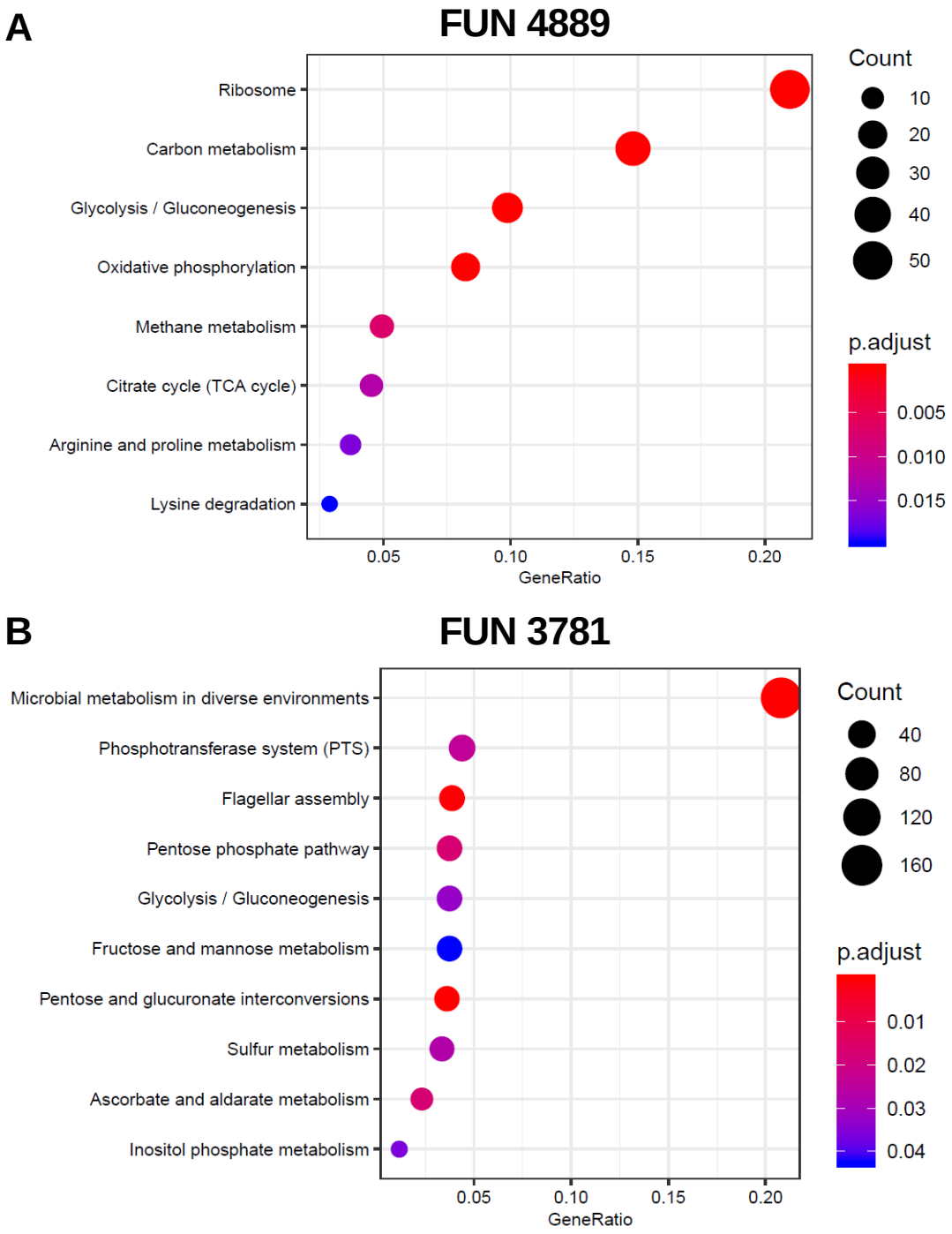

Figure 9.8: Pathway analysis of FUN 4889 and 3781 using KEGG: Gene differentially expressed between wildtype and A. FUN 4889, B. 3781 were grouped in several categories using KEGG pathway data base.

FUN 5331

FUN 5331 (STM14_5331 – 5341) is an eleven gene operon that is negatively repressed by auto regulator STM14_5340. FUN 5331 was found to be conserved in 4 of 6 *S.* enterica subspecies and seven genes (5331, 5332, 5333, 5334, 5335, 5336 and 5337) were found to be highly expressed in multicellular aggregates. Comparative transcriptome analysis suggests that FUN 5331 is involved in ABC transport, and in proline and arginine metabolism (Fig 9.9A). Future analysis of this operon will focus on examining FUN 5331 strains with mutation in STM14_5340 under various conditions, such as biofilm formation and dessication survival.

FUN 1977

FUN 1977 (STM14_1977 – 1981) is a five gene operon negatively regulated by transcription factor LeuO. Genes in FUN 1977 have homologs to periplasmic binding proteins (1977), amino acids ABC transporter permease (1978), ABC transporter ATP binding subunit (1979), ABC transporter membrane protein (1980) and putative inner membrane protein (1981). The homologues of genes in FUN 1977 agrees with transcriptome analysis, which suggests that genes in FUN 1977 are involved in ABC transport, and in arginine biosynthesis. In addition, LeuO involved in the biosynthesis of leucine was found to regulate 1977 expression (Fig 9.9B).

Genes in FUN 1977 are upregulated in SPI-2 media [325] and predicted to interact with SPI-2 genes SsrB and SprB [320]. FUN 1977 has a GC content of 42% (Table 1), since genes with low GC contents are often involved in virulence, and SPI-2 is one of the virulence determinant of *Salmonella*, we wanted to determine if FUN 1977 is involved in virulence. There was no competitive difference between wildtype and FUN 1977 in all organs analyzed (Fig 9.10B), suggesting that FUN 1977 may have no role in *Salmonella* virulence. Based on our results we predict that FUN 1977 is likely involved in the ATP dependent transport of amino acids.

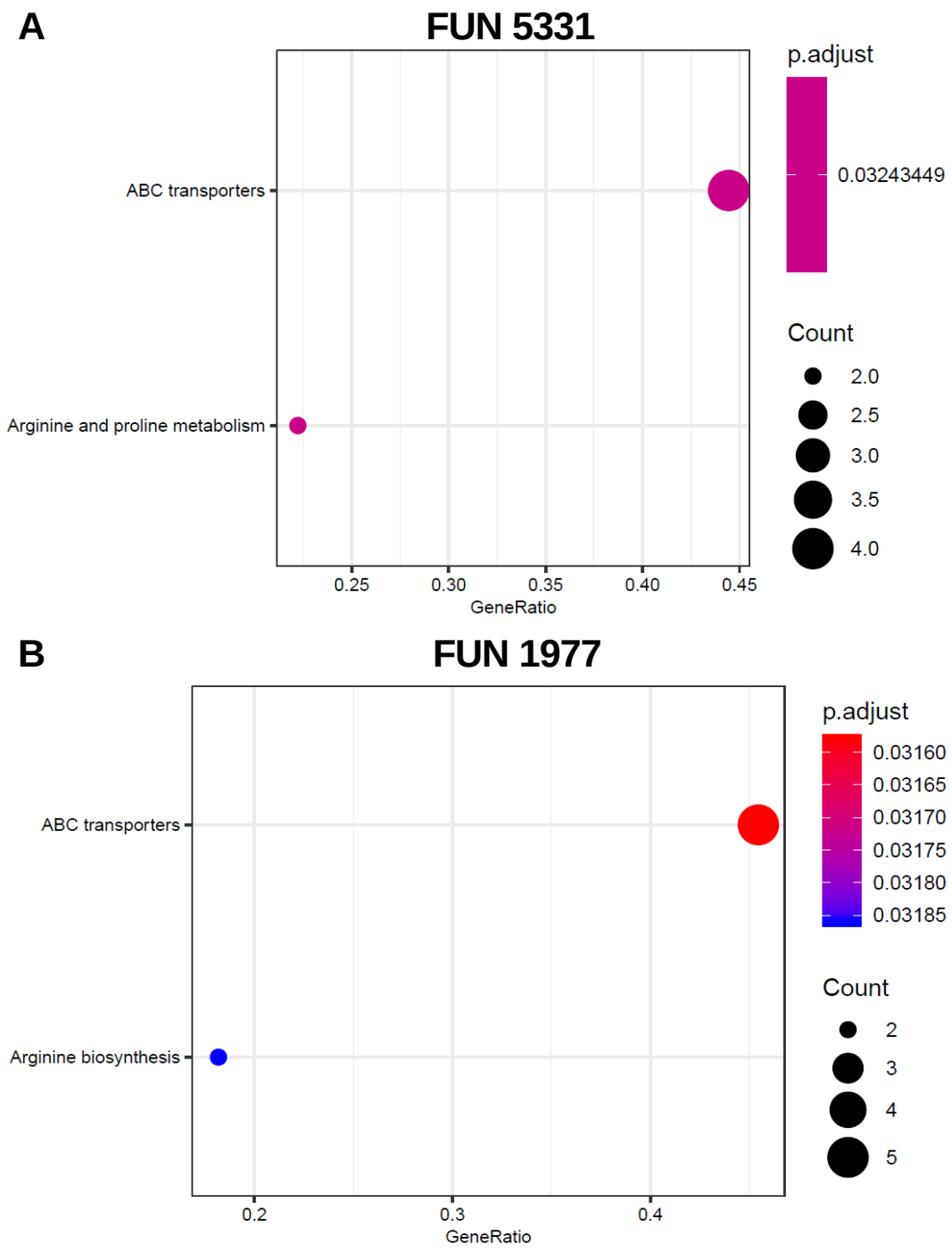

Figure 9.9: Pathway of FUN 5331 and 1977 using KEGG. Genes differentially expressed between wildtype and A. FUN 5331 or B. FUN 1977 were grouped in several categories using KEGG pathway data base.

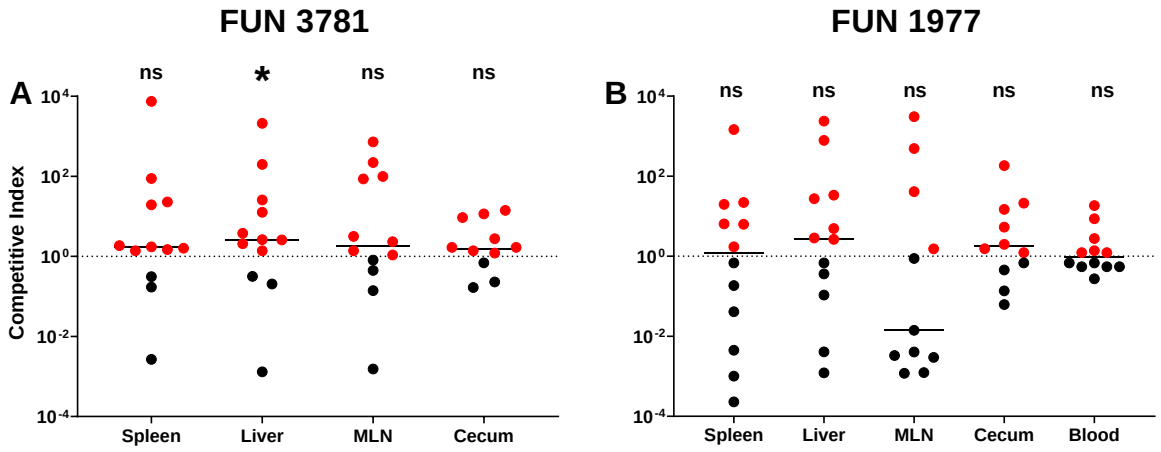

Figure 9.10: Competitive infection between wildtype and FUN operon mutants. C57BL/6 mice were infected with a 1:1 ratio of S. Typhimurium 14028 wildtype and FUN 3781 (A) or 14028 and FUN 1977 (B). At 4-7 days post-infection bacteria, were enumerated and competitive index (CI) values were calculated for each mouse in each organ. CI = (CFU FUN 3781 or 1977)/wildtype)output/(CFU FUN 3781 or 1977/14028)input. The horizontal dotted line represents a CI value of 1, or equal virulence between strains. Red circles represent mice where FUN 3781 or 1977 won the competition. Statistical significance. *p < 0.05 or ns p > 0.05.

9.4 Conclusion

With the recent advances in sequencing technologies, FUN genes are being identified with greater frequency, with the promise of new discoveries that will increase our understanding of pathogens. Transcriptome comparison identified 798 FUN genes, differentially expressed between *S.* Typhimurium multicellular aggregates and planktonic cells [176]. FUN genes were mapped to operons, and 23 operons with potential roles in *Salmonella* virulence and persistence were selected for further analysis. Most of the operons identified have no known homologs in current databases of functionally annonated genes; therefore, some of the selected FUN operons may be involved in novel processes that will enhance our understanding of *Salmonella* virulence and persistence.

By integrating information obtained from several experiments, we were able to predict the function of some of the selected FUN operons. Multicellular aggregates and planktonic cells produced by *S.* Typhimurium population divergence is a form of bet hedging that allows *Salmonella* survival in various environments [176]. We predict that some the FUN operons

analyzed (primarily FUN 4889, 3781 and 1977) are involved in *Salmonella* metabolism under different conditions. It is possible that some of the FUN operons analyzed enable *Salmonella* to utilize uncommon sugars found in diverse environments. No phenotype was identified for most of the selected FUN operons. Identifying conditions under which FUN operons display altered phenotypes compared to wildtype is one of the major challenges associated with identifying the roles of FUN operons (genes), due to functional redundancy and transcriptional priming. And this provides an explanation for why most FUN operon mutants had no altered phenotypes under the conditions tested.

Funtional redundancy is a major challenge of identifying the roles of FUN genes because, multiple genes (operons) can independently perform a particular biological process. Hence in the presence of genes that perform similar functions, FUN operon mutants will display no altered phenotypes. The expression of FUN operons may be repressed under the conditions tested due to functional redundancy. Therefore, repressor identification can aid in the analysis of FUN operons. This is demonstrated by mutation of *STM14_5340* and *leuO* which led to increased expression of FUN 5331 and FUN 1977, respectively.

Transcriptional priming is another challenge for the identification of the roles of FUN genes. It has been proposed that SPI-2 genes are expressed (in low levels) under non-inducing conditions in preparation for later stages of *Salmonella* life cycle [176,326,327]. If FUN genes are been primed for later stages of *Salmonella* life cycle, it is conceivable that mutants will exhibit no phenotype under the conditions where the transcript was identified. Some FUN genes may be involved in novel processes that are yet to be discovered, hence conditions where mutants show altered phenotypes are yet to developed. With the increasing number of FUN genes identified with every new sequenced genome, a framework is required for analyzing the roles of FUN genes.

A framework that addresses some of the key issues associated with reverse genetics would be invaluable for the identification of the roles of FUN genes (Fig 9.11). Homology searches are indispensable for FUN genes analysis, and should first be used to determine if the roles of the FUN genes have been described in other species. Since the acquisition or loss of genetic material can play an important role in bacterial evolution, the level of conservation of FUN genes can be used to determine if the gene has been selectively maintained among bacterial species. Some

FUN genes may be orphan genes or map to operons comprised of genes with known function. However, if FUN genes map to operons comprised entirely of FUN genes, operon analysis can increase the odds of identifying a role, because proteins encoded in an operon usually have coordinated functions or participate in the same or related biological processes. Using reporter assays to identify conditions where FUN operons are expressed is recommended, because subsequent analysis can be performed under this condition. Some transcription factors are global regulators that regulate the expression of several genes under specific conditions. Therefore, the identification of transcription factors that regulate the expression of FUN operons can also be indicators of conditions under which FUN mutants can be analyzed. Finally, genes differentially expressed between FUN mutants and wildtype can be used to identify pathways or biological processes altered as a result of FUN operon deletion. This can help to address functional redundancy because if well known pathways/ biological processes are identified, FUN mutants with mutation in key gene(s) in the identified pathways can be used to analyze the roles of FUN operons. Since mRNA expression does not always lead to protein synthesis, perhaps proteomic analysis under selected conditions is the best strategy for identifying FUN genes in the sequencing era.

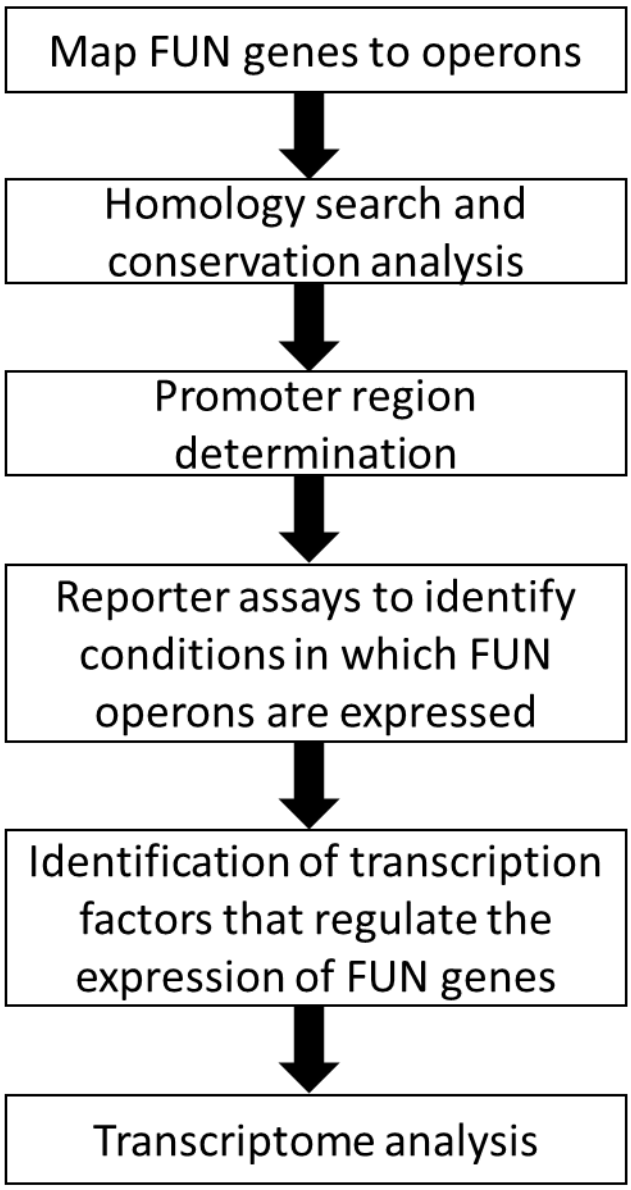

Figure 9.11: Proposed workflow for the analysis of FUN genes.

Table 9.1. List of selected FUN operons

Operons	Name (FUN)	% GC content	Conser-vation	Invasion/ Survival assays	Rdar	CPS	Predicted function based on pathway analysis
Planktonic							
STM14_3221-3227	3221	51.7	1/7			10^1	
STM14_5063-5047	5063	52.7	2/7	ns/ns	+	10^2	
STM14_3072-3068	3072	46.1	5/7	ns/ns	ns	10^1	ABC transport and Arginine metabolism
STM14_1425-1418	1425	50.8	2/7	ns/ns	ns	10^2	
STM14_1434-1437	1434	51.4	1/7	ns/ns	ns	10^1	
STM14_1156-1162	1156	50.2	3/7	ns/ns	ns	10^3	
STM14_1977-1981	1977	42.6	4/7	ns/ns	ns	10^1	ABC transporter and Arginine biosynthesis
STM14_1149-1154	1149	50.6	2/7	ns/ns	+	10^4	
STM14_1148-1140	1148	50.6	1/7			10^4	
STM14_2342-2429	2342	48.2	2/7	ns/ns	ns	10^1	
STM14_1526-1528	1526	43.2	5/7	ns/ns	ns	10^4	
STM14_3192-3186	3192	54.7	1/7	ns/ns	ns	10^1	
STM14_1455-1461	1455	54.1	1/7			10^1	
Multicellular aggregates							
STM14_1951-1952	1951	53.2	4/7	ns/ns	ns	10^2	
STM14_1953-1958	1953	53.5	4/7	ns/ns	+	10^1	
STM14_4847-4848	4748	44.6	3/7	ns/ns	ns	10^2	
STM14_3208-3201	3208	52.1	4/7	ns/ns	+	10^1	
STM14_5331-5341	5331	55.2	4/7	ns/ns	ns	10^2	ABC transporter, Arginine, and proline metabolism
STM14_2472-2463	2472	51.5	1/7		ns	10^2	
Planktonic and Multicellular aggregates							
STM14_4768-4772	4768	54.9	5/7	+/ns	ns	10^2	
STM14_3781-3776	3781	49.4	5/7	ns/ns	+	10^1	Microbial metabolism
STM14_4889-4894	4889	54.1	1/7	ns/+	ns	10^3	

STM14_2475-2484	2475	49.1	1/7	ns/+	ns	10^1

Ns – not significant.

9.5 Materials and methods

9.5.1 Bacterial strains, media, and growth conditions

The bacterial strains used in this study are listed in Table S12.2. For standard growth, strains were inoculated from frozen stocks onto LB agar (lysogeny broth, 1% NaCl, 1.5% agar) supplemented with an appropriate antibiotic (50 µg mL^{-1} kanamycin (Kan), 34 µg mL^{-1} chloramphenicol (Cam) or 5 µg mL^{-1} tetracycline (Tet)) and grown overnight at 37˚C. Isolated colonies were used to inoculate 5 mL LB broth and the culture was incubated for 18 hours at 37˚C with agitation at 200 rpm.

To visualize cellulose production, 2 µl aliquots of overnight culture were spotted on 1% tryptone agar supplemented with calcofluor white (fluorescent brightener 28; Sigma-Aldrich; 200 µg mL^{-1}). To evaluate cellulose production by crystal violet staining, 5 x 10^6 cells were inoculated into 5 mL LB broth and incubated at 37°C for 18 hours with agitation at 200 rpm. For Caco-2 invasion and macrophage survival assays, cells from overnight cultures were diluted 1:100 into fresh LB broth and grown at 37°C until the cultures reached an optical density of 0.7 at 600 nm. For murine infection experiments, overnight cultures grown in LB broth were diluted to the desired CFU concentration in 100 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), pH 8, and used to infect mice.

9.5.2 Generation of *S.* Typhimurium 14028 mutant strains

The *S.* Typhimurium 14028 FUN operon mutants were generated using the lambda red recombinase knockout procedure [170]. Primers containing 50-nucleotide sequence on either side of the operons (Table S12.3) were used to amplify the *cat* gene from pKD3 using Phusion High-Fidelity DNA polymerase (New England BioLabs). The PCR products were purified and electroporated into *S.* Typhimurium 14028 cells containing pKD46. Mutant strains were first selected by growth at 37˚C on LB agar supplemented with 7 µg mL^{-1} Cam, before re-streaking onto LB agar containing 34 µg mL^{-1} Cam. The mutations were moved into a clean background strains by transduction with P22 phage [171]. The *cat* gene was resolved from the chromosome

using pCP20. Sanger DNA sequencing of PCR products amplified from the chromosome of mutant strain using primers listed in table S12.3, confirmed the loss of operons.

9.5.3 Generation of reporter plasmids and luciferase reporter assays

To generate a luciferase reporter to measure genes expression, the intergenic regions upstream the first genes in selected FUN operons, were PCR amplified from *S*. Typhimurium 14028 using primers listed in table S12.3, and Phusion high-fidelity DNA polymerase (New England BioLabs), with reaction conditions outlined by the manufacturer. The resulting PCR products were purified, sequentially digested with *Xho*I and *Bam*HI (New England BioLabs), and ligated using T4 DNA ligase (New England BioLabs) into the pCS26 reporter plasmid containing a promoterless *luxCDABE*, cut with *Xho*I and *Bam*HI. Primers pZE05 and pZE06 were used to verify the successful fusion of FUN operon promoter region to *luxCDABE*. *S*. Typhimurium 14028 were transformed with the resulting pCS26 FUN::*luxCDABE* plasmid.

For bioluminescence assays, overnight cultures of *S*. Typhimurium 14028 transformed with pCS26 FUN::*luxCDABE* plasmid were diluted 1 in 600 in 1% tryptone or M9-glucose supplemented with 50 µg mL^{-1} Kan, to a final volume of 150 µl per well, in 96 well clear bottom black plates (9520 Costar; Corning Inc). To minimize evaporation of the medium during the assay, cultures were overlaid with 50 µL of mineral oil. Cultures were assayed for absorbance (600 nm, 0.1 s) and luminescence (1s; in counts per second [CPS]), every 30 min during growth at 28˚C or 37°C with agitation in a Victor X3 multilabel plate reader (Perkin-Elmer).

9.5.4 Generation of Transposon mutants

Random mutagenesis with Tn*10* transposon was used to identify regulators of FUN operon expression [251,252]. *S*. Typhimurium 14028 harbouring pNK972 was infected with P22 lysate of *S*. Typhimurium TT10605 containing Tn10Cam on F'. The resulting transductants (~100,000 colonies) were pooled and a P22 *S*. Typhimurium 14028 Tn*10*dCam transducing fragment library was generated, following the method outlined by Maloy [171]. The resulting P22 phage lysate was used to infect *S*. Typhimurium 14028 transformed with FUN operon promoter::luciferase fusion plasmids and plated on 1% tryptone agar supplemented with 20 µg/ml CAM. The resulting transductants (10, 000 to 20, 000) were screened for light production using IVIS lumina II imager. Luminate colonies were isolated and used for luciferase assays. To identify the sites of transposon insertions nested PCR was performed on purified genomic DNA

using primers CAT01 and ARB1 or ARB6. The product of the first reaction was further amplified using primers CAT02 and ARB2. PCR products were purified and sequenced. To identify the site of Tn*10* insertion DNA sequence was mapped to *S.* Typhimurium 14028 using Geneious.

9.5.5 Invasion Assay using polarized Caco-2 cells

Invasion of polarized Caco-2 cells were assessed in triplicate wells for *S.* Typhimurium wildtype and FUN operon knock out strains. Caco-2 cells were grown at 37°C in the presence of 5% CO_2 in complete Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum and 1% nonessential amino acids. To obtain polarized monolayers, Caco-2 cells were seeded onto a transwell insert for approximately 21 days. The cells were used for invasion assays once the transepithelial resistance (TER) was 700 to 900 Ω cm^{-2}. *S.* Typhimurium strains grown to late exponential phase (at an OD_{600} of 0.7 in LB broth at 37°C) were resuspended in complete DMEM and applied in 100 µl aliquots to Caco-2 cells, at a multiplicity of infection (MOI) of 10. After 1 h exposure to *S.* Typhimurium, Caco-2 cells were washed three times with 200 µl PBS and incubated for 2 h with complete DMEM containing 1.2 mg/ml gentamicin to kill any remaining extracellular bacteria. The Caco-2 cells were washed two times with 200 µl of PBS and lysed by exposure to 1% Triton X-100. To determine the rates of invasion, serial dilutions of the *Salmonella* containing lysate were plated in 4 µl drops on LB agar and incubates overnight at 37°C.

9.5.6 Macrophage survival assay

RAW 264.7 macrophages were grown in DMEM supplemented with 10% heat denatured FBS, 1mM sodium pyruvate and 50 µg mL^{-1} gentamicin. Prior to seeding cells into multi-well plates, the cells were washed with versene, centrifuged at 1200 rpm, and resuspended in DMEM with 10% FBS (without antibiotics). RAW 264.7 cells were seeded at 1.25 x 10^5 *S.* Typhimurium cells per 0.5mL per well in a 48-well plate. The cells were allowed to adhere for at least 3 hours before infection.

Approximately 1.25 x 10^5 macrophages were infected with *S.* Typhimurium at a multiplicity of infection (MOI) of ~100. Infected macrophages were centrifuged at 1000 rpm for 1 min and incubated at 37°C for 30 mins. Following *S.* Typhimurium internalization, macrophages were washed twice with warm PBS, and medium containing 20 µg mL-1 gentamicin was added to kill

extracellular bacteria. Macrophages were lysed with 0.1% Triton X-100, at 21 hours post-infection to enumerate intracellular *S.* Typhimurium. To determine the number of intramacrophage bacteria, lysed cells were serial diluted, plated on LB agar and incubated overnight at 37°C.

9.5.7 Biofilm screening of *S.* Typhimurium strains

Biofilm cell mass was quantified by crystal violet (CV) staining. Approximately 10^7 CFU of overnight cultures of *S.* Typhimurium wildtype or FUN strains were added to 96 well MBEC plates and incubated at 30°C for 48 hours. Plates were washed twice by being submerged into a water tray, followed by being shaken into a waste tray to remove non-attached cells. After air drying for 10 mins, 125 µl of 0.1% (wt/vol) CV solution was added to each well, and plates were incubated for 10 to 15 mins at RT. After staining, plates were washed twice with water and vigorously tapped on paper towels to remove any excess liquid, followed by air drying for 5 to 10 mins. 200 µl of 95% ethanol was added to each well and the plates were covered and incubated for 15 mins at RT. 125 µl of solution from each well was transferred into new clear flat bottom 96 well plate and the optical density at 590 nm was measured.

9.5.8 Murine infection Experiments

Six to eight-week-old female C57BL/6 mice purchased for Jackson Laboratory (Bar Harbor, ME) were assigned to cage groups using a randomization table prepared in Microsoft Excel, and individual mice were marked with ear notches.

For competitive index (CI) experiments 2 groups of six mice were challenged with a mixed inoculum consisting of ~1:1 ratio of Kan^R and Cam^R *S.* Typhimurium 14028 strain containing the sig70_16 *luxCDABE* construct [175]. Mice were challenged via oral gavage with a total CFU of ~10^7. Infected mice were weighed daily and monitored for clinical signs of infections. Mice that had a >20% drop in weight were humanely euthanized. All mice were euthanized 4-7 days post infection, and spleen, live, mesenteric lymph nodes (MLN) and cecum collected from each mouse. Collected organs were placed in a 2 ml Eppendorf Safe-Lock tube containing 1 ml of phosphate-buffered saline (PBS), and a 5-mm steel bead (Qiagen product 69989), and homogenized using a mixer mill (Retsch; MM400) for 5 min at 30 Hz. To determine the number of CFU, initial challenges or organ homogenates were serially diluted and plated on LB agar

supplemented with 50 µg mL^{-1} Kan and 20 µg mL^{-1} Cam. The CI values were calculated as follows: $(CFU\ Kan^R/CFU\ Cam^R)_{Output}/(CFU\ Kan^R/CFU\ Cam^R)_{input}$.

9.5.9 Ethics Statement

All animals were cared for and used 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 20190071, which was approved by the University of Saskatchewan's Animal Research Ethics Board.

9.5.10 RNA purification procedure

Total RNA was isolated from multicellular aggregates or planktonic cells. 1% tryptone was inoculated with $1OD_{600}$ of *S*. Typhimurium overnight and incubated that 28°C for 13 hours in a shaking water bath at 200 RPM. Aggregate and planktonic cells were separated by low speed centrifugation (1000 RPM, 2 minutes, 9 acceleration, 5 brake). Planktonic cells (Supernatant) were transferred into new tubes and OD equivalent to 20 OD_{600} was pelleted by centrifugation at 10 000 x g, for 2 minutes, and resuspended in 1 ml PBS. Approximately 20 to 30 mg of aggregate were transferred to 1.5 ml Eppendorf using sterile loop and culture medium was removed by centrifugation at 10 000 x g for 2 minutes. Aggregates were resuspended in 1 ml PBS. Planktonic cells and aggregate resuspended in PBS were placed in 2 ml Eppendorf Safe-Lock tube containing a 5- mm steel bead (Qiagen product 69989) and homogenized using a mixer mill (Retsch; MM400) for 5 minutes at 30 Hz. Homogenized samples were centrifuged at 13200 RPM for 1 minutes and resuspend in lysis buffer. Lysed cells were placed in Eppendorf tubes containing zirconia beads and homogenized in a mixer mill for 5 minutes at 30 Hz. RNA purification was performed using Qiagen according to the manufacture's instruction.

9.5.11 RNA-seq data processing and gene expression analysis

FastQC was used for quality control for the raw sequencing data. The adapters and low-quality reads were further filtered with Trim Galore to get the cleaned data. The filtering criteria included: sequences with phred score<=20, adapters and unmatched paired-end reads. FastQC was also used to observe the quality of cleaned data. After filtering, the cleaned data were mapped to the *Salmonella* typhimurium 14028S reference genome sequences with Bowtie2 by default alignment parameters [328]. The generated SAM files were used for gene expression

quantification with TrBorderExt [315]. EdgeR was used for gene expression comparison [329]. The False Discovery Rate (FDR) of <0.05 were present as the significance level.

9.5.12 Functional annotation and enrichment analysis

Gene Ontology (GO) overrepresentation analysis was performed using PANTHER GO_Slim, to the genes with significantly differential expression [330]. Each gene was analyzed for the three basic oncology terms - 'molecular function', 'biological process' and 'cellular component', respectively. A package, clusterProfiler, was also used to make the KEGG pathway enrichment analysis for the significant genes. For KEGG enrichment analysis, the significance level was set as FDR < 0.05.

10.0 General conclusions and future directions

Salmonella are important pathogens worldwide and have a significant effect on global health. My PhD projects were focused on *Salmonella* pathogenesis, vaccine development, biofilm formation and the analysis of function unknown genes. Understanding different aspects of *Salmonella* biology is important for the development of mitigation strategies to combat *Salmonella* infections.

In section 3, I described a conserved SNP in invasive *S.* Typhimurium that results in a missense mutation in the sensory domain of a diguanylate cyclase enzyme, STM1987. STM1987 catalyzes the formation of c-di-GMP, which positively regulates cellulose production [106]. Previous research has shown that *Salmonella* produces cellulose inside macrophages as an antiviurlence factor [107]. Mutation in STM1987 reduced cellulose production and increased *Salmonella* survival inside macrophages, and virulence in a mouse model of infection. Future studies will use enzyme assays to directly determine the effect of the missense mutation on STM1987 activity. Since STM1987 has been shown to respond to intramacrophage signals such as L-arginine [134], future studies will also determine if STM1987 is the main diguanylate cyclase required for c-di-GMP production inside macrophages. Traditionally, cellulose is associated with biofilm formation [87], my work highlights a more complex role of cellulose production in the *Salmonella* life cycle.

Current vaccine development strategies against NTS are focused on EPS-based vaccines. Immunological studies showed that the O-Ag portion of LPS is the main target of protective immunity against (i)NTS serovars [71]. O-Ag capsule has similar repeating units as LPS O-Ag, hence our initial objective was to develop O-Ag capsule as a potential vaccine candidate. However, work done to boost O-Ag capsule production lead to increased colanic acid production. Immunization with colanic acid did not protect against *Salmonella*. However, GMMAs purified from colanic acid overproducing strains induced a partially protective immune response against *Salmonella* in mice. Future work on this project will focus on developing GMMA-EPS based vaccines against NTS, and identifying the protective antigens present in GMMAs. Work done by Gibson et al., identified the operons *yihUTSRQPO* and *yihVW* as the putative O-Ag capsule biosynthesis operon [96]. I speculate that the *yih* operon is not involved in O-Ag capsule biosynthesis, but may have dual functionality, where it is involved in SQ

catabolism and the synthesis of an unknown EPS or the modification of the antigenic content of the outer membrane. Further analysis will also focus on identifying novel EPS, that can be developed as a vaccine against NTS. With the rise in antibiotic resistant isolates, a vaccine is urgently needed to reduce the incidence of NTS.

Biofilm formation, which is critical for *Salmonella* survival and transmission, is regulated by transcription factor CsgD [87]. Understanding the fined-turned regulation of CsgD expression can help us to piece together how regulation occurs in the natural environment and develop strategies that will reduce transmission especially through food outbreaks. In section 7, I showed that *csgD* respond differently to some regulatory inputs once activated. When introduced at the beginning of growth, increasing concentrations of NaCl and casamino acids reduce *csgD* expression. However, when introduced after *csgD* expression has been induced, NaCl has no effect, while casamino acids have a dose dependent effect on *csgD* expression. In contrast, the introduction of glucose and increased temperature above 30° repress *csgD* expression before and after induction. Future work on this will focus on using proteomics to correlate expression data. This work adds an interesting new observation to the existing body of knowledge surrounding the regulation of *csgD* expression in *Salmonella*.

Current genome databases are filled with numerous sequenced bacterial genomes that contain hundreds of FUN genes. With the rise in antibiotic resistant isolates, FUN genes can provide new targets for the development of the next generation of antimicrobials. Therefore, it is important to develop a framework that can be used to identify the roles of FUN genes. Using FUN genes differentially expressed between multicellular aggregates and planktonic cells, I showed that it is difficult to identify conditions in which FUN operons (gene) mutants exhibit altered phenotypes. I propose a framework that can be used for the analysis of FUN genes. This framework includes mapping FUN genes to operons, using operon mutants for FUN genes analysis, identifying regulators, and comparative transcriptomic analysis. This framework can lead to the design of experimental conditions under which FUN genes will exhibit altered phenotypes. Future work will focus on using this framework to identify the roles of FUN genes differentially expressed between *S* Typhimurium multicellular aggregates and planktonic cells.

My work adds to the existing body of knowledge on *Salmonella* pathogenesis, biofilm formation and vaccine development. The biological relevance is high because a combined knowledge of

these aspects of *Salmonella* biology will be relevant in the development of strategies to reduce the global burden of *Salmonella* infections.

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12.0 Supplementary information

Table S12.1. List of *S.* Typhimurium isolates corresponding to figure 3.3 that were analyzed for the presence of *stm1987* SNP

	Isolate	Accession Number	Year of isolation	Place of isolation	Continent	Source	MLST	Phylogenetic lineage	STM1987 G215C	Represented in Figure
1	5576	ERS004955	2005	Kenya	Africa	Human	313	I	C	*
2	5577	ERS004958	2004	Kenya	Africa	Human	313	I	C	*
3	5580	ERS004896	2004	Kenya	Africa	Human	394	I	C	*
4	5597	ERS004898	2005	Kenya	Africa	Human	313	I	C	*
5	5912	ERS004963	2005	Kenya	Africa	Human	313	I	C	*
6	100DRC	ERS009030	1989	DRC	Africa	Human	313	I	C	*
7	1905U	ERS009036	1996	Uganda	Africa	Human	313	I	C	*
8	2017U	ERS004902	1996	Uganda	Africa	Human	313	I	C	*
9	228DRC	ERS009033	1991	DRC	Africa	Human	313	I	C	*
10	265DRC	ERS009034	1991	DRC	Africa	Human	313	I	C	*
11	269DRC	ERS009048	1991	DRC	Africa	Human	313	I	C	*
12	270DRC	ERS009046	1991	DRC	Africa	Human	313	I	C	*
13	334U	ERS009044	2002	Uganda	Africa	Human	313	I	C	*
14	5897U	ERS009037	1997	Uganda	Africa	Human	313	I	C	*
15	6325U	ERS009042	1998	Uganda	Africa	Human	313	I	C	*
16	868U	ERS009041	2002	Uganda	Africa	Human	313	I	C	*
17	958U	ERS009035	2003	Uganda	Africa	Human	313	I	C	*
18	A018	ERS007446	1997	Malawi	Africa	Human	313	I	C	*
19	A082	ERS007447	1997	Malawi	Africa	Human	313	I	C	*
20	A130	ERS007468	1997	Malawi	Africa	Human	313	I	C	*
21	A13198	ERS007457	2001	Malawi	Africa	Human	313	I	C	*
22	A13212	ERS007458	2001	Malawi	Africa	Human	313	I	C	*
23	A16083	ERS007460	2002	Malawi	Africa	Human	313	I	C	*
24	A357	ERS007448	1998	Malawi	Africa	Human	313	I	C	*
25	A3800	ERS007451	1999	Malawi	Africa	Human	313	I	C	*
26	A4283	ERS007452	1999	Malawi	Africa	Human	313	I	C	*
27	A4447	ERS007453	1999	Malawi	Africa	Human	313	I	C	*
28	A680	ERS007450	1998	Malawi	Africa	Human	313	I	C	*
29	C2110	ERS007454	2000	Malawi	Africa	Human	313	I	C	*
30	C2167	ERS007455	2000	Malawi	Africa	Human	313	I	C	*
31	C2364	ERS007456	2000	Malawi	Africa	Human	313	I	C	*
32	D11578	ERS007459	2001	Malawi	Africa	Human	313	I	C	*
33	D15040	ERS007462	2002	Malawi	Africa	Human	313	I	C	*
34	D25248	ERS004899	2004	Malawi	Africa	Human	313	I	C	*
35	D25646	ERS004895	2004	Malawi	Africa	Human	313	I	C	*

36	M1111568	ERS009021	2001	Mozambique	Africa	Human	313	I	C	*
37	M1174516	ERS007629	2001	Mozambique	Africa	Human	313	I	C	*
38	M1253289	ERS009022	2001	Mozambique	Africa	Human	313	I	C	*
39	M1438312	ERS007631	2001	Mozambique	Africa	Human	313	I	C	*
40	M1553285	ERS009020	2001	Mozambique	Africa	Human	313	I	C	*
41	M1560153	ERS007623	2001	Mozambique	Africa	Human	313	I	C	*
42	M1776464	ERS007624	2002	Mozambique	Africa	Human	313	I	C	*
43	5575	ERS004953	2004	Kenya	Africa	Human	313	I	C	
44	5581	ERS004957	2003	Kenya	Africa	Human	313	I	C	
45	356DRC	ERS004905	1991	DRC	Africa	Human	313	I	C	
46	5597B	ERS004898	2005	Kenya	Africa	Human	313	I	C	
47	A24924	ERS184455	2003	Malawi	Africa	Human	313	I	C	
48	D22889	ERS005403	2003	Malawi	Africa	Human	313	I	C	
49	M1175849	ERS009051	2001	Mozambique	Africa	Human	313	I	C	
50	Q175F6	ERS033126	2003	Malawi	Africa	Human	313	I	C	
1	5579	ERS004962	2005	Kenya	Africa	Human	313	II	C	*
2	5582	ERS004957	2005	Kenya	Africa	Human	313	II	C	*
3	146U	ERS009045	2002	Uganda	Africa	Human	313	II	C	*
4	198U	ERS009032	2002	Uganda	Africa	Human	313	II	C	*
5	254DRC	ERS009040	1991	DRC	Africa	Human	313	II	C	*
6	373DRC	ERS009047	1991	DRC	Africa	Human	313	II	C	*
7	415DRC	ERS009039	1992	DRC	Africa	Human	313	II	C	*
8	666U	ERS004903	2002	Uganda	Africa	Human	313	II	C	*
9	A24906	ERS007463	2006	Malawi	Africa	Human	313	II	C	*
10	A24910	ERS007464	2006	Malawi	Africa	Human	313	II	C	*
11	A32751	ERS007465	2005	Malawi	Africa	Human	313	II	C	*
12	A32773	ERS007466	2005	Malawi	Africa	Human	313	II	C	*
13	A38589	ERS007469	2005	Malawi	Africa	Human	313	II	C	*
14	A38596	ERS007470	2005	Malawi	Africa	Human	313	II	C	*
15	A39051	ERS007472	2002	Malawi	Africa	Human	313	II	C	*
16	A39129	ERS007473	2006	Malawi	Africa	Human	313	II	C	*
17	A39155	ERS007474	2006	Malawi	Africa	Human	313	II	C	*
18	C13184	ERS007471	2002	Malawi	Africa	Human	313	II	C	*
19	D14916	ERS007461	2006	Malawi	Africa	Human	313	II	C	*
20	D23580	FN424405	2004	Malawi	Africa	Human	313	II	C	*
21	D23769A	ERS007467	2003	Malawi	Africa	Human	313	II	C	*
22	D25023	ERS004897	2004	Malawi	Africa	Human	313	II	C	*
23	D25734	ERS004900	2004	Malawi	Africa	Human	313	II	C	*
24	D26275	ERS023828	2004	Malawi	Africa	Human	313	II	C	*

25	I3	ERS007483	2002	Mali	Africa	Human	313	II	C	*
26	I32	ERS007484	2002	Mali	Africa	Human	313	II	C	*
27	I45	ERS007485	2002	Mali	Africa	Human	313	II	C	*
28	I7	ERS007486	2002	Mali	Africa	Human	313	II	C	*
29	J17	ERS007480	2002	Mali	Africa	Human	313	II	C	*
30	J20	ERS007482	2003	Mali	Africa	Human	313	II	C	*
31	J27	ERS007481	2002	Mali	Africa	Human	313	II	C	*
32	J3	ERS007479	2005	Mali	Africa	Human	313	II	C	*
33	M1605206	ERS007627	2002	Mozambique	Africa	Human	313	II	C	*
34	M2907772	ERS007619	2003	Mozambique	Africa	Human	313	II	C	*
35	P51	ERS007478	2006	Mali	Africa	Human	313	II	C	*
36	P62	ERS007476	2005	Mali	Africa	Human	313	II	C	*
37	P73	ERS007477	2005	Mali	Africa	Human	313	II	C	*
38	P78	ERS007475	2005	Mali	Africa	Human	313	II	C	*
39	PO1124	ERS009025	2010	Nigeria	Africa	Human	313	II	C	*
40	PO1140	ERS009026	2010	Nigeria	Africa	Human	313	II	C	*
41	PO1276	ERS009027	2010	Nigeria	Africa	Human	313	II	C	*
42	PO609	ERS009024	2009	Nigeria	Africa	Human	313	II	C	*
43	Q134A	ERS033111	2002	Malawi	Africa	Human	313	II	C	*
44	Q175A	ERS033114	2002	Malawi	Africa	Human	313	II	C	*
45	Q18A	ERS033110	2002	Malawi	Africa	Human	313	II	C	*
46	Q255A	ERS033115	2002	Malawi	Africa	Human	313	II	C	*
47	Q255F4	ERS033127	2003	Malawi	Africa	Human	313	II	C	*
48	Q258A	ERS033116	2002	Malawi	Africa	Human	313	II	C	*
49	Q258F4	ERS033128	2003	Malawi	Africa	Human	313	II	C	*
50	Q285A	ERS033117	2002	Malawi	Africa	Human	313	II	C	*
51	Q285F5	ERS033129	2005	Malawi	Africa	Human	313	II	C	*
52	Q303F5	ERS033130	2004	Malawi	Africa	Human	313	II	C	*
53	Q340A	ERS033119	2002	Malawi	Africa	Human	313	II	C	*
54	Q340F4	ERS033131	2004	Malawi	Africa	Human	313	II	C	*
55	Q363A	ERS033120	2002	Malawi	Africa	Human	313	II	C	*
56	Q363F3	ERS033132	2003	Malawi	Africa	Human	313	II	C	*
57	Q367A	ERS033121	2002	Malawi	Africa	Human	313	II	C	*
58	Q367F2	ERS033133	2003	Malawi	Africa	Human	313	II	C	*
59	14DRC	ERS009029	1988	DRC	Africa	Human	313	II	C	
60	A20	ERS005114	2002	Mali	Africa	Human	313	II	C	
61	A21	ERS005117	2002	Mali	Africa	Human	313	II	C	
62	A40	ERS005115	2002	Mali	Africa	Human	313	II	C	
63	D71	ERS005118	2002	Mali	Africa	Human	313	II	C	
64	D83	ERS005120	2002	Mali	Africa	Human	313	II	C	
65	M2815310	ERS009050	2004	Mozambique	Africa	Human	313	II	C	

66	P30	ERS005116	2005	Mali	Africa	Human	313	II	C	
67	Q134F9	ERS033123	2002	Malawi	Africa	Human	313	II	C	
68	Q18A_S	ERS033112	2002	Malawi	Africa	Human	313	II	C	
69	Q18F3_S	ERS033124	2005	Malawi	Africa	Human	313	II	C	
1	LA137	ERS009058	2008	Laos	South-east Asia	Human	19	Non-African	G	*
2	LT1	ERS007487	1946	United Kingdom	Europe	Human	19	Non-African	G	*
3	LT10	ERS007496	1945	Chile	South America	Human	19	Non-African	G	*
4	LT11	ERS007497	1938	Sweden	Europe	Rats	19	Non-African	G	*
5	LT12	ERS007498	1946	United Kingdom	Europe	Human	19	Non-African	G	*
6	LT2	ERS007488	1946	United Kingdom	Europe	U	19	Non-African	G	*
7	LT3	ERS007489	1946	Denmark	Europe	Human	19	Non-African	G	*
8	LT4	ERS007490	1946	Sweden	Europe	animal	19	Non-African	G	*
9	LT5	ERS007491	1946	Switzerland	Europe	calf	128	Non-African	G	*
10	LT6	ERS007492	1946	Sweden	Europe	pigeon	19	Non-African	G	*
11	LT7	ERS007493	1946	USA-CO	North America	lamb	19	Non-African	G	*
12	LT8	ERS007494	1943	Denmark	Europe	mouse	19	Non-African	G	*
13	LT9	ERS007495	1946	Denmark	Europe	goose	19	Non-African	G	*
14	PVI	ERS007513	1973	Uruguay	South America	Human	19	Non-African	G	*
15	THA14	ERS009072	1997	Thailand	South-east Asia	Human	19	Non-African	G	*
16	5578	ERS004959	2005	Kenya	Africa	Human	19	Other African, invasive	G	*
17	5632	ERS004961	2003	Kenya	Africa	Human	19	Other African, invasive	G	*
18	5634	ERS004963	2003	Kenya	Africa	Human	19	Other African, invasive	C	*
19	5647	ERS004964	2005	Malawi	Africa	Human	19	Other African, invasive	G	*
20	6079U	ERS009038	1998	Uganda	Africa	Human	19	Other African, invasive	G	*
21	Q303A	ERS033118	2002	Malawi	Africa	Human	19	Other African, invasive	G	*
22	*ST4/74	CP002487	1966	United Kingdom	Europe	Bovine	19	Non-African	G	
23	88-138-DT2	ERS184454	1988	Germany	Europe	Pigeon	128	Non-African	G	
24	DT1	ERS007598	2009	United Kingdom	Europe	Human	19	Non-African	G	
25	DT12	ERS007564	2009	United Kingdom	Europe	Human	19	Non-African	G	

26	DT120	ERS007566	2009	United Kingdom	Europe	Human	34	Non-African	G	
27	DT135	ERS007567	2009	United Kingdom	Europe	Human	19	Non-African	G	
28	DT177	ERS007572	2008	United Kingdom	Europe	Human	19	Non-African	G	
29	DT191A	ERS007574	2009	United Kingdom	Europe	Human	19	Non-African	G	
30	DT193	ERS007576	2009	United Kingdom	Europe	Human	34	Non-African	G	
31	DT195	ERS007578	2009	United Kingdom	Europe	Human	19	Non-African	G	
32	DT24	ERS007582	2009	United Kingdom	Europe	Human	19	Non-African	C	
33	DT56	ERS007588	2009	United Kingdom	Europe	Human	19	Non-African	G	
34	DT7	ERS007590	2009	United Kingdom	Europe	Human	34	Non-African	G	
35	DT8	ERS007592	2009	United Kingdom	Europe	Human	19	Non-African	G	
36	DT97	ERS007594	2008	United Kingdom	Europe	Human	34	Non-African	G	
37	DT99	ERS007596	2009	United Kingdom	Europe	Human	19	Non-African	G	
38	SARA1	ERS007499	NA	Mexico	North America	Human	19	Non-African	G	
39	SARA10	ERS007508	1987	USA-CA	North America	Opposum	19	Non-African	G	
40	SARA11	ERS007509	NA	Thailand	South-east Asia	unspecified	19	Non-African	G	
41	SARA12	ERS007510	1987	USA-LA	North America	Horse	19	Non-African	G	
42	SARA2	ERS007500	1946	United Kingdom	Europe	Laboratory strain	19	Non-African	G	
43	SARA3	ERS007501	1987	USA-RI	North America	Horse	19	Non-African	G	
44	SARA4	ERS007502	1986	USA-IN	North America	Rabbit	19	Non-African	G	
45	SARA5	ERS007503	NA	Mongolia	Europe	unspecified	19	Non-African	G	
46	SARA9	ERS007507	1987	USA-CA	North America	Parrot	98	Non-African	G	
47	SL1344	FQ312003	1981	United Kingdom	Europe	Bovine	19	Non-African	G	
48	U276	ERS007600	2009	United Kingdom	Europe	Human	19	Non-African	G	
49	U277	ERS007602	2009	United Kingdom	Europe	Human	568	Non-African	G	
50	U288	ERS007604	2009	United Kingdom	Europe	Human	19	Non-African	G	
51	U302	ERS007606	2009	United Kingdom	Europe	Human	19	Non-African	G	
52	U310	ERS007608	2009	United Kingdom	Europe	Human	19	Non-African	G	
53	U313	ERS007611	2008	United Kingdom	Europe	Human	19	Non-African	G	
54	U319	ERS007613	2008	United Kingdom	Europe	Human	19	Non-African	G	
55	249DRC	ERS004904	1991	DRC	Africa	Human	19	Other African, invasive	G	
56	M2815013	ERS009049	2003	Mozambique	Africa	Human	19	Other African, invasive	G	

Table S12.2. Strains and plasmids used in this study.

Strains or Plasmids	Genotype	Reference
Strains		
S. Typhimurium 14028	Wild-type strain	ATCC
Δ*csgD*	Deletion of *csgD* ORF	[176]
Δ*bcsA*	Deletion of *bcsA* ORF	[118]
Δ*csgA*	Deletion of *csgA* ORF	[176]
ΔSPI-1	Deletion of SPI-1	[176]
ΔSTM14_2475 - 2484	Deletion of STM14_2475 – 2484 operon	This study
ΔSTM14_4768 - 4772	Deletion of STM14_4768 – 4772 operon	This study
ΔSTM14_4889 - 4894	Deletion of STM14_4889 – 4894 operon	This study
ΔSTM14_1149 - 1154	Deletion of STM14_1149 – 1154 operon	This study
ΔSTM14_1156 - 1162	Deletion of STM14_1156 – 1162 operon	This study
ΔSTM14_1425 - 1418	Deletion of STM14_1425 – 1418 operon	This study
ΔSTM14_1434 - 1437	Deletion of STM14_1434 – 1437 operon	This study
ΔSTM14_1455 - 1461	Deletion of STM14_1455 – 1461 operon	This study
ΔSTM14_1526 – 1528	Deletion of STM14_1526 – 1528 operon	This study
ΔSTM14_3072 - 3068	Deletion of STM14_3072 – 3068 operon	This study
ΔSTM14_3192 - 3186	Deletion of STM14_3192 – 3186 operon	This study
ΔSTM14_3221 - 3227	Deletion of STM14_3221 – 3227 operon	This study
ΔSTM14_5063 - 5047	Deletion of STM14_5063 – 5047 operon	This study
ΔSTM14_2432 - 2429	Deletion of STM14_2432 – 2429 operon	This study
ΔSTM14_4847 - 4848	Deletion of STM14_4847 – 4848 operon	This study
ΔSTM14_1977 - 1981	Deletion of STM14_1977 – 1981 operon	This study
ΔSTM14_1951 - 1952	Deletion of STM14_1951 – 1952 operon	This study
ΔSTM14_1953 - 1958	Deletion of STM14_1953 – 1958 operon	This study
ΔSTM14_3208 - 3201	Deletion of STM14_3208 – 3201 operon	This study
ΔSTM14_5331 - 5341	Deletion of STM14_5331 – 5341 operon	This study
ΔSTM14_2472 - 2463	Deletion of STM14_2472 – 2463 operon	This study
ΔSTM14_4552 - 4554	Deletion of STM14_4552 – 4554 operon	This study
ΔSTM14_3781 - 3776	Deletion of STM14_3781 – 3776 operon	This study
Plasmids		
pCS26-2475::*luxCDABE*	STM14_2475 – 2484 promoter	This study

pCS26-4768::*luxCDABE*	STM14_4768 – 4772 promoter	This study
pCS26-4889::*luxCDABE*	STM14_4889 – 4894 promoter	This study
pCS26-1148::*luxCDABE*	STM14_1148 – 1140 promoter	This study
pCS26-1149::*luxCDABE*	STM14_1149 – 1154 promoter	This study
pCS26-1156::*luxCDABE*	STM14_1156 – 1162 promoter	This study
pCS26-1425::*luxCDABE*	STM14_1425 – 1418 promoter	This study
pCS26-1434::*luxCDABE*	STM14_1434 – 1437 promoter	This study
pCS26-1455::*luxCDABE*	STM14_1455 – 1461 promoter	This study
pCS26-1526::*luxCDABE*	STM14_1526 – 1528 promoter	This study
pCS26-3072::*luxCDABE*	STM14_3072 – 3068 promoter	This study
pCS26-3192::*luxCDABE*	STM14_3192 – 3186 promoter	This study
pCS26-3221::*luxCDABE*	STM14_3221 – 3227 promoter	This study
pCS26-5063::*luxCDABE*	STM14_5063 – 5047 promoter	This study
pCS26-2432::*luxCDABE*	STM14_2432 – 2429 promoter	This study
pCS26-4847::*luxCDABE*	STM14_4847 – 4848 promoter	This study
pCS26-1977::*luxCDABE*	STM14_1977 – 1981 promoter	This study
pCS26-1951::*luxCDABE*	STM14_1951 – 1952 promoter	This study
pCS26-1953::*luxCDABE*	STM14_1953 – 1958 promoter	This study
pCS26-3208::*luxCDABE*	STM14_3208 – 3201 promoter	This study
pCS26-5331::*luxCDABE*	STM14_5331 – 5341 promoter	This study
pCS26-2472::*luxCDABE*	STM14_2472 – 2463 promoter	This study
pCS26-4552::*luxCDABE*	STM14_4552 – 4554 promoter	This study
pCS26-3781::*luxCDABE*	STM14_3781 – 3776 promoter	This study

Table S12.3: Oligonucleotides used in this study

Primer	Sequence (5’ – 3’)[a]	Purpose
STM14_2475 - 2484 sense	GTGAAGTCTTGTCGGCGTCCGGCT CTTCCAACAACAGGAGGAAGGCG ACAGTGTAGGCTGGAGCTGCTTC	To amplify *cat* gene product from pKD3 to generate ΔSTM14_2474 – 2484 strain by lambda red recombination
STM14_2475 - 2484 antisense	ATTCGGTGAGAAAAAAACGCCAG AATTTTAACTGGCGCACATCGAAA AGCCCTCCTTAGTTCCTATTCCG	

STM14_2475 - 2484 verify F	TCTGGCGTGCAACCTTCG	To confirm deletion of STM14_2474 – 2484 from *S*. Typhimurium 14028
STM14_2475 - 2484 verify R	AAGCACTTACGTGATTTTCTTATAGTG	
STM14_3781 - 3776 sense	TGCCTGAATTAATACCACTCTCTG ATCTGATGCCTCAATAAAATGTC ATAGTGTAGGCTGGAGCTGCTTC	To amplify *cat* gene product from pKD3 to generate ΔSTM14_3781 – 3776 strain by lambda red recombination
STM14_3781 - 3776 antisense	TACCGTGCTGTGATAGCGGTCTT AGTCAAGCGTGATGACAAGGTA ACGCGCCTCCTTAGTTCCTATTCCG	
STM14_3781 - 3776 verify F	GTGAAAGCGTGCTTTTTAACCC	To confirm deletion of STM14_3781 – 3776 from *S*. Typhimurium 14028
STM14_3781 - 3776 verify R	GCCTGGAACTGAATATCGTTCC	
STM14_4768 - 4772 sense	TATTCCGCAATGCCTGGAGTGTT TTCAGATGTTTAATTTCGATCATG AAAGTGTAGGCTGGAGCTGCTTC	To amplify *cat* gene product from pKD3 to generate ΔSTM14_4768 – 4772 strain by lambda red recombination
STM14_4768 - 4772 antisense	TACGAGACCGGAGTCGGTGTAG GCTCAACAAAGCGTCATCGCCAC GTTAGCCTCCTTAGTTCCTATTCCG	
STM14_4768 - 4772 verify F	AGAGCGGATTGGGTCTGGT	To confirm deletion of STM14_4768 – 4772 from *S*. Typhimurium 14028
STM14_4768 - 4772 verify R	GGCTAACCACAGCGCATTAC	
STM14_4889 - 4894 sense	TCATTAATTGTTCACCAGATTTG CGCTGTTAAGCGTAAAGGAATAT CAACGTGTAGGCTGGAGCTGCTTC	To amplify *cat* gene product from pKD3 to generate ΔSTM14_4889 – 4894 strain by lambda red recombination
STM14_4889 - 4894 antisense	AGAGCCTTGTCCGGCCTCCAAACG GTATTCGTTAATGCAATATCAAGCT GCCTCCTTAGTTCCTATTCCG	
STM14_4889 - 4894 verify F	AGCCTCCATAGATTGTCTGGTG	To confirm deletion of STM14_4889 – 4894 from *S*. Typhimurium 14028
STM14_4889 - 4894 verify R	TCTGGAAAATGACAGCCACCC	
STM14_1953 - 1958 sense	CGCATTAAAACAAGAAATCAAGG CGCTGTTAACACAATAAAGGGGG	To amplify *cat* gene product from pKD3 to

STM14_1953 - 1958 antisense	CATTGTGTAGGCTGGAGCTGCTTC GTAATATAAAGGTCGGGTACTAT ACCCGACCTTTTCCTGTATGATTT CCCCCTCCTTAGTTCCTATTCCG	generate ΔSTM14_1953 – 1958 strain by lambda red recombination
STM14_1953 - 1958 verify F	ACAGTGACTATGGCATCCCG	To confirm deletion of STM14_1953 – 1958 from *S*. Typhimurium 14028
STM14_1953 - 1958 verify R	CAGAAATATTGAACAAGTGAAAA CGAC	
STM14_1977 - 1981 sense	CTAATGCTTAATTTATAATACGC ATTACCTTACTACAGGAAATAGA TAGCGTGTAGGCTGGAGCTGCTTC	To amplify *cat* gene product from pKD3 to generate ΔSTM14_1977 – 1981 strain by lambda red recombination
STM14_1977 - 1981 antisense	GTCTGGCAGTGGCAGTACTGTTT ATCACTGCACTGGATAGAAAACG GTAACCTCCTTAGTTCCTATTCCG	
STM14_1977 - 1981 verify F	TTAACAGCGATGGAATACATAACGT	To confirm deletion of STM14_1977 – 1981 from *S*. Typhimurium 14028
STM14_1977 - 1981 verify R	CTGGAGGCGATATGATTGAGGT	
STM14_2472 - 2463 sense	GGCGCAGCTTTGCCAAGCATGG CTGAATCATAGACTTTTTTTACCT GTTTGTGTAGGCTGGAGCTGCTTC	To amplify *cat* gene product from pKD3 to generate ΔSTM14_2472 – 2463 strain by lambda red recombination
STM14_2472 - 2463 antisense	GCTTAGTGATTCGGCGATTTGAC CGCCACCACTGCAGAAGGAGTAG ATCCCCTCCTTAGTTCCTATTCCG	
STM14_2472 - 2463 verify F	CTGTTTACCATCTTGCATTACAGGC	To confirm deletion of STM14_2472 – 2463 from *S*. Typhimurium 14028
STM14_2472 - 2463 verify R	GATGCGAGCAGGCTCCTTC	
STM14_3208 - 3201 sense	GACGATATCAGATTTACATAAAA TATAGCCGTTTTAATCCAGTTTTG CAAGTGTAGGCTGGAGCTGCTTC	To amplify *cat* gene product from pKD3 to generate ΔSTM14_3208 – 3201 strain by lambda red recombination
STM14_3208 - 3201 antisense	AAAAGTCAGAAGATAGCTGGTA TGACATTGTAAGAAGATCTGATG GCTGCCCTCCTTAGTTCCTATTCCG	

STM14_3208 - 3201 verify F	AAAACCCCGCACGATGGC	To confirm deletion of STM14_3208 – 3201 from *S*. Typhimurium 14028
STM14_3208 - 3201 verify R	TTTTTAACCGCGTCTGTACATCG	
STM14_4847 - 4848 sense	CATTGACGTATAGATTATTTTCA CTACTATAAGCCAATGGCGTATG GAATGTGTAGGCTGGAGCTGCTTC	To amplify *cat* gene product from pKD3 to generate ΔSTM14_4847 – 4848 strain by lambda red recombination
STM14_4847 - 4848 antisense	ATAAGTTCTCTGGTATACCGGCT AGCAATCTACGTTAGCCGGATCA TTGCCCTCCTTAGTTCCTATTCCG	
STM14_4847 - 4848 verify F	GACCAGTTCGGCAATTCCTT	To confirm deletion of STM14_4847 – 4848 from *S*. Typhimurium 14028
STM14_4847 - 4848 verify R	CTGGAAAATGAGCAGGCTGC	
STM14_5331 -5341 sense	TACAAACTTTTAACCCTTACCG CTAGTTTTATCTCATTGCCATTA ACGGGGTGTAGGCTGGAGCTGCTTC	To amplify *cat* gene product from pKD3 to generate ΔSTM14_5331 – 5341 strain by lambda red recombination
STM14_5331 -5341 antisense	TTATCTGGCGCGGTAGCAGCA ACCAGGTAGTGCATCATTTGCG TTGATCGCCTCCTTAGTTCCTATTCCG	
STM14_5331 -5341 verify F	TCTCACACTTTCCCGGCGAT	To confirm deletion of STM14_5331 – 5341 from *S*. Typhimurium 14028
STM14_5331 -5341 verify R	GCTACAAGCTCGATGAACTCA	
STM14_1148 -1140 sense	GTTATAAAAATGTAGCGATGC GACTGCTAACCCCTTGAATTTA AGGATTTGTGTAGGCTGGAGCTGCTTC	To amplify *cat* gene product from pKD3 to generate ΔSTM14_1148 – 1140 strain by lambda red recombination
STM14_1148 -1140 antisense	AATCTAATAGTTCCAATGAATA AAGAAACTGAAGGTTGTAAAAT GTGAATCCTCCTTAGTTCCTATTCCG	
STM14_1148 -1140 verify F	ATTGAACCCCGATCACACCA	To confirm deletion of STM14_1148 – 1140 from *S*. Typhimurium 14028
STM14_1148 -1140 verify R	TCTCCAGTTCTACGAGTTGGT	
STM14_1149 - 1154 sense	AGTTTTACGTGATAATCTCCAG CTACCAACTCGTAGAACTGGAG	To amplify *cat* gene product from pKD3 to

	AGCCAAGTGTAGGCTGGAGCTGCTTC	generate ΔSTM14_1149 – 1154 strain by lambda red recombination
STM14_1149 - 1154 antisense	TGATTGTACCTAATAATGCTTT	
	TGCTTTCTGCTGTGGATTTAAC	
	CAGCTACCTCCTTAGTTCCTATTCCG	
STM14_1149 - 1154 verify F	TGGCGAAAATGGGATGAAGA	To confirm deletion of STM14_1149 – 1154 from *S*. Typhimurium 14028
STM14_1149 - 1154 verify R	TTGAATACAACCTTGGCTCTTTTCC	
STM14_1156 - 1162 sense	TCTAACAGGTATACTGTGTTTA	To amplify *cat* gene product from pKD3 to generate ΔSTM14_1156 – 1162 strain by lambda red recombination
	TATACAGTTGTTGAATGTAGAG	
	GGAATTGTGTAGGCTGGAGCTGCTTC	
STM14_1156 - 1162 antisense	AATCAGATTAGCCATTTTCCG	
	TTAATTTTGCAATAGCTAAAT	
	TATTTTGGCCTCCTTAGTTCCTATTCCG	
STM14_1156 - 1162 verify F	TTTTATCTGAACCCGCTGCG	To confirm deletion of STM14_1156 – 1162 from *S*. Typhimurium 14028
STM14_1156 - 1162 verify R	ATTTGACACCAAGGGAATATACCAC	
STM14_1425 - 1418 sense	AGATTCGGAATTGAAATTTT	To amplify *cat* gene product from pKD3 to generate ΔSTM14_1425 – 1418 strain by lambda red recombination
	GTGAGAACGTGGTCTAGCAGCCAT	
	CTGCAAGTGTAGGCTGGAGCTGCTTC	
STM14_1425 - 1418 antisense	GCTACCTGCTGGCGCAGAAAG	
	GAGATCCGGATGCATAACCAAAA	
	GACATGCCTCCTTAGTTCCTATTCCG	
STM14_1425 - 1418 verify F	CTGTGAAAGCGTCCGGAAAC	To confirm deletion of STM14_1425 – 1418 from *S*. Typhimurium 14028
STM14_1425 - 1418 verify R	GGTGAGGATGATTGATTTCGCA	
STM14_1434 - 1437 sense	AAATACAAACAACGCAAAG	To amplify *cat* gene product from pKD3 to generate ΔSTM14_1434 – 1437 strain by lambda red recombination
	ACGCCGGTCTGATTTAACAGGAGTC	
	CGCCCAGTGTAGGCTGGAGCTGCTTC	
STM14_1434 - 1437 antisense	TTATTATTCACACTTCATTAAT	
	GCAAGCATCTTTATGCTATGC	
	TACTAATCCTCCTTAGTTCCTATTCCG	

STM14_1434 - 1437 verify F	AAAGCGAGTTCTTGCAGGTA	To confirm deletion of STM14_1434 – 1437 from *S*. Typhimurium 14028
STM14_1434 - 1437 verify R	AACATCTATGCGTCTGTCTATTTGC	
STM14_1455 - 1461 sense	AGTTAAAAACGAAGATCGCA GATCCTTTCCTGTTTCCGGGA GACTTTTCCGTGTAGGCTGGAGCT GCTTC	To amplify *cat* gene product from pKD3 to generate ΔSTM14_1455 – 1461 strain by lambda red recombination
STM14_1455 - 1461 antisense	CCTCCCGCAATTTGTAACAAT AAAGGCCTGAATCAGGCCTTTT GGGATGCCCTCCTTAGTTCCTATTCCG	
STM14_1455 - 1461 verify F	TCCTTTCCGGGAATTCTGCC	To confirm deletion of STM14_1455 – 1461 from *S*. Typhimurium 14028
STM14_1455 - 1461 verify R	TTTTCCACAGCAGGCAAATGC	
STM14_1526 - 1528 sense	GGGCCATCGAATGGATATTCC CACATGGCTCTCGTTTTGTTGAG GTGGATGTGTAGGCTGGAGCTGCTTC	To amplify *cat* gene product from pKD3 to generate ΔSTM14_1526 – 1528 strain by lambda red recombination
STM14_1526 - 1528 antisense	TCGAGTTGCTTAAAGGCGGCA AGGGAGTGAGTCCCCAGAAGCAT AGATAACCTCCTTAGTTCCTATTCCG	
STM14_1526 - 1528 verify F	GGCGTTCCTCAACTACTACTT	To confirm deletion of STM14_1526 – 1528 from *S*. Typhimurium 14028
STM14_1526 - 1528 verify R	CTTAGAAGCCGTTTTGTTTCAGAC	
STM14_3072 - 3068 sense	GAGATCGCCGCGTAAATTGTGA GCCAGACGCCGCGCCGCGGCGT CTGTCTGTGTAGGCTGGAGCTGCTTC	To amplify *cat* gene product from pKD3 to generate ΔSTM14_3072 – 3068 strain by lambda red recombination
STM14_3072 - 3068 antisense	ACGTTGGTAATCTGATCCCAGG TGGCAGTTTATAGTTCAACCAAT TACTGCCTCCTTAGTTCCTATTCCG	
STM14_3072 - 3068 verify F	GGTTCAGCCAGAATGCGCT	To confirm deletion of STM14_3072 – 3068 from *S*. Typhimurium 14028
STM14_3072 - 3068 verify R	CCCAACCGAGCCAGGGTA	

STM14_3192 - 3186 sense	CCTCCCGCAATTTGTAACAATA AAGGCCTGAATCAGGCCTTTTG GGATGCGTGTAGGCTGGAGCTGCTTC	To amplify *cat* gene product from pKD3 to generate ΔSTM14_3192 – 3186 strain by lambda red recombination
STM14_3192 - 3186 antisense	AGTTAAAAACGAAGATCGCAG ATCCTTTCCTGTTTCCGGGAGAC TTTTCCCCTCCTTAGTTCCTATTCCG	
STM14_3192 - 3186 verify F	GCTGTATATTCTCTTCTTTTGTTGCC	To confirm deletion of STM14_3192 – 3186 from *S*. Typhimurium 14028
STM14_3192 - 3186 verify R	AAAAAGAAAGGATCTGCTGGATAACG	
STM14_3221 - 3227 sense	CGCGCAGAAGGTGATAAACGT TCCGCTGGCCGGCGATAAGGCAA ACGAGGGTGTAGGCTGGAGCTGCTTC	To amplify *cat* gene product from pKD3 to generate ΔSTM14_3221 – 3227 strain by lambda red recombination
STM14_3221 - 3227 antisense	CGAGCCCGATGTTCTGGAGT TCACGCCACGTCAGACCGGATGAG TCGACCCCTCCTTAGTTCCTATTCCG	
STM14_3221 - 3227 verify F	CCACGACCACCCAGGCAG	To confirm deletion of STM14_3221 – 3227 from *S*. Typhimurium 14028
STM14_3221 - 3227 verify R	AAACTGCCTGGAGTGTGGTC	
STM14_5063 - 5047 sense	AGGGTATTATTTTAATTCAACACC GCCGGGCTTTATACCCGGCGAGAGA TGTGTAGGCTGGAGCTGCTTC	To amplify *cat* gene product from pKD3 to generate ΔSTM14_5062 – 5047 strain by lambda red recombination
STM14_5063 - 5047 antisense	TCAGTCCATGCTTAGCATG GCTACAGAACCCACAGCAACTGTGG CAGGCACCTCCTTAGTTCCTATTCCG	
STM14_5063 - 5047 verify F	CTATGCAAACTGAACATTTAATGCTAA	To confirm deletion of STM14_5063 – 5047 from *S*. Typhimurium 14028
STM14_5063 - 5047 verify R	TGGTAACCGAAGATCTGGCG	
STM14_2432 – 2429 sense	AACATATTTTTATGGATACCAGC AGTTTGACTATCAGCTCCTGGAG ATAAGTGTAGGCTGGAGCTGCTTC	To amplify *cat* gene product from pKD3 to generate ΔSTM14_2432 – 2429 strain by lambda red recombination
STM14_2432 – 2429 antisense	GTCCTGGGGAATATAACCTG GGCCACCTATACCGGGGAGAAC	

	GGATAACTCCTCCTTAGTTCCTATTCCG	
STM14_2432 – 2429 verify F	ATTGTTCTATACGGCACAATTATGAA	To confirm deletion of STM14_2432 – 2429 from *S*. Typhimurium 14028
STM14_2432 – 2429 verify R	GTGGCACATCAGCTGCGT	
STM14_4552-4554 sense	ACGGTCTACACTTACTCTTGAAAAA GTGCAAACCGATAAGGATACCGTT TGTGTAGGCTGGAGCTGCTTC	To amplify *cat* gene product from pKD3 to generate ΔSTM14_4552 – 4554 strain by lambda red recombination
STM14_4552-4554 antisense	CATTTGTAGGCCGGATACGACGTT AGTCGTCATCCGGCAATAATAAAT GCCCTCCTTAGTTCCTATTCCG	
STM14_4552-4554 verify F	TACACTATTCTGCCACGCAAGA	To confirm deletion of STM14_4552 – 4554 from *S*. Typhimurium 14028
STM14_4552-4554 verify R	TGGATCACTCGTTCTCTTGCC	
Fun 4552 lux F	GATCCTCGAGCGTTCCAGCTCT CCCAGTAC	To amplify STM14_4552 – 4554 promoter region from *S*. Typhimurium 14028
Fun 4552 lux R	GATCGGATCCGTAGCGCGACAA GTGTGGC	
FUN 1951 Lux for	GATCCTCGAGTGCAGCGCCGGA ACATGA	To amplify STM14_1951 – 1958 promoter region from *S*. Typhimurium 14028
FUN 1951 Lux Rev	GATCGGATCCCCCGTTTAGCCGC AGTAAAG	
FUN 1953 Lux for	GATCCTCGAGCTACACAAGAGG CACGACCA	To amplify STM14_1953 – 1958 promoter region from *S*. Typhimurium 14028
FUN 1953 Lux Rev	GATCGGATCCGCCGCCGAGAGA CAGGAT	
FUN 1977 Lux for	GATCCTCGAGGAAGATCAGGAGT AAATA ACAAAAGCG	To amplify STM14_1977 – 1981 promoter region from *S*. Typhimurium 14028
FUN 1977 Lux Rev	GATCGGATCCGAAGACGACATTATT CCCAACACT	
FUN 2472 Lux For	GATCGGATCCTTCCATTCAGGT TCATGTCCCA	To amplify STM14_2472 – 2463 promoter region

FUN 2472 Lux Rev	GATCCTCGAGGTTGTCTGACAC GGTTTCCT	from *S*. Typhimurium 14028
Fun 3208 Lux For	GATCGGATCCAGCCGAGACGACGAAT AAAATGC	To amplify STM14_3208 – 3201 promoter region from *S*. Typhimurium 14028
Fun 3208 Lux Rev	GATCCTCGAGAAACGTATACAGAGCA TCCTGC	
Fun 4847 Lux For	GATCCTCGAGGCGATAAACCCA GCGACCAG	To amplify STM14_4847 – 4848 promoter region from *S*. Typhimurium 14028
Fun 4847 Lux Rev	GATCGGATCCATGGTATTCATCATCA TCGAGCA	
Fun 5331 Lux For	GATCCTCGAGGCTAAAAACCACCC GCAATACA	To amplify STM14_5331 – 5341 promoter region from *S*. Typhimurium 14028
Fun 5331 Lux Rev	GATCGGATCCACCTGTTCAGCCAGT TCGTT	
Fun 2475 Lux For	GATCCTCGAGGGCAAGGTTCT GATAAGCCAG	To amplify STM14_2475 – 2484 promoter region from *S*. Typhimurium 14028
Fun 2475 Lux Rev	GATCGGATCCTGTAAAAAGGT CGGCGTCAC	
Fun 3781 Lux For	GATCGGATCCCGCGCCGTAGT AATTCAACG	To amplify STM14_3781 – 3776 promoter region from *S*. Typhimurium 14028
Fun 3781 Lux Rev	GATCCTCGAGCTTCGGCAAGTA ATGTCACGT	
Fun 4768 Lux For	GATCCTCGAGGATGATCGCAG GCGTGAT	To amplify STM14_4768 – 4772 promoter region from *S*. Typhimurium 14028
Fun 4768 Lux Rev	GATCGGATCCTATTCCTCAAGCGCAA TTTGTTCA	
Fun 4889 Lux For	GATCCTCGAGCAGGATCATAG TTGCGCCAT	To amplify STM14_4889 – 4894 promoter region from *S*. Typhimurium 14028
Fun 4889 Lux Rev	GATCGGATCCTCGCCATATCTC CCGCGC	
Fun 1148 Lux For	GATCGGATCCTCAGCCATAGAC CAGCCGTT	To amplify STM14_1148 – 1140 promoter region from *S*. Typhimurium 14028
Fun 1148 Lux Rev	GATCCTCGAGCAGCCACTTGCCT	

	ACAGCTT	
Fun 1149 Lux For	GATCCTCGAGGAGGTCAGCCATA GACCAGC	To amplify STM14_1149 – 1154 promoter region from *S*. Typhimurium 14028
Fun 1149 Lux Rev	GATCGGATCCCCGATGCTCATTTG AGTCATGT	
Fun 1156 Lux For	GATCCTCGAGGGCTATTGATGAGCT AAAGCCA	To amplify STM14_1156 – 1162 promoter region from *S*. Typhimurium 14028
Fun 1156 Lux Rev	GATCGGATCCTCCGGAAGCTGT TTTGCCC	
Fun 1425 Lux For	GATCGGATCCCAGGTGGTAAGCG CATGTCT	To amplify STM14_1425 – 1418 promoter region from *S*. Typhimurium 14028
Fun 1425 Lux Rev	GATCCTCGAGCACTGCGCCGC CAATCTC	
Fun 1434 Lux For	GATCCTCGAGACCGCAACAAGCAA CAGACT	To amplify STM14_1434 – 1437 promoter region from *S*. Typhimurium 14028
Fun 1434 Lux Rev	GATCGGATCCGCTACTGTCTCTA TTGCTACAGC	
Fun 1455 Lux For	GATCCTCGAGGCGATACTCTTGA ATTTCATAT	To amplify STM14_1455 – 1461 promoter region from *S*. Typhimurium 14028
	TTAAGACC	
Fun 1455 Lux Rev	GATCGGATCCTTGTTCACGTTCATG GAAAAGTCTC	
Fun 1526 Lux For	GATCCTCGAGTCGTACGCTTTGT GGATTTTGC	To amplify STM14_1526 – 1528 promoter region from *S*. Typhimurium 14028
Fun 1526 Lux Rev	GATCGGATCCTTCGCTCAATAGTCT TGTATCTGTCT	
Fun 2432 Lux For	GATCGGATCCAGGTGATAAAGTAAC TGCGTATATTCG	To amplify STM14_2432 – 2429 promoter region from *S*. Typhimurium 14028
Fun 2432 Lux Rev	GATCCTCGAGGTGACGATATATC AGCGGGAC	
Fun 3072 Lux For	GATCGGATCCGGGACAACTCCAG TAACATTAATTGC	To amplify STM14_3072 – 3068 promoter region from *S*. Typhimurium 14028
Fun 3072 Lux Rev	GATCCTCGAGTCTCAATATCTCTCA	

	TTAGAAGTACGT	
Fun 3192 Lux For	GATCGGATCCATTCACGTTCATG GAAAAGTCTCC	To amplify STM14_3192 – 3186 promoter region from *S*. Typhimurium 14028
Fun 3192 Lux Rev	GATCCTCGAGTTTTTACCAGTTGC AAAGCCAGC	
Fun 3221 Lux For	GATCCTCGAGGCGAGTACATTTTA TAGATCCATGGC	To amplify STM14_3221 – 3227 promoter region from *S*. Typhimurium 14028
Fun 3221 Lux Rev	GATCGGATCCTCGAGCTGGTTT ACGTGCG	
Fun 5063 Lux For	GATCGGATCCGCGGAAAGCGAA ACCACCTC	To amplify STM14_5068 – 5047 promoter region from *S*. Typhimurium 14028
Fun 5063 Lux Rev	GATCCTCGAGTGGCGCGGATCAA TTAAATGC	
pZE05	CCAGCTGGCAATTCCGA	Used to verify promoter fusion to *luxCDABE*
pZE06	AATCATCACTTTCGGGAA	
CAT01	CAGGGTCGTTAAATAGCCGC	CAT01 is used with ARB1 or ARB6 for nested PCR (first reaction) to identify the site of Tn10Cam insertion
ARB1	GGCCACGCGTCGANNNNNNNNGATAT	
ARB6	GGCCACGCGTCGANNNNNNNNACGCC	
CAT02	CCGTGTGCTTCTCAAATGCC	Used for nested PCR (second reaction) to identify the site of Tn10Cam insertion
ARB2	GGCCACGCGTCGACTAGTAC	

[a] Nucleotide sequences corresponding to restriction enzyme sites are underlined.