Role of *Salmonella enterica* subspecies *enterica* serovar Enteritidis pathogenicity island-2 in chickens

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By

Amanda Lynn Stacy Wisner

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ABSTRACT

Salmonella enterica subspecies *enterica* serovar Enteritidis (*S.* Enteritidis) has been identified as a significant cause of salmonellosis in humans. *Salmonella* pathogenicity islands 1 and 2 (SPI-1 and SPI-2) each encode a specialized type III secretion system (T3SS) that enables *Salmonella* to manipulate host cells at various stages of the invasion/infection process. The SPI-2 T3SS has been identified as vital for survival and replication of *S.* Typhimurium and *S.* Enteritidis in mouse macrophages, as well as full virulence in mice. In order to test the ability of SE SPI-2 mutants to survive *in vivo* we used a chicken isolate of SE (Sal18). In one study, we orally co-challenged 35-day-old specific pathogen free (SPF) chickens with two bacterial strains per group. The control group received two versions of the wild-type (WT) strain Sal18: Sal18 *att*Tn7::*tet* and Sal18 *att*Tn7::*cat*, while the other two groups received the WT strain (Sal18 *att*Tn7::*tet*) and one of two mutant strains (Sal18 *att*Tn7::*cat* Δ*spaS*Δ*ssaU* or Sal18 ΔSPI-1ΔSPI-2::*cat*). From this study we conclude that *S.* Enteritidis deficient in the SPI-1 and SPI-2 systems are out-competed by the WT strain. In a second study, groups of SPF chickens were challenged at 1 week of age with four different strains; a WT strain and three other strains missing either one or both of the SPI-1 and SPI-2 regions. On days 1 and 2 post-challenge (PC) we observed a reduced systemic spread of the SPI-2 mutants, but by day 3 the mutants' systemic distribution levels matched that of the WT strain. Based on these two studies, we conclude that the SPI-2 T3SS facilitates invasion and systemic spread of *S.* Enteritidis in chickens, but alternative mechanisms for these processes appear to exist.

Several structural components of the T3SSs encoded by SPI-1 and SPI-2 are exposed to the host's immune system prior to/during the infection/invasion process, making them potential vaccine candidates. Several of these candidates genes were cloned, the proteins overproduced, purified, and formulated as vaccines for use in further studies. SPI-2 T3SS proteins used for vaccine studies included the secretin, SsaC, the needle, SsaG, the filament, SseB, and a part of the translocon, SseD, as well as a number of effectors, SseI, SseL, SifA, and SifB. The first vaccine study involved vaccination of SPF chickens with SseB and SseD, followed by challenge with the WT *S.* Enteritidis strain Sal18. Additional studies evaluated whether hens vaccinated with SPI-2 T3SS structural or effector components could mount a significant humoral immune response (as measured by serum immunoglobulin Y [IgY] titres), whether these antibodies could

be transferred to progeny (as measured by egg yolk IgY titres), and whether vaccinates and progeny of vaccinates could be protected against challenge with the WT *S.* Enteritidis strain Sal8. The results of our studies show that vaccinated chickens do produce high levels of SPI-2 T3SS specific serum IgY that they are able to transfer to their progeny. It was demonstrated that vaccinates and progeny of vaccinates had lower overall countable recovered SE per bird in most situations.

In order to better identify the role of the SPI-2 T3SS in chickens, we used the well-known gentamicin protection assay with activated HD11 cells. HD11 cells are a macrophage-like chicken cell line that can be stimulated with phorbol 12-myristate 13-acetate (PMA) to exhibit more macrophage-like morphology and greater production of reactive oxygen species (ROS). Activated HD11 cells were infected with a WT *S.* Typhimurium strain, a SPI-2 mutant *S.* Typhimurium strain, a WT *S.* Enteritidis strain, a SPI-2 mutant *S.* Enteritidis strain, or a non-pathogenic *Escherichia coli* (*E. coli*) strain. SPI-2 mutant strains were found to survive as well as their parent strain at all time points post-infection (PI) up to 24 h PI, while the *E. coli* strain was no longer recoverable by 3 h PI. We can conclude from these observations that the SPI-2 T3SS is not important for survival of *Salmonella* in the activated macrophage-like HD11 cell line, and that *Salmonella* must employ other mechanisms for survival in this environment as *E. coli* is effectively eliminated.

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DEDICATION

For my mom,
Valerie Joyce Murray.

Thanks :)

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ABBREVIATIONS

Å	Angström
AI-3	Auto Inducer 3
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphatase
BBS-TB	Borate buffered saline with Tween-20 and BSA
BCIP	5-Bromo-4-chro-3-indolyl phosphate
BG	Brilliant Green
BFP or bfp	Bundle forming pilus
bp	Base pairs
BSA	Bovine serum albumin
c.	Centisome
Caco-2	Human intestinal epithelial cell line
Caspase	Cysteine dependent aspartate specific protease
CDC	Center for Disease Control
CFIA	Canadian Food Inspection Agency
CFU	Colony forming unit
CXC3CR1	CX3 chemokine receptor 1
ddH_2O	Double distilled water
DNA	Deoxyribonucleic acid
E. coli	*Escherichia coli*
EE-1	Early endosomal marker 1
ELISA	Enzyme-linked immunosorbant assay
ETEC	Enterotoxigenic *Escherichia coli*
EU	European Union
FBS	Fetal bovine serum
FIM or fim	Fimbriae
Fis	Factor for inversion stimulation
GALT	Gut-associated lymphoid tissue

H-NS	Histone-like nucleoid structuring
HACCP	Hazard analysis of critical control points
HD11	Chicken immortal macrophage like cell line
Hha	Hemolysin expression-modulating protein
HRP	Horseradish peroxidase
IFNγ	Interferon gamma
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IgY	Immunoglobulin Y
IHF	Integration host factor
IκB	Inhibitor of kappa B
IL	Interleukin
IM	Inner membrane
iNOS	Inducible nitric oxide synthase
Ipaf	ICE protease-activating factor
IPTG	Isopropyl-β-D-thiogalactopyranoside
IRAK-4	Interleukin receptor associated kinase 4
kb	Kilobases
kDa	Kilodaltons
LAMP-1	Lysosomal-associated membrane protein
LB	Luria-Bertani
LEE	Locus of enterocyte effacement
lgps	Lysosomal glycoproteins
lpf or LPF	Long polar fimbriae
LPM	Low phosphate medium
LPS	Lipopolysaccharide
M cell	Microfold cell
MAP	Mitogen activated protein
MAPK	Mitogen activated protein kinase
Mbp	Megabases

MES	2-(N-morpholino)ethanesulfonic acid
MHC	Major histocompatibility complex
MLN	Mesenteric lymph nodes
MOI	Multiplicity of infection
MOS	Mannan oligosaccharides
mRNA	Messenger RNA
MyD88	Myeloid differentiation primary response gene 88
NADPH	Nicotinamide adenine dinucleotide phosphate
NAP	Nucleoid-associated protein
NBT	Nitroblue tetrazolium
NEB	New England Biolabs
NF-κB	Nuclear factor kappa B
NK	Natural killer
NLR	Nucleotide-binding oligomerization domain-like receptors
NO	Nitric oxide
OD	Optical density
OL	Oligo
OM	Outer membrane
ORF	Open reading frame
OS	Oxygen species
PAMP	Pathogen associated molecular pattern
PBS	Phosphate buffered saline
PBSA	Phosphate buffered saline A
PBI	Plant Biotechnology Institute
PC	Post-challenge
PCR	Polymerase chain reaction
PEF or pef	Plasmid encoded fimbriae
PG	Peptidoglycan
phox	NADPH phagocytic oxidase
PI	Post-infection
PMA	Phorbol 12-myristate 13-acetate

PMN	Polymorphonuclear leukocytes
PNPP	Diethanolamine phosphate
PP	Peyer's patch
PRR	Pattern recognition receptors
PT4	Phage type 4
Raji B	Human B lymphocyte cell line
ROS	Reactive oxygen species
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
RTX	Repeats in toxin
S. Choleraesuis	*Salmonella enterica* subspecies *enterica* serovar Choleraesuis
SCV	*Salmonella* containing vesicle
S. Dublin	*Salmonella enterica* subspecies *enterica* serovar Dublin
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
S. Enteritidis	*Salmonella enterica* subspecies *enterica* serovar Enteritidis
SEF or sef	*Salmonella* Enteritidis fimbriae
S. Gallinarum	*Salmonella enterica* subspecies *enterica* serovar Gallinarum
Sif	*Salmonella* induced filament
S. Pullorum	*Salmonella enterica* subspecies *enterica* serovar Pullorum
SPF	Specific pathogen free
SPI	*Salmonella* pathogenicity island
S. Paratyphi	*Salmonella enterica* subspecies *enterica* serovar Paratyphi
spv	*Salmonella* virulence plasmid
S. Typhimurium	*Salmonella enterica* subspecies *enterica* serovar Typhimurium
S. Typhi	*Salmonella enterica* subspecies *enterica* serovar Typhi
T1SS	Type I secretion system
T2SS	Type 2 secretion system
T3S	Type III secretion
T3SS	Type III secretion system
T4SS	Type IV secretion system

T5SS	Type V secretion system
T6SS	Type VI secretion system
Tafi	Thin aggregative fimbriae
TBS	Tris buffered saline
TCA	Trichloro-acetic acid
TGFβ	Tumour growth factor beta
TLR	Toll-like receptor
TNFα	Tumour necrosis factor alpha
TRIP6	Thyroid receptor-interacting protein 6
Trx1	Thioredoxin-1
TSLP	Thymic stromal lymphopoeitin
UCAC	University Council on Animal Care
UK	United Kingdom
US or USA	United States of America
USDA	United States Department of Agriculture
UPEC	Uropathogenic *Escherichia coli*
VIDO	Vaccine and Infectious Disease Organization
VSA	VIDO special antigen (Emulsigen D)
WHO	World Health Organization
WT	Wild-type

1.0 LITERATURE REVIEW

1.1 *Salmonella*

Salmonellae are motile, facultatively anaerobic, Gram-negative rods measuring 0.3-1.5 by 1.0-2.5 μm in size. The genus Salmonella was named for Dr. Daniel Salmon, a veterinary bacteriologist at the United States Department of Agriculture (USDA) [7, 8]. The *Salmonella* species are closely related to *Escherichia*, *Yersinia*, and *Shigella*, and contain a circular chromosome approximately 4.7 Mbp in size with an overall GC content of 52% [6, 7, 9].

1.1.1 Nomenclature

The genus *Salmonella* lies within the kingdom Eubacteria, class Gammaproteobacteria, order Enterobacteriales, and family Enterobacteriaceae. *Salmonella* is divided into two species, *Salmonella bongori* and *Salmonella enterica*. Within *Salmonella enterica* there are 6 subspecies: *salamae*, *arizonae*, *diarizonae, houtenae, indica* and *enterica* [10]. These subspecies can be further classified into approximately 50 serogroups based on their lipopolysaccharide (LPS) O antigen component [11]. *Salmonella bongori* and most subspecies of *Salmonella enterica* colonize the environment and cold-blooded animals, and in some cases can cause disease in these animals. The exception is *Salmonella enterica* subspecies *enterica*, which finds its niche in warm-blooded animals [12]. *Salmonella enterica* subspecies *enterica* can be further divided into over 2500 serovars based on their flagellar (H) antigen and LPS O antigen structures [10-13]. Many of these serovars are host-adapted, although some are non-host-adapted. Host-adapted serovars tend to cause life-threatening systemic disease in their host, while non-host-adapted strains tend to cause gastroenteritis in many different host species. For the purposes of this document, serovars within *Salmonella enterica* subspecies *enterica* will be identified by an italicized S followed by a period and the serovar name. For example, *Salmonella enterica* subspecies *enterica* serovar Enteritidis will be referred to simply as *S.* Enteritidis. Of note, serovars Typhi and Paratyphi (*S.* Typhi and *S.* Paratyphi) cause systemic disease in humans and some primates, while serovars Gallinarum and Pullorum (*S.* Gallinarum and *S.* Pullorum) produce systemic disease in chickens. Serovar Dublin (*S.* Dublin) causes systemic disease in cattle, and Choleraesuis (*S.* Choleraesuis) in pigs. Serovars Typhimurium (*S.* Typhimurium) and Enteritidis (*S.* Enteritidis) are non-host-adapted and are able to cause different disease outcome in

various host species [14-18]. *S.* Typhimurium and *S.* Enteritidis will be discussed further in section 1.1.2, and specific disease outcomes of *S.* Typhimurium and *S.* Enteritidis in various hosts will be discussed in section 1.4.4 Worldwide distributions of common *Salmonella* isolates from humans and animals are depicted in Figures 1.1 and 1.2, respectively.

1.1.2 *Salmonella enterica* subspecies *enterica* serovars Typhimurium and Enteritidis

Salmonella enterica subspecies *enterica* serovars Typhimurium and Enteritidis (*S.* Typhimurium and *S.* Enteritidis) are non-host-adapted strains, and can cause disease in a wide variety of host species. They are able to induce a systemic infection in mice, young calves, chicks, and piglets. However, they are also able to colonize poultry and adult cattle without symptoms [14-18]. In humans, infection with either of these serovars results in a self-limiting gastroenteritis (salmonellosis) involving fever, diarrhea, and abdominal pain. In rare cases, typically in the very young or immunocompromised, the infection can become systemic and lead to hospitalization and even death. A very small proportion of humans with salmonellosis can develop reactive arthritis (previously referred to as Reiter's syndrome), which is initially characterized by joint pain, eye irritation, and pain during urination [18-20].

1.1.3 Human disease, animal reservoirs, and modes of transmission

Infections by *S. enterica* are one of the most common causes of bacterial food-borne gastroenteritis (food poisoning) in the world, along with *E. coli* and *Campylobacter* infections [21]. Of the *S. enterica* serovars, *S.* Enteritidis and *S.* Typhimurium are the leading cause of salmonellosis in humans in most countries (Figure 1.1). *S.* Enteritidis and *S.* Typhimurium are passed to humans via consumption of contaminated poultry meat, water and eggs. *S.* Enteritidis is more often associated with salmonellosis acquired from eggs, as it has a greater tendency to colonize eggs and reproductive organs of poultry than *S.* Typhimurium [22]. Because chickens mostly do not show symptoms of disease, entire flocks can become colonized quite quickly and shed bacteria in their feces for extended periods of time [23-26]. Loss of consumer confidence in products because of *Salmonella* contamination can result in substantial economic loss to the poultry industry. Additionally, human cases of salmonellosis place a significant burden on the health care system [18]. There are approximately 1.4 million cases of salmonellosis per year resulting in about 15,000 hospitalizations and 400 deaths per year in the United States of America

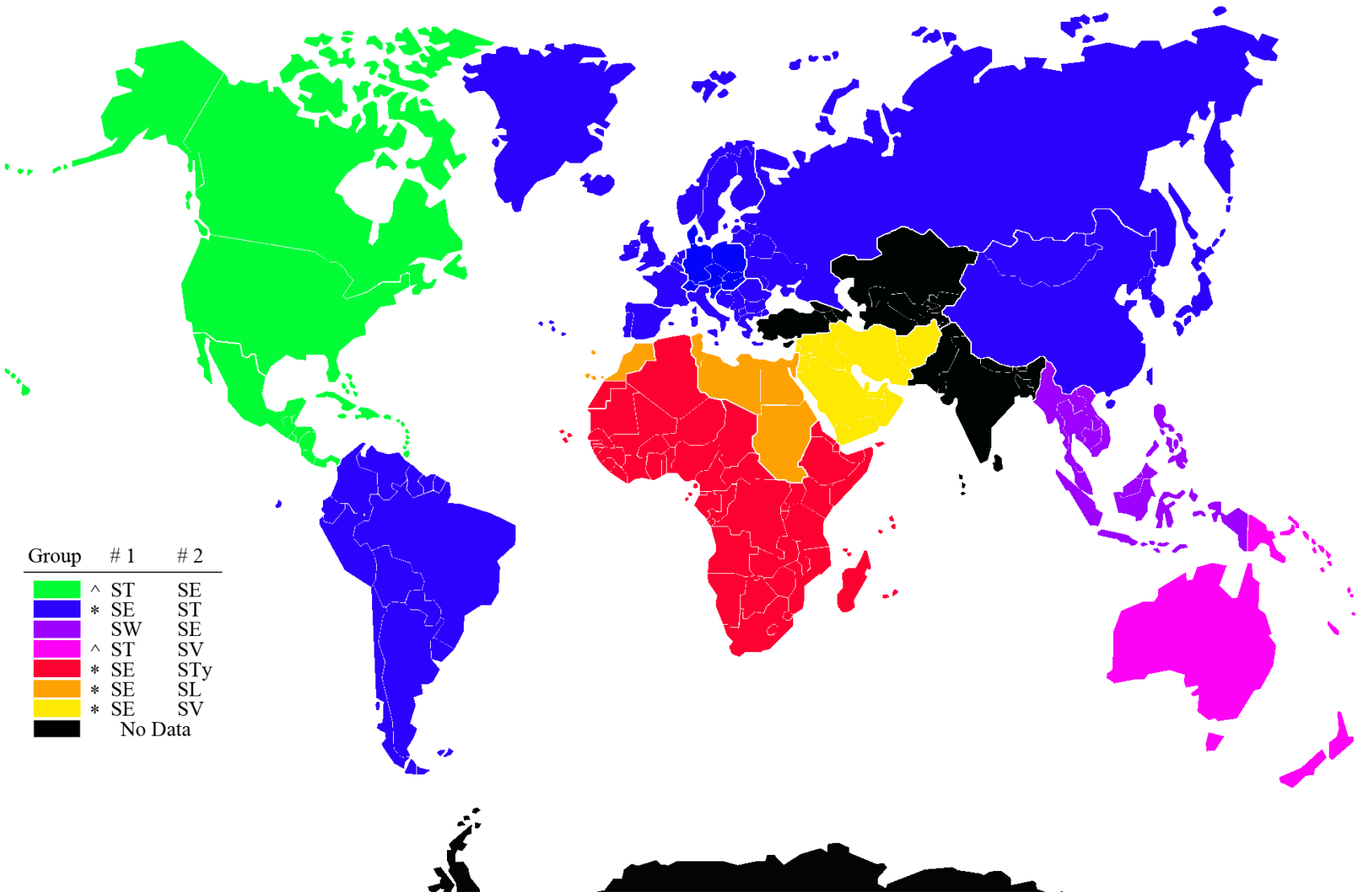

Figure 1.1 Human isolates of *Salmonella*

Figure 1.1 Human isolates of *Salmonella* (previous page)

Distributions of the first and second most common human isolates of *Salmonella* are shown here. Regions that have the same top two isolates are similarly coloured. All strains are isolates of *Salmonella enterica* subspecies *enterica*, and are represented by and S followed by the first letters of its serovar name. Serovars shown are: Enteritidis (SE – marked by * when found as most common isolate), Typhimurium (ST – marked by ^ where found as most common isolate), Weltevreden (SW), Virchow (SV), Typhi (STy), Livingston (SL). Adapted from [27].

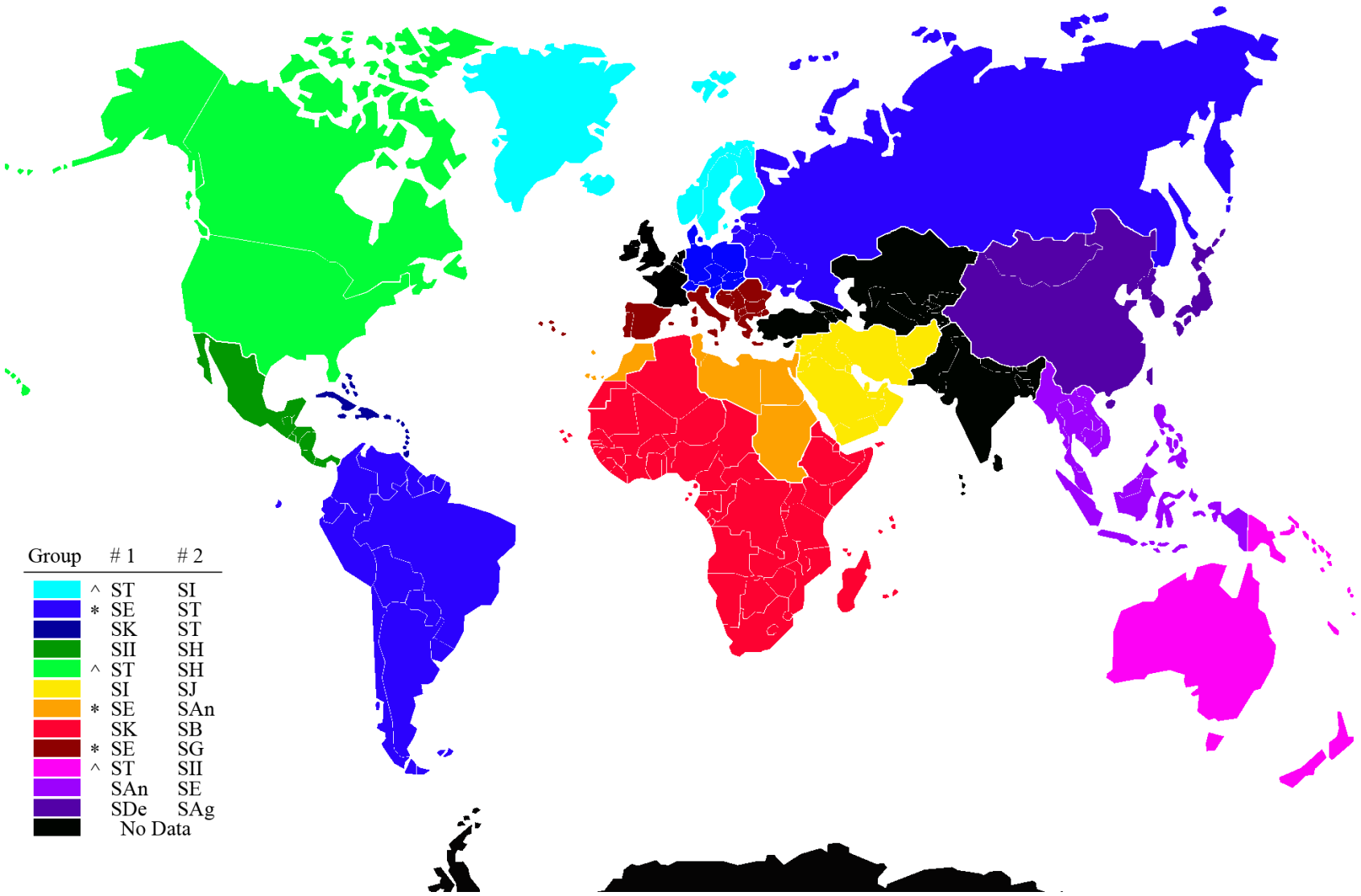

Figure 1.2 Animal isolates of *Salmonella*

Figure 1.2 Animal isolates of *Salmonella* (previous page)

Distributions of the first and second most common animal isolates of *Salmonella* are shown here. Regions that have the same #1 and #2 isolates are similarly coloured. All strains are isolates of *Salmonella enterica* subspecies *enterica*, and are represented by and S followed by the first letters of its serovar name. Serovars shown are: Enteritidis (SE – marked by * when found as most common isolate), Typhimurium (ST – marked by ^ where found as most common isolate), Kentucky (SK), II 1,4,12,.27 (SII), Infantis (SI), Anatum (SAn), Derby (SDe), Heidelberg (SH), Jedburgh (SJ), Bredeney (SB), Gallinarum (SG), Agona (SAg). Adapted from [27].

(USA) [28]. Around 95% of these cases are caused by consumption of contaminated food products, and *S.* Enteritidis is responsible for at least 15% of these cases. *S.* Enteritidis is the second most commonly isolated serovar in North America after *S.* Typhimurium, while *S.* Enteritidis is number one in the European Union (EU) (Figures 1.1 and 1.2) [15, 19, 27, 29, 30].

1.2 *Salmonella* virulence factors

1.2.1 Flagella

Flagella are complex motility structures found in members of Prokarya, Archaea, and Eukarya [31]. The presence of flagella has been associated with virulence in many pathogens, including *Salmonella,* which usually expresses between five and ten flagella at random on the cell surface [32, 33]. However, there is conflicting evidence for the contribution of flagella to virulence in *S.* Enteritidis. Flagellar mutants have been shown to be less proficient in colonizing eggs than WT *S.* Enteritidis [34]. In 20-day-old chickens, Parker *et al.* observed that removal of the gene encoding FlhD (important for transcription of flagellar genes) caused enhanced invasiveness in these chickens when challenged orally [33]. Other studies have shown that *S.* Enteritidis strains with deletions in major flagellar genes had decreased adherence to chicken intestinal explants and human intestinal epithelial cell lines, suggesting that flagella are important in adherence of *S.* Enteritidis to intestinal epithelial cells prior to invasion [35, 36]. Allen-Vercoe *et al.* [37] also demonstrated that strains defective for production of flagella were recovered at lower numbers from the spleens and livers of 1-day-old orally challenged chicks than the WT strain, implicating a role for flagella in invasion. This group also showed that flagellar mutants performed similarly to the WT strain in colonization of the ceca of 1- and 5-day-old chickens following oral challenge. However, when mutant strains were given in conjunction with WT *S.* Enteritidis in a competition experiment, there was much greater shedding of the WT strain than the mutants, suggesting that flagella do provide a competitive survival advantage [38]. The structure of the flagellar system of *Salmonella enterica* resembles that of the T3SS; similarities and differences between the flagellar and T3S systems will be discussed further in section 1.3.

1.2.2 Fimbriae

Fimbriae, or pili, are typically 2-8 nm in width and extend 0.5-10 μm from the cell

surface. Fimbriae play an important role in many bacteria, including biofilm formation and the persistence of bacteria in the environment, as well as contribute to colonization and invasion of the host. Many fimbriae are conserved between the *Salmonella* serovars, while some are unique. As each fimbria is typically specific to a given receptor, the differences in fimbrial distribution among serovars may contribute to host specificity. There are many known and predicted fimbrial operons in *S.* Enteritidis [11, 32, 39]. Fimbriae are composed of fimbrin proteins arranged in a helical fashion and can be divided into three classes based on their method of assembly. Fimbriae of the type IV class are assembled via a type IV secretion system (T4SS). The only known type IV pilus in *S.* Enteritidis is encoded by the *bfp* (bundle forming pilus) operon and may be important in the formation of bacterial colonies on the intestinal epithelium prior to entry [32, 40]. Fimbrins belonging to the chaperone-usher class are bound by chaperones in the periplasm to prevent aggregation and ushered to the outer membrane where the fimbria is formed, while those belonging to the nucleator-dependent pathway are assembled extracellularly [32, 39].

There are many known operons encoding fimbriae of the chaperone-usher class in *S.* Enteritidis: *bcf*, *fim*, *lpf*, *pef*, *saf*, *sef*, *stb*, *std*, *ste*, *peg*, *sti*, *stf* and *sth* [6, 32]. The fimbrial usher BcfC is important in colonization in cattle and *S.* Enteritidis missing *bcfC* have a decreased ability to survive in chicken egg albumen [6, 23]. The fimbria encoded by the *fim* operon (SEF21) has been shown to be important for invasion of human and mouse intestinal epithelial cells in both *S.* Enteritidis and *S.* Typhimurium [36, 41]. In *S.* Typhimurium, SEF21 is important for cecal colonization of chicks, but not cattle [42]. The presence of SEF21 has also been shown to be important for *S.* Enteritidis colonization of eggshells, while the absence of SEF21 resulted in prolonged bacteremia, ovary colonization, and cecal shedding in chickens [43]. SEF14, encoded by the *sef* (*Salmonella* Enteritidis fimbriae) operon on *Salmonella* pathogenicity island (SPI) 10 (described in section 1.2.3.3), was not found to be involved in the invasion of human intestinal epithelial cells, and is only expressed by *S.* Enteritidis and poultry-associated serovars such as *S.* Dublin, *S.* Gallinarum, and *S.* Pullorum [32, 36]. The fact that SEF14 is only expressed by poultry-associated *Salmonella* serovars indicates that it may have some function in *Salmonella* survival and/or pathogenesis in poultry. However, *S.* Enteritidis strains defective for SEF14 were just as adherent to chicken intestinal explants as the WT strain, and were recovered from liver and spleen of 1-day-old orally challenged chickens in similar numbers to the WT strain. The strains missing SEF14 were shown to colonize the cecum of 1- to 5-day old chickens

at similar levels to the WT strain, so the role of SEF14 in poultry remains unclear. The same results were observed for strains with defects in SEF21, plasmid encoded fimbriae (PEF, encoded by the *pef* operon) and long polar fimbriae (LPF, encoded by the *lpf* operon) [35-38]. The PEF of *S.* Typhimurium has been found to contribute to adhesion of bacteria to the small intestine of mice, but not human intestinal cell lines [32]. In accordance with these observations, Rajashekara *et al.* [44] observed similar results when testing SEF14 and SEF21 mutants in comparison to a WT *S.* Enteritidis strain, using two human intestinal epithelial cell lines, two chicken macrophage cell lines, and cecal colonization of 5-day-old chicks. In their studies SEF14 and SEF21 mutants performed comparably to the WT strain in all situations both *in vitro* and *in vivo*. A study by Clayton *et al.* [45] showed no difference in cecal colonization by fimbrial *std*, *sti*, *stf*, *stf*, *sth*, *bcf*, *lpf*, *sef*, *fim*, *saf*, or *ste* mutants compared to WT *S.* Enteritidis in orally infected 18-day-old chickens. They did, however, observe a reduction in cecal colonization by fimbrial *peg* and *stb* mutants, in comparison with WT *S.* Enteritidis. As in *S.* Enteritidis, *stb* of *S.* Typhimurium has been shown to be important for cecal colonization in chicks, as was *sth*, which is contrary to what is observed with *S.* Enteritidis [42].

There is only one nucleator-dependent type fimbriae expressed in *S.* Enteritidis, known as SEF17; previously known as thin aggregative fimbriae (tafi). SEF17, which is encoded by the *agf* operons, is homologous to curli of *E. coli* (encoded by the *csg* operons), and is essential for biofilm formation and persistence of *Salmonella* in the environment [32, 39, 46]. Thin aggregative fimbriae of *S.* Typhimurium have been shown to be essential in the induction of a pro-inflammatory response in a bovine model, to enhance adherence and invasion of *S.* Enteritidis to human intestinal epithelial cells *in vitro,* and contribute to *S.* Enteritidis colonization of eggs [34, 36, 47]. However, results from a study by Rajashekara *et al.* indicated no difference between SEF17.

mutants and WT *S.* Enteritidis in regards to invasion of human intestinal epithelial cell lines, phagocytosis by chicken macrophage cell lines, or colonization of chicken ceca [44]. As with SEF14, SEF21, PEF, and LPF; SEF17 mutants did not differ in adherence to chicken intestinal epithelial explants or recovery from liver and spleen of 1-day-old chickens [35, 37]. Furthermore, Clayton *et al.* did not observe a difference in SEF17 mutants compared to WT *S.* Enteritidis in cecal colonization of orally infected 18-day-old chickens [45]. *Salmonella* have developed a diverse set of fimbrial genes that vary from serovar to serovar. Fimbriae contribute

to environmental survival and persistence, as well as virulence, and harbouring a unique set of fimbrial operons likely contributes to host specificity among different serovars.

1.2.3 *Salmonella* pathogenicity islands

Pathogenicity islands were first identified in uropathogenic *E. coli* (UPEC) in the late 1980s, and have since been described in a wide variety of bacteria [48-50]. Pathogenicity islands have been identified in both Gram-negative and Gram-positive species, and are associated with plant, animal, and human pathogens, as well as non-pathogenic bacteria. They typically harbour large clusters of genes (10 – 200 kb) related to virulence and/or survival and fitness, and have a different GC content in comparison to the rest of the genome. Pathogenicity islands can often be mosaic in structure and are often bordered by transposon insertion sequences and direct repeats, as well as bacteriophage genes, indicating their insertion into the genome through single or multiple horizontal gene transfer events [50, 51]. To date there have been 21 *Salmonella* pathogenicity islands (SPIs) identified. As expected, SPIs are often bordered by transposon insertion sequences or bacteriophage genes, and tend to have a different GC content in comparison to the rest of the genome. SPIs also often contain clusters of genes that are associated with virulence [32, 52].

1.2.3.1 *Salmonella* pathogenicity island 1

In *S.* Enteritidis, SPI-1 is located at the end of centisome (c.) 62. It is 40.2 kb in length and has a GC content of 47% [9, 32]. There are 41 genes encoding a T3SS, T3SS regulatory genes, T3SS effectors, and a metal transport system encoded on SPI-1 (Figure 1.3) [6, 50, 53]. SPI-1 is known to be important for cell invasion of intestinal epithelial cells as well as apoptosis of macrophages [54-56]. In *S.* Typhimurium, strains defective for InvC (a major structural component of the SPI-1 T3SS) have a 50% higher lethal dose when given orally to Balb/c mice, but perform similarly to WT strains when given intraperitoneally, indicating a role for SPI-1 in colonization and invasion during the initial phase of infection, but not during the systemic phase [57]. SPI-1 will be discussed further in section 1.3.2.

1.2.3.2 *Salmonella* pathogenicity island 2

In *S.* Enteritidis, SPI-2 is located at the end of c. 37. At 39.8 kb in length it is similar in

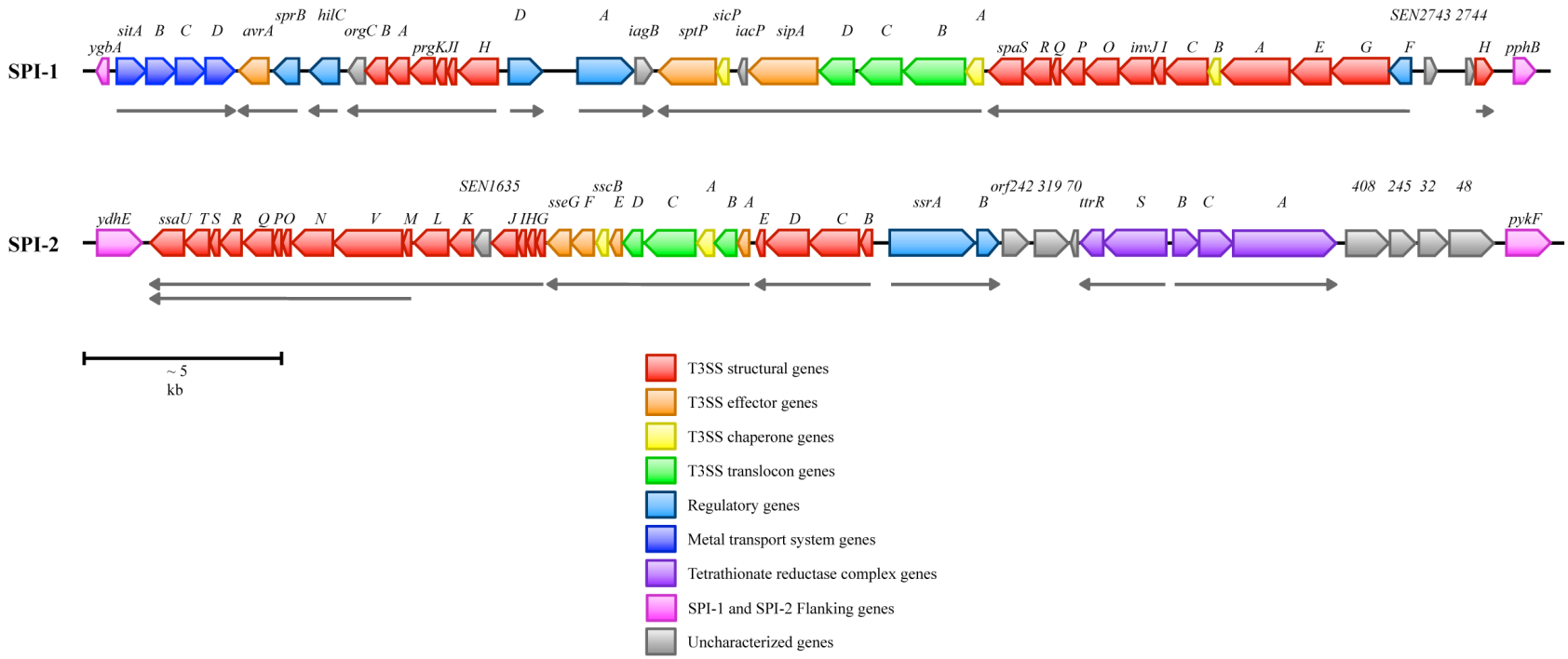

Figure 1.3 Genetic organization of *Salmonella* pathogenicity islands 1 and 2

The organization of the ~40kb regions of the *S.* Enteritidis chromosome harbouring SPI-1 and SPI-2 is shown above. Gray arrows represent known or predicted transcriptional units and genes are coloured based on the function of the encoded protein [1-5]. Genetic organization based on the *S.* Enteritidis PT4 (phage type 4) sequence provided by Wellcome Trust Sanger Institute (UK), GenBank accession number AM933172 [6].

size to SPI-1, with a GC content of 43%. It is thought that SPI-2 is important for survival within the *Salmonella* containing vacuole (SCV) and the systemic phase of infection, and is known to be necessary for systemic infection in mice [58-61]. There are 44 genes encoded on SPI-2 including a T3SS, T3SS regulatory genes, T3SS effectors, and a tetrathionate reductase system (Figure 1.3) [6, 50]. SPI-2 will be discussed further in section 1.3.3.

1.2.3.3 Other *Salmonella* pathogenicity islands

To date, there have been 21 SPIs described in *Salmonella enterica* subspecies *enterica.* SPIs 1-5 have been fairly well characterized and are present in most serovars, while the majority of the others have only been characterized in *S.* Typhimurium and *S.* Typhi. The distribution and composition of these other SPIs also varies among different serovars [6, 11, 60]. Following are brief descriptions of the 21 known SPIs, in order from SPI-3 to SPI-21. For a complete list of these SPIs and their locations in the *S.* Enteritidis PT4 genome see Table 1.1.

There are 10 genes contained on SPI-3, including *mgtCB, misL, rmbA* and *slsA*. The whole of SPI-3 is necessary for *S.* Typhimurium virulence in mice, while different genes within SPI-3 are important for *S.* Typhimurium infection of calves and chicks [42, 50, 62]. MgtC and B are involved in magnesium (Mg^{2+}) transport and are required for growth in low Mg^{2+} environments such as occurs in the SCV. MisL is a large autotransporter protein, also known as a type V secretion system (T5SS), that has been shown to be important for infection of chicks, but not calves, by *S.* Typhimurium. RmbA is a putative cytoplasmic protein that is important for *S.* Typhimurium colonization of calves, but not chicks, and SlsA is a putative inner membrane protein that is important for *S.* Typhimurium colonization of both calves and chicks [42]. SPI-4 encodes both a large non-fimbrial adhesion protein (SiiE) encoded by *siiE*, and it's type I secretion system (T1SS), encoded by *siiC, D* and *F*. SPI-4 is co-regulated with SPI-1, and, along with SPI-1, is important for *S.* Typhimurium to be able to facilitate uptake by polarized human epithelial cell *in vitro* by inducing membrane ruffles. It has also been shown to be important for *S.* Typhimurium infection of calves, but not chicks [11, 42, 63]. SPI-5 harbours the genes for SopB (a SPI-1 T3SS secreted protein), PipA and PipB (SPI-2 T3SS secreted proteins), PipC (SopB's chaperone), and PipD [9, 32]. SPI-5 has been shown to be important for *S.* Dublin infection and enteritis in cattle, but not systemic infection in mice [64]. SPI-5 is also not required for *S.* Typhimurium infection and disease in cattle. However, mutations in *pipB* decrease

Table 1.1 ***Salmonella*** **pathogenicity islands**

SPI	Centisome	Size (kb)	Function	Reference
SPI-1	62/63	40.2	Invasion of the intestinal epithelium 47% GC	[6, 9, 32]
SPI-2	37/38	39.8	Systemic infection of mice, survival in intestinal epithelial cells and macrophages 43% GC	[6, 9, 32]
SPI-3	82/83	16.6	MgtC and B Mg^{2+} transporter, MisL T5SS Implicated in intramacrophage survival Certain components important *S.* Typhimurium infection of mice, calves and/or chicks 47.5% GC	[6, 32, 42, 50, 62]
SPI-4	93/94	25.0	T1SS (*siiCDF*) and large non-fimbrial adhesin SiiE Co-regulated with SPI-1 Important for induction of membrane ruffling and entry of polarized epithelial cells in conjunction with the SPI-1 T3SS Implicated in *S.* Typhimurium infection of calves	[6, 11, 42, 63]
SPI-5	23	6.6	SPI-1 T3SS effector SopB and its chaperone PipC SPI-2 T3SS effectors PipA and PipB PipD Important for *S.* Dublin (but not *S.* Typhimurium) induced enteritis in cattle Important for *S.* Typhimurium systemic infection in chicks	[6, 9, 32, 42, 64, 65]
SPI-6	7/8	17.6	The *saf* fimbrial operon of chaperone usher class A T6SS and *tcf* fimbrial operon that are absent in *S.* Enteritidis Up to 44 kb in other serovars	[6, 11, 32, 66]
SPI-7	Absent	Up to 134	Vi capsule biosynthetic genes A type IV fimbrial operon SopE in *S.* Typhi Only present in *S.* Typhi, *S.* Paratyphi and some strains of *S.* Dublin Largest PI identified so far, varies in size between serovars	[6, 11, 32, 67]
SPI-8	Absent	6 - 8	Resistance to bacteriocins Also absent in *S.* Typhimurium	[6, 11, 32]
SPI-9	60	16.3	T1SS, and a RTX-like protein The RTX protein is complete in *S.* Enteritidis, but not *S.* Typhimurium	[6, 32]

SPI-10	98	10.0	*Sef* fimbrial operon (chaperone-usher class) in *S.* Enteritidis Larger in other serovars (up to 33 kb)	[6, 11, 32]
SPI-11	41	6.7	PagC, PagD and MsgA important for survival of *S.* Typhimurium in macrophages	[6, 11]
SPI-12	50	5.8	SPI-2 T3SS effector sspH2 Important for full virulence of *S.* Typhimurium in mice	[6, 11, 68]
SPI-13	68/69	25.3	Important for systemic infection in mice by *S.* Typhimurium	[6, 68, 69]
SPI-14	19/20	6.8	Electron transfer and putative regulatory genes	[6, 11]
SPI-15	Absent	N/A	5 hypothetical proteins Not present in either *S.* Enteritidis or *S.* Typhimurium	[6, 11]
SPI-16	13	3.3	LPS modification High homology to SPI-17	[6, 11]
SPI-17	54	3.6	LPS modification Present in *S.* Enteritidis and *S.* Typhi, but not *S.* Typhimurium High homology to SPI-16	[6, 11]
SPI-18	Absent	Absent	In *S.* Typhi encodes 2 genes for the cytolysin HlyE and the invasion TaiE Not present in either *S.* Enteritidis or *S.* Typhimurium	[6, 11]
SPI-19	24/25	14.1	T6SS likely non-functional in *S.* Enteritidis as most of island has been deleted Up to 45 kb in other serovars 54.3% GC	[6, 66]
SPI-20	Absent	34 kb	T6SS Only identified in *Salmonella enterica* subsp. *arizonae* 53.1% GC	[6, 66]
SPI-21	Absent	55 kb	T6SS Only identified in *Salmonella enterica* subsp. *arizonae* 49.6% GC	[6, 66]

virulence of *S.* Typhimurium in chicks [42, 65].

In *S.* Enteritidis, SPI-6 is 17.6 kb, but in other serovars can be 40 - 60 kb in length. A full version of SPI-6 contains genes encoding a type VI secretion system (T6SS), as well as the *saf* and *tcf* fimbrial operons. The shortened form of SPI-6 found in *S.* Enteritidis has only the *saf* fimbrial operon [11, 32, 66]. SPI-7 is only present in *S.* Typhi, *S.* Paratyphi, and some strains of *S.* Dublin. It is a very large PI, up to 134 kb in length, with a mosaic structure. SPI-7 includes Vi capsule biosynthesis genes, a type IV fimbrial operon, and *sopE*. Although SPI-7 is not present in *S.* Typhimurium and *S.* Enteritidis, SopE is encoded elsewhere in the chromosomes of these serovars [11, 32, 67]. SPI-8 contains genes important for resistance to bacteriocins, but is also absent in *S.* Enteritidis and *S.* Typhimurium [11, 32].

A T1SS and an exported repeats in toxin (RTX) protein are encoded by SPI-9, although the gene encoding the RTX protein is complete only in *S.* Enteritidis, and not *S.* Typhimurium [6, 32]. SPI-10 is 10 kb long in *S.* Enteritidis, and only contains the *sef* fimbrial operon. In *S.* Typhimurium and *S.* Typhi, SPI-10 is much larger (up to 33 kb) and harbours many more genes [6, 11, 32]. SPI-11 is approximately 7 kb in *S.* Enteritidis, and contains *pagC, pagD, msgA, envE,* and the pseudogene *envF*. In *S.* Typhimurium, the full *envF* gene is present. EnvE and EnvF are lipoproteins, while PagC, PagD and MsgA are associated with *S.* Typhimurium survival in macrophages [6, 11]. SPI-12 is approximately 6 kb in *S.* Enteritidis, and contains the gene for the SPI-2 secreted effector SspH2. It is over twice the size in *S.* Typhimurium, and is required for full virulence of *S.* Typhimurium in mice [6, 11, 68].

At 25kb, SPI-13 is important for systemic infection of mice by *S.* Typhimurium, as well as intracellular replication inside mouse macrophages [11, 68, 69]. SPI-14 encodes putative regulatory and electron transfer proteins and SPI-15 is a small pathogenicity island encoding five hypothetical proteins that is absent in both *S.* Enteritidis and *S.* Typhimurium [6, 11]. Both SPI-16 and SPI-17 are quite small (3.3 and 3.6 kb respectively) and contain genes involved in LPS modification. SPI-17 is present in *S.* Enteritidis and *S.* Typhi, but not *S.* Typhimurium [6, 11]. SPI-18 encodes only two proteins in *S.* Typhi: the cytolysin HlyE, and TaiE, an invasin, but is not present in either *S.* Enteritidis or *S.* Typhimurium [11].

All three of the SPIs 19, 20, and 21 encode T6SSs. SPI-19 is 45 kb long and also encodes two Hcp-like proteins and a VgrG homologue. Most of SPI-19 has been deleted in *S.* Enteritidis, leaving only 16 kb. Like SPI-19, SPI-20 also encodes a T6SS, Hcp-like and VgrG-like proteins,

and three ImpA homologues. SPI-21 encodes T6SS components, three VgrG-like proteins, and includes some genes that are involved in resistance to bacteriocins. Both SPIs 20 and 21 are absent in *S.* Enteritidis, and are, in fact, only present in *Salmonella enterica* subspecies *arizonae* [66].

1.2.4 Prophages

There are many prophage-like elements within the *S.* Typhimurium and *S.* Enteritidis genomes, many of them encoding T3SS effectors. These include genes for SopE, SopE2, and SspH1, which are secreted by the SPI-1 T3SS, as well as GogB, SseK3, SseI, and SspH2, which are secreted by the SPI-2 T3SS (Tables 1.2 and 1.3). Of note, one prophage encodes SodCI, a superoxide dismutase that is important for survival of *S.* Typhimurium in macrophages [6].

1.2.5 *Salmonella* virulence plasmid

The *Salmonella* virulence plasmid is found as a low copy (one to two copies per bacterial cell) plasmid of varying size (60 kb in *S.* Enteritidis and 90-96kb in *S.* Typhimurium). This plasmid harbours the *spv* locus comprised of five genes (*spvRABCD*). SpvR is a positive regulatory protein belonging to the LysR/MetR family that is responsible for the expression of the rest of the *spv* genes. SpvB acts to ADP-ribosylate actin, contributing to the survival of *Salmonella* in the intracellular environment via manipulation of the host cell cytoskeleton [32, 70]. The plasmid also harbours gens for *pef* (plasmid encoded fimbriae [PEF]), *tlpA* (a thermo sensor regulator), *rck* (resistance to complement killing), and *rsk* (regulation of serum killing) [32].

1.2.6 Other virulence determinants

LPS is a major component of the outer membrane of Gram-negative bacteria, including *Salmonella*. LPS is made up of a hydrophobic region (lipid A) that anchors it to the bacterial outer membrane, a non-repeated core oligosaccharide, and a repeated polysaccharide (O antigen). LPS helps to protect *Salmonella* from the harsh environment of the gastrointestinal tract and is responsible for a multitude of host immune responses. *Salmonella* is capable of modifying the structure of LPS under certain environmental conditions. These modifications may aid in protection of the bacteria from certain antimicrobial peptides produced by the host, and/or may

reduce the immunostimulatory potential. When genes needed for the production of LPS are defective, *S.* Typhimurium virulence is reduced in both calves and chicks [32, 42, 71, 72]. *S.* Enteritidis also expresses a heat labile cytotoxin similar to the cholera toxin, and a *Shigella dysenteriae* 1-like toxin [32].

1.3 Type III Secretion Systems

T3SSs act as 'injectisomes' and are used by bacteria to deliver effector proteins directly into the host cells cytoplasm. The first T3SS was isolated in 1998, although it was first visualized in the 1980s and was initially thought to be an intermediate complex of the flagellar system during its biosynthesis [73, 74]. All T3SSs share significant genetic and protein homology and can be divided into five phylogenetic groups: **1)** the Ysc group (such as the plasmid-encoded T3SSs of *Yersinia* species and *Pseudomonas aeruginosa*); **2)** the Hrp1 group (plant pathogens *Pseudomonas syringae* and *Erwinia* species); **3)** the Hrp2 group (such as the mega-plasmid-encoded T3SSs of the plant bacteria *Ralstonia* and *Xanthamonas* species, and one of the T3SSs of *Burkholderia* species); **4)** the Inv/Mxi/Spa group (the SPI-1 T3SS of *Salmonella enterica*, the chromosomally-encoded T3SS of *Shigella,* the non-functional ETT2 T3SS of enterotoxigenic *Escherichia coli* [ETEC], and the second T3SS of *Burkholderia* species); and **5)** the Esa/Ssa group (the locus of enterocyte effacement [LEE] T3SS of ETEC, the SPI-2 T3SS of *Salmonella enterica*, the chromosomally-encoded T3SS of *Yersinia* species, and the plasmid-encoded T3SS of *Shigella* species) [75, 76]. T3SSs are encoded on large pathogenicity islands, located either within the chromosome or on a plasmid. Flagellar genes, while clustered together in the same area of the chromosome, are not as tightly packed and are not located on pathogenicity islands [52, 77].

T3SSs are mainly found in pathogenic Gram-negative bacteria; however, there are a few exceptions. For instance, T3SSs have been found in the *Chlamydia*/*Verrucomivrobia* super-phylum that does not resemble either Gram-negative or Gram-positive bacteria. As well, there are a few examples of non-pathogenic symbiotic bacteria of plants having T3SSs, and even a T3SS used for virulence by unicellular Protozoa [31, 78]. Flagella are associated with both pathogenic and non-pathogenic bacteria and are most often not involved in direct virulence as T3SSs are. However, there are some cases where the flagellar apparatus is responsible for the secretion of virulence factors. For instance, the flagellar apparatus of *Campylobacter jejuni* is

essential for its virulence and secretes *Campylobacter* invasion antigens (Cia), and the flagellar system of *Bacillus thuringiensis* can secrete the virulence factors hemolysin BL and phosphatidylcholine-preferring phospholipase C [73].

1.3.1 Flagella

1.3.1.1 Structural components of flagella

The flagellum of *Salmonella enterica* is made up of 22 structural proteins, six cytoplasmic proteins, four structural chaperones and three regulatory proteins (Figure 1.4). The structure consists of a C ring (FliG, FliM and FliN) and an MS ring (FlgF and FliF) embedded in the cytoplasmic (inner) membrane. An ATPase is located on the cytoplasmic side of the apparatus (FliI). The P ring (FlgI) is located in the peptidoglycan layer and the L ring (FlgH) is in the outer membrane. A rod spanning the two bacterial membranes made up of FliF connects the inner membrane and outer membrane rings, and other proteins (FliE, FlgB, FlgC, FlgF and FlgG) are also associated with the basal body. A type three secretion (T3S) apparatus is located within the basal body structure (FliO, FliP, FliQ, FliR, FlhA, FlhB, FliH and the FliI ATPase). The motor-stator (MotA and MotB), which is the driving force for motion, is also located within the basal body. MotA is located within the inner membrane and connects to MotB, which extends into the periplasm. A hook (FlgE) extends from the L/P rings. FliK acts as a 'molecular ruler' to control the length of the hook. The hook is followed by the hook-filament junction (FlgK and FlgL) and is extended by a long filament (flagellin). *Salmonella* encodes for two flagellin proteins (FliC and FljB) that make up the filament, but these two proteins are never expressed at the same time. This differential expression may aid *Salmonella* in escaping the host immune defenses by antigenic variation, and/or contribute to host specificity. Finally, the filament is topped off by the capping protein, FlgD [32, 42, 77, 79-82].

1.3.1.2 Assembly and regulation of flagella

Flagellar genes can be organized into three classes: early, middle and late. The early genes *flhDC* encode the master regulators FlhD and FlhC, which positively regulate gene expression of the middle genes. The middle genes consist of those that make up the MS, C, P and L rings, the T3S export apparatus, the motor-stator, the rod and the hook [77, 83, 84]. The

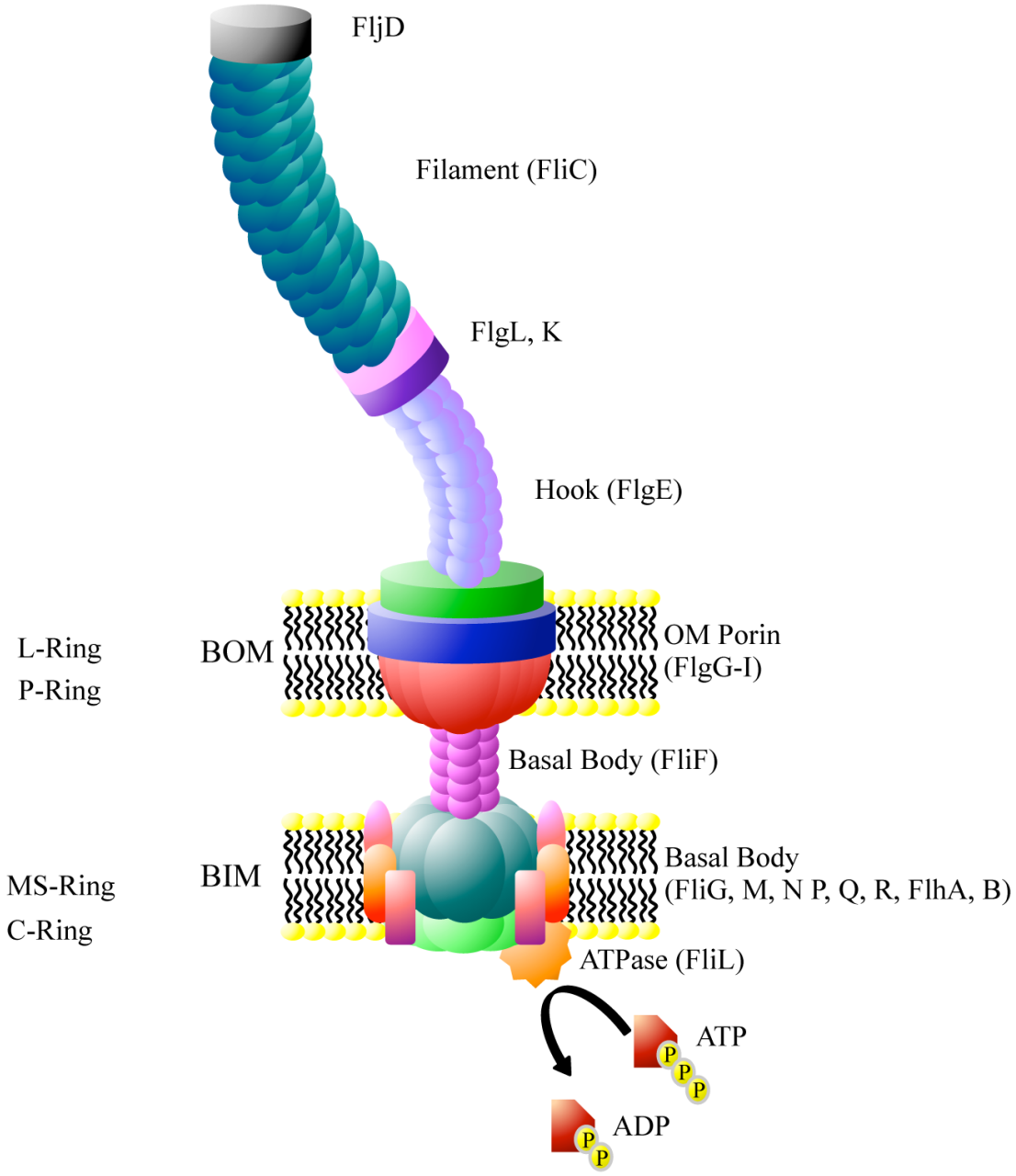

Figure 1.4 Schematic representation of the flagellar system of *Salmonella*

The molecular organization of the flagellar system is depicted above. Stoichiometry of proteins was followed where known. BIM – Bacterial inner membrane. BOM – bacterial outer membrane. HCM – host cell membrane. Adapted and modified from [85].

MS ring is assembled first, followed by the T3S export apparatus and the motor-stator. These steps are dependent on the Sec secretion system. Next, the rod is formed (T3S-dependent), followed by the L and P rings (Sec-dependent). Following the formation of the hook (T3S-dependent), there is a switch to late gene expression including genes encoding the hook-filament junction, filament, and cap proteins [77, 84]. FliA (a flagella-specific σ^{28}) activates expression of the late genes. FlgN act as a chaperone for the hook-filament junction proteins FlgK and FlgL, while FliT acts as the chaperone for FliD (the cap). Once the hook-filament junction and the cap proteins have been depleted from the cytoplasm, indicating a completed flagellum, FlgN and FliT are freed. FliT is then able to bind FlhC, which inhibits middle gene transcription, while free FlgN acts to enhance transcription of FlgM, an anti-σ^{28} factor [84]. FlgM is actively transcribed during both middle and late gene expression. During middle gene expression, FlgM binds FliA, resulting in the repression of late gene expression. After assembly of the hook and hook-filament junction, FlgM is exported by the T3S apparatus, freeing FliA and allowing for late gene expression. Stimulation of further expression of FlgM by free FlgN allows late gene expression to be terminated quickly upon completion of the flagella [84, 85].

1.3.2 *Salmonella* pathogenicity island 1 type III secretion system

1.3.2.1 Structural components and effectors of the *Salmonella* pathogenicity island 1 type III secretion system

The basal body of the SPI-1 T3SS (Figure 1.5) is composed of an inner membrane ring formed by PrgH and PrgK, many inner membrane proteins (SpaP, SpaQ, SpaR, SpaS and InvA), an ATPase (InvC) and an outer membrane secretin (InvG). Extending from the outer membrane secretin is the needle formed by PrgI, topped by the translocon made up of SipB and SipC [74]. The SPI-1 T3SS is responsible for the secretion of a specific set of effectors. AvrA, SipA, SipB, SipC, SipD and SptP are all encoded on SPI-1, while the genes encoding GogB, SopE, SopE2 and SspH1 are located on bacteriophages, the gene for SopB is located on SPI-5, and the genes for SopA, SopD, SlrP, SteA and SteB are located elsewhere within the chromosome. GogB, SlrP, SspH1, SteA and SteB are also secreted by the SPI-2 T3SS [6, 7, 86, 87]. Chaperones of both the SPI-1 and SPI-2 T3SS tend to contain an amphipathic α-helix near the C-terminal end and are very small and acidic in nature. There is usually no sequence similarity between

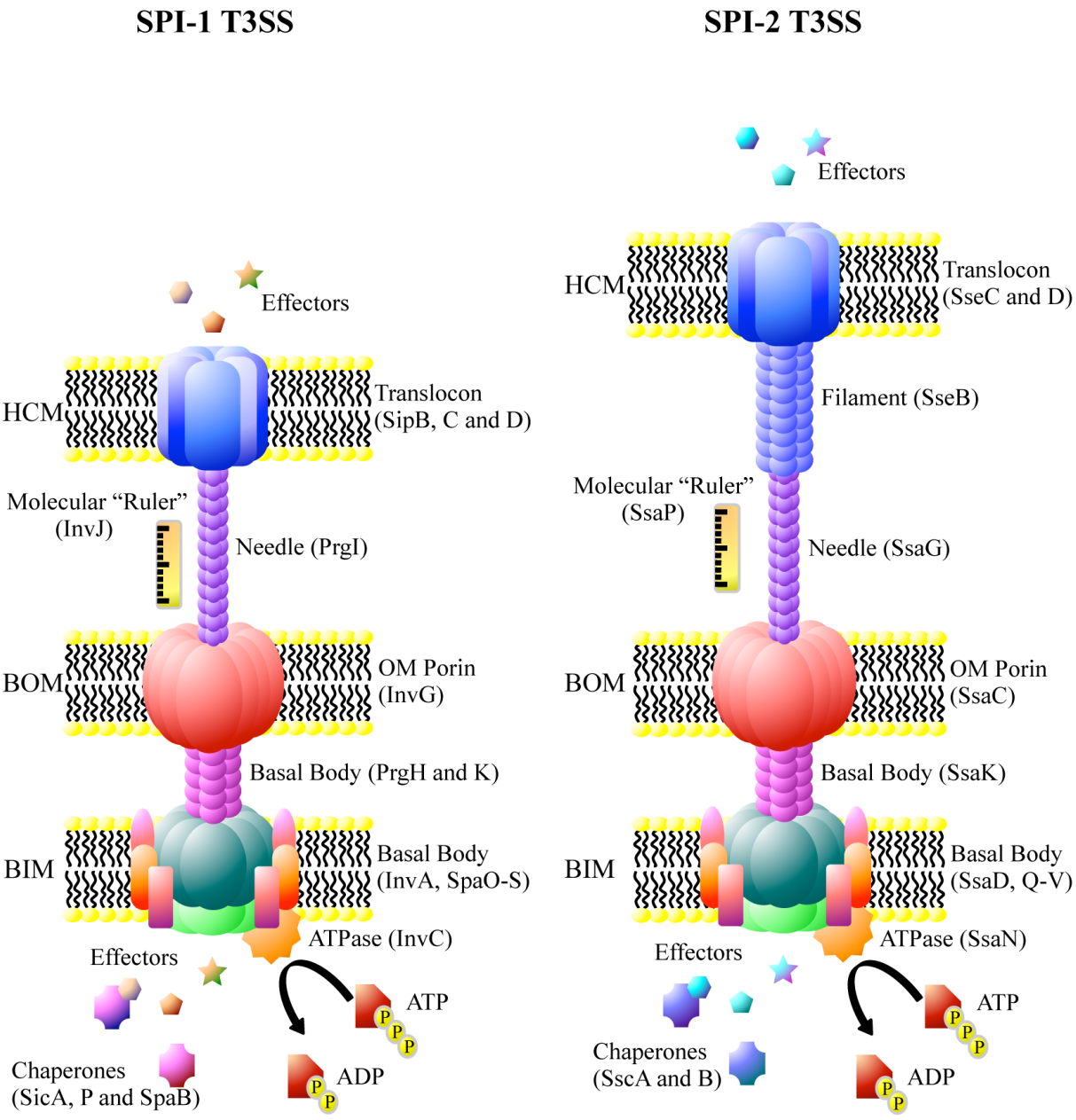

Figure 1.5 Schematic representation of the *Salmonella* pathogenicity islands 1 and 2 type III secretion systems

The molecular organization of the SPI-1 T3SS is shown on the left, and SPI-2 on the right. Stoichiometry of proteins was followed where known. BIM – Bacterial inner membrane. BOM – bacterial outer membrane. HCM – host cell membrane. Adapted and modified from [74, 78].

chaperones or signal sequences of SPI-1 and SPI-2 T3SS effector proteins [75, 88]. See Table 1.2 for a list of all structural and effector components of the SPI-1 T3SS.

1.3.2.2 Assembly and regulation of the *Salmonella* pathogenicity island 1 type III secretion system

The assembly of the SPI-1 T3SS proceeds in a similar manner to the assembly of the flagella. The inner membrane and outer membrane rings are formed first in a sec-dependent manner, followed by the association of the rings and formation of the remaining basal body components, including the ATPase. Formation of the needle and translocon is T3S-dependent, and needle length is controlled by InvJ, which acts as a 'molecular ruler' [74, 75, 89].

Expression of the SPI-1 T3SS is regulated by many environmental and genetic signals. Environmental signals include pH, osmolarity, the presence of bile, Mg^{2+} concentration, and the presence of short chain fatty acids [90]. The preferred invasion site of *Salmonella* is the M-cells of the distal small intestine. When bile is present, indicating the beginning of the small intestine, or when short-chain fatty acids are present, which are produced by microflora of the large intestine, SPI-1 expression is repressed. These environmental signals indicate that the bacterium is not near its preferred site of entry. SPI-1 expression is induced at near neutral pH, and high osmolarity [90, 91]. In the presence of Iron (Fe^{2+}), the ferric uptake regulator (Fur) acts to increase the expression of HilD (A SPI-1 regulator, discussed further in the following text) in an unknown manner. Once in the SCV, where there is limited Fe^{2+}, this indirect activation of HilD by Fur is stopped [90, 92]. See Figure 1.6 for a diagram of the interaction of the regulation pathways outlined below, along with those outlined in sections 1.3.3.2 and 1.3.4.

Nucleoid associated proteins (NAPs) affect supercoiling of DNA, and are thus able to alter gene expression. The NAPs Hha and H-NS both repress transcription of many genes, including *rtsA* and the SPI-1 gene *hilA* under conditions of low osmolarity [70, 90, 93]. Hu, IHF and Fis are also NAPs, and are important for expression of SPI-1 genes [90, 94].

PhoP/PhoQ and BarA/SirA belong to two-component global regulatory systems that respond to environmental conditions. In low Mg^{2+} conditions, for example within the SCV, PhoP can act to negatively regulate HilA, leading to the down regulation of the SPI-1 T3SS. SirA positively regulates HilA, by regulating the expression of HilD [2, 51, 52, 90]. BarA/SirA also controls the *csr* system. CsrA can bind mRNAs at their ribosomal binding site, thus stabilizing,

Table 1.2 *Salmonella* pathogenicity island 1 type III secretion system components

Protein	Gene	Location	Function	Reference
AvrA	*avrA*	SPI-1	SPI-1 T3SS effector protein: deubiquitinates of IκBα and β-catenin, thereby limiting inflammatory response	[95, 96]
HilA	*hilA*	SPI-1	Major SPI-1 transcriptional activator, acts on *prg*, *sip*, and *inv/spa* operons	[90, 97]
HilC	*hilC*	SPI-1	Regulatory protein: acts on *hilA*, *hilC*, and *hilD*	[2, 90]
HilD	*hilD*	SPI-1	Regulatory protein: acts on *hilA*, *hilC*, and *hilD*	[2, 90]
HilE	*hilE*	Outside SPI-1	Regulatory protein, negatively regulates HilA through HilD	[2, 90, 98]
IacP	*iacP*	SPI-1	Putative acyl carrier protein, involved in regulating translocation of other SPI-1 T3SS effector proteins including SopA, SopB, and SopD	[99]
IagB	*iagB*	SPI-1	Invasion protein	[6, 100]
InvA	*invA*	SPI-1	SPI-1 T3SS structural component: needle complex export protein	[75]
InvB	*invB*	SPI-1	SPI-1 T3SS secretion chaperone: chaperone to SopA and SopE	[101]
InvC	*invC*	SPI-1	SPI-1 T3SS structural component: ATPase	[102, 103]
InvE	*invE*	SPI-1	Invasion protein: controls protein translocation and order of effector translocation	[104]
InvF	*invF*	SPI-1	Regulatory protein: acts on *sip* operon and *sopB*	[90, 105]
InvG	*invG*	SPI-1	SPI-1 T3SS structural component: forms outer membrane pore	[2, 105]
InvH	*invH*	SPI-1	SPI-1 T3SS structural component, important for efficient assembly of InvG, PrgH, and PrgK	[4]
InvI	*invI*	SPI-1	Needle complex assembly protein, chaperone to InvJ	[6, 106]
InvJ	*invJ*	SPI-1	Molecular ruler: controls length of SPI-1 T3SS needle	[107]
OrgA	*orgA*	SPI-1	Needle complex assembly protein	[3, 6]
OrgB	*orgB*	SPI-1	Needle complex export protein, interacts with InvC ATPase	[3, 108]
OrgC	*orgC*	SPI-1	Putative SPI-1 effector	[3, 108]
PphB	*pphB*	SPI-1	Serine/threonine protein phosphatase 2	[6]

PrgH	*prgH*	SPI-1	SPI-1 T3SS structural component: Inner membrane protein	[75, 109]
PrgI	*prgI*	SPI-1	SPI-1 T3SS structural component: needle complex major subunit	[75, 110]
PrgJ	*prgJ*	SPI-1	SPI-1 T3SS structural component: needle complex minor subunit (rod)	[75, 109]
PrgK	*prgK*	SPI-1	SPI-1 T3SS structural component: needle complex inner membrane lipoprotein	[4, 109]
SicA	*sicA*	SPI-1	SPI-1 T3SS secretion chaperone	[6]
SicP	*sicP*	SPI-1	SPI-1 T3SS secretion chaperone: chaperone to SptP	[111]
SitA	*sitA*	SPI-1	Metal transport system: putative periplasmic binding protein	[6, 53]
SitB	*sitB*	SPI-1	Metal transport system: putative ATP-binding protein	[6, 53]
SitC	*sitC*	SPI-1	Metal transport system: putative permease	[6, 53]
SitD	*sitD*	SPI-1	Metal transport system: putative permease	[6, 53]
SipA	*sipA*	SPI-1	SPI-1 T3SS effector protein: involved in actin cytoskeleton rearrangement resulting in membrane ruffles during initial entry, disruption of intestinal epithelial cell tight junctions, and in SCV maturation and positioning through cooperation with SifA	[96, 112-114]
SipB	*sipB*	SPI-1	SPI-1 T3SS structural component: forms part of the translocon of the SPI-1 T3SS along with SipC and SipD, involved in autophagy, required for attachment to host epithelial cells Binds caspase 1 induces apoptosis	[51, 115-117]
SipC	*sipC*	SPI-1	SPI-1 T3SS structural component: forms part of the translocon of the SPI-1 T3SS along with SipB and SipD, involved in actin bundling, required for attachment to host epithelial cells	[115, 116, 118]
SipD	*sipD*	SPI-1	SPI-1 T3SS structural component: forms part of the translocon of the SPI-1 T3SS along with SipB and SipC, required for attachment to host epithelial cells	[106, 112, 115]
SlrP	*slrP*	Outside SPI-1	SPI-1 T3SS effector protein: Ubiquitination of Trx1, involved in cell death	[86, 95]

SopA	*sopA*	Outside SPI-1	SPI-1 T3SS effector protein: involved in migration of PMN's and escape of *Salmonella* from the SCV, can also be secreted by the flagellar system	[119, 120]
SopB	*sopB*	SPI-5	SPI-1 T3SS effector protein: involved in actin polymerization during invasion, induction of pro-inflammatory cytokines, disruption of intestinal epithelial cell tight junctions, and SCV maturation and positioning	[64, 96, 114, 120]
SopD	*sopD*	Outside SPI-1	SPI-1 T3SS effector protein: involved in membrane fission, actin rearrangement during entry and SCV positioning	[96, 120]
SopE	*sopE*	Prophage ΦSE12, similar to Gifsy-2	SPI-1 T3SS effector protein: involved in actin polymerization, induction of inflammation, disruption of intestinal epithelial cell tight junctions, SCV maturation, and activation of caspase-1 in macrophages	[6, 114, 121]
SopE2	*sopE2*	Bacteriophage	SPI-1 T3SS effector protein: involved in actin polymerization, disruption of intestinal epithelial cell tight junctions, and induction of inflammation	[96, 114]
SpaO	*spaO*	SPI-1	SPI-1 T3SS structural component: needle complex export	[6, 75]
SpaP	*spaP*	SPI-1	SPI-1 T3SS structural component: needle complex export	[6, 75]
SpaQ	*spaQ*	SPI-1	SPI-1 T3SS structural component: needle complex export	[6, 75]
SpaR	*spaR*	SPI-1	SPI-1 T3SS structural component: needle complex export	[6, 75]
SpaS	*spaS*	SPI-1	SPI-1 T3SS structural component: important for export of needle protein and switch between needle and translocon export	[122]
SptP	*sptP*	SPI-1	SPI-1 T3SS effector protein: tyrosine phosphatase/GTPase activating protein, involved in down regulation of inflammation and induces cell to regain original state after invasion, preferentially expressed in the spleen of mice	[96, 111, 123, 124]
SprB	*sprB*	SPI-1	Transcriptional regulator	[6, 125]

SspH1	*sspH1*	Gifsy-3 prophage in *S.* Typhimurium Remnant on ΦSE20 in *S.* Enteritidis	SPI-1 T3SS effector: E3 ubiquitin ligase, downregulates inflammation by inhibition of NF-κB expression Also secreted by SPI-2, not present in *S.* Enteritidis	[6, 126, 127]
SteA	*steA*	Outside SPI-1	SPI-1 T3SS effector: unknown function, localizes to Golgi	[95]
SteB	*steB*	Outside SPI-1	SPI-1 T3SS effector: unknown function, required for full virulence	[95]
SEN2743	*stm2904*	SPI-1	Putative ABC-type transporter	[6]
SEN2744	*stm2905*	SPI-1	Putative acetyltransferase	[6]

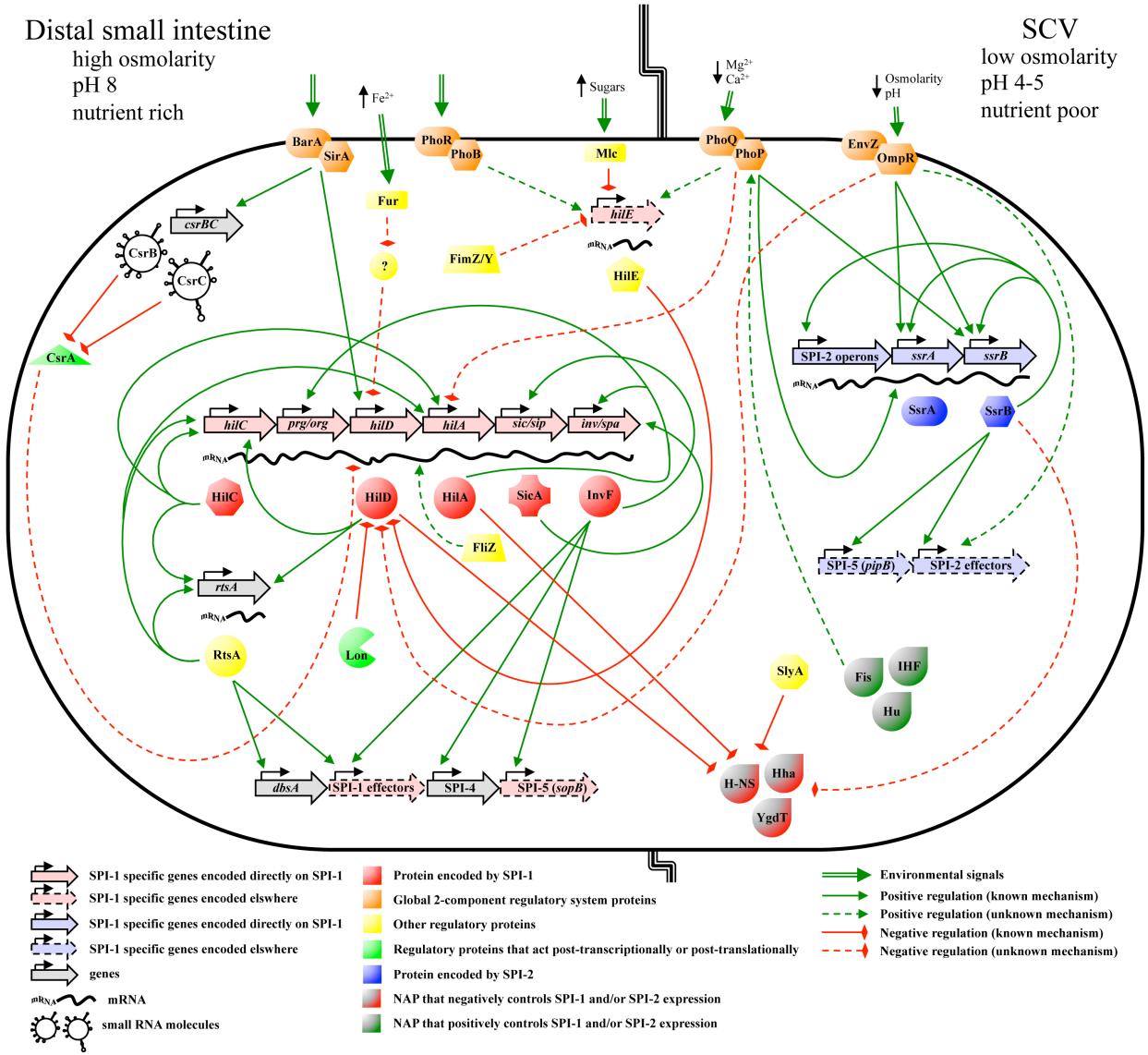

Figure 1.6 Regulation of *Salmonella* pathogenicity islands 1 and 2

The major modes of SPI-1 and SPI-2 regulation are depicted above; see text for details (Section 1.3).

or alternatively, reducing, translation of SPI-1 T3SS proteins, likely at the level of HilD. CsrB and C are small RNA molecules that bind and stop the action of CsrA. BarA/SirA activate CsrB and C, keeping CsrA levels in check. Optimal levels of all three molecules are needed for proper expression of SPI-1. The EnvZ/OmpR system senses osmolarity and may act to regulate HilD post-translationally. The PhoP/PhoQ and PhoR/PhoB systems can activate expression of HilE, which then acts to repress expression of SPI-1 genes, through direct binding to HilD. The type 1 fimbriae regulators FimZ and FimY have also been shown to negatively regulate transcription of SPI-1 genes, likely through activation of *hilE*, while the flagella regulator FliZ positively regulates expression of HilA post-transcriptionally [2, 90]. Mlc is a global regulator that detects the presence of sugars such as glucose and mannose, whereby Mlc can repress expression of *hilE* when sugars are readily available, such as in the small intestine [128]. The Lon protease (controlled by DnaK and σ^{32}), negatively regulates SPI-1 by degrading HilD in response to the stress of the SCV environment [129].

HilA belongs to the OmpR/ToxR family of transcriptional regulators, while InvF, HilC and D are in the AraC/XylS family. The genes encoding these proteins (*hilA, hilC, hilD* and *invF*) are located on SPI-1 [50, 51]. Expression of HilD is likely induced by environmental conditions, and leads to expression of HilC and RtsA. RtsA and HilC can also activate expression of themselves, and each other. RtsA activates *hilA* expression directly, as well as the expression of *slrP* (a SPI-1 T3SS effector) and *dsbA*, which is needed for assembly of T3SS. HilC and D act to derepress transcription of *hilA* and *rtsA* by relieving silencing by H-NS and Hha. HilA is then free to activate transcription of the *prg*/*org* and *inv*/*spa* operons (including *invF*). RtsA, HilD and HilC can also activate transcription of the *inv*/*spa* operon independently of HilA, but to a lower degree than HilA. InvF activates transcription of the *sic*/*sip* (including *sicA*) operon of SPI-1, as well as genes within SPI-4 and SPI-5 (*sopB*) [2, 50, 51, 70, 90, 93]. SicA is the chaperone for the translocator proteins SipB and C. Once the translocon has been secreted, SicA is free and can activate expression of *invF,* creating a positive feedback loop of secreted effector gene expression once the SPI-1 T3SS is fully formed [70, 75].

1.3.3 *Salmonella* pathogenicity island 2 type III secretion system

1.3.3.1 Structural components and effectors of the *Salmonella* pathogenicity island 2 type III secretion system

The SPI-2 T3SS (Figure 1.5) is composed of an inner membrane ring that, in conjunction with many other inner membrane proteins, makes up the basal body. These include SsaD, SsaR, SsaS, SsaT, SsaU and SsaV. The cytoplasmic ATPase is SsaN. The outer membrane secretin is made up of SsaC, and is connected to the inner membrane components via SsaJ. A small needle extends from the outer membrane secretin (SsaG) and is extended by a larger filament (SseB); in comparison, many other T3SSs do not have a filament extension. The filament is topped off by the translocon proteins SseC and SseD [74, 78, 79]. SsaP, which acts as a 'molecular ruler', controls the length of the needle, just as InvJ of the SPI-1 T3SS [130].

The SPI-2 T3SS has been shown to secrete many effectors (GogB, PipB, PipB2, SifA, SifB, SopD2, SseF, SlrP, SseG, SseI, SseJ, SseK1, SseK2, SseL, SspH1, SspH2, SteA, SteB and SteC), although the functions of many are still unknown at this time. Some of the genes encoding these proteins are located directly on the chromosome in the SPI-2 region, but some are located elsewhere on the chromosome, within lysogenic phages (*e.g.* Gifsy-1, -2 and -3) or on the *Salmonella* virulence plasmid. While these proteins are secreted by the SPI-2 T3SS, GogB, SlrP, SspH1, SteA and SteB are also known to be secreted by the SPI-1 T3SS [7, 86, 87]. The functions of these effectors in *Salmonella* pathogenesis will be discussed further in section 1.4.1. A complete list of SPI-2 T3SS components, including effectors known to be secreted by the SPI-2 T3SS, can be found in Table 1.3.

1.3.3.2 Assembly and regulation of the *Salmonella* pathogenicity island 2 type III secretion system

As with the assembly of the flagellar apparatus and the SPI-1 T3SS, The SPI-2 T3SS is assembled in a step-wise manner involving first the insertion of the inner membrane ring (SsaU) and outer membrane secretin in a sec-dependent manner. Association of the inner membrane and outer membrane rings, placement of further basal body components and recruitment of the ATPase takes place, followed by the subsequent assembly of the rest of the apparatus in a T3S-dependent manner [75, 84, 89].

Table 1.3 *Salmonella* pathogenicity island 2 type III secretion system components

Protein	Gene	Location	Function	Reference
GogB	*gogB*	Gifsy-1 prophage	SPI-2 T3SS effector protein, expressed intracellularly	[131]
Orf32	*orf32*	SPI-2	Putative proline iminopeptidase	[6]
Orf48	*orf48*	SPI-2	Putative amino acid permease	[6]
Orf70	*orf70*	SPI-2	Putative cytoplasmic protein	[6]
Orf242	*orf242*	SPI-2	Putative regulatory protein	[6]
Orf245	*orf245*	SPI-2	Putative cytoplasmic protein	[6]
Orf319	*orf319*	SPI-2	Putative inner membrane protein	[6]
Orf408	*orf408*	SPI-2	Putative regulatory protein	[6]
PipB	*pipB*	SPI-5	SPI-2 T3SS effector protein: associated with Sif formation and the SCV, but not required for virulence	[64, 95, 132]
PipB2	*pipB2*	Outside SPI-2	SPI-2 T3SS effector protein: recruits kinsin-1 to SCV, involved in sif formation	[95, 133, 134]
SifA	*sifA*	Outside SPI-2	SPI-2 T3SS effector protein: required for positioning of Sifs and positioning of the SCV	[135, 136]
SifB	*sifB*	Outside SPI-2	SPI-2 T3SS effector protein: involved (but not essential) in the formation of Sifs	[137, 138]
SopD2	*sopD2*	Outside SPI-2	SPI-2 T3SS effector protein: Involved in Sif and SCV formation	[95, 139]
SsaB (SpiC)	*ssaB (spiC)*	SPI-2	SPI-2 T3SS effector protein: prevents fusion of late endosomes with the SCV, acts with SsaM to initiate switch between secretion of translocon components and effectors	[140-142]
SpvB	*spvB*	*Salmonella* virulence plasmid (*spv*)	SPI-2 T3SS effector protein: inhibits actin polymerization associated with the SCV	[96, 143]
SpvC	*spvC*	*spv* Plasmid	SPI-2 T3SS effector protein: Phosphothreonine lysase of MAPK proteins, involved in down regulation of inflammation	[95, 144]
SrfH	*srfH*	Outside SPI-2	SPI-2 T3SS effector protein: involved in macrophage motility; important for early dissemination of *Salmonella* to spleens of mice	[95]
SsaC	*ssaC*	SPI-2	SPI-2 structural component: forms outer membrane pore of the SPI-2 T3SS	[58, 130]
SsaD	*ssaD*	SPI-2	SPI-2 T3SS structural component	[6]
SsaE	*ssaE*	SPI-2	SPI-2 T3SS chaperone to SseB and PipB	[145]

SsaG	*ssaG*	SPI-2	SPI-2 structural component: forms the needle of the SPI-2 T3SS	[79, 146]
SsaH	*ssaH*	SPI-2	SPI-2 T3SS structural component	[6, 147]
SsaI	*ssaI*	SPI-2	SPI-2 T3SS structural component: needle complex minor subunit (rod)	[6, 148]
SsaJ	*ssaJ*	SPI-2	SPI-2 T3SS structural component: needle complex inner membrane lipoprotein, required for systemic infection of mice	[1, 6, 149]
SsaK	*ssaK*	SPI-2	SPI-2 T3SS structural component	[1, 6]
SsaL	*ssaL*	SPI-2	SPI-2 T3SS structural component: required for secretion of SPI-2 encoded effectors, but not for effectors encoded outside SPI-2	[6, 150]
SsaM	*ssaM*	SPI-2	SPI-2 T3SS structural component: acts with SsaM to initiate switch between secretion of translocon components and effectors	[6, 142]
SsaV	*ssaV*	SPI-2	SPI-2 T3SS structural component: needle complex export protein	[1, 6]
SsaN	*ssaN*	SPI-2	SPI-2 T3SS structural component: ATPase	[1, 6]
SsaO	*ssaO*	SPI-2	SPI-2 T3SS structural component	[1, 6]
SsaP	*ssaP*	SPI-2	SPI-2 T3SS structural component	[1, 6]
SsaQ	*ssaQ*	SPI-2	SPI-2 T3SS structural component: needle complex export	[1, 6]
SsaR	*ssaR*	SPI-2	SPI-2 T3SS structural component: needle complex export	[1, 6]
SsaS	*ssaS*	SPI-2	SPI-2 T3SS structural component: needle complex export	[1, 6]
SsaT	*ssaT*	SPI-2	SPI-2 T3SS structural component: needle complex export	[1, 6]
SsaU	*ssaU*	SPI-2	SPI-2 T3SS structural component: similar to SpaS, so likely also involved in switch between needle and translocon export	[1, 6]
SscA	*sscA*	SPI-2	SPI-2 T3SS secretion chaperone: chaperone of SseC	[140]
SscB	*sscB*	SPI-2	SPI-2 T3SS secretion chaperone: chaperone of SseF	[151]
SseA	*sseA*	SPI-2	SPI-2 T3SS secretion chaperone: chaperone of SseB	[152]
SseB	*sseB*	SPI-2	SPI-2 structural component: forms the needle filament of the SPI-2 T3SS	[146, 153]

SseC	*sseC*	SPI-2	SPI-2 structural component: forms part of the SPI-2 T3SS translocon along with SseD	[146, 153]
SseD	*sseD*	SPI-2	SPI-2 structural component: forms part of the SPI-2 T3SS translocon along with SseC	[146, 153]
SseE	*sseE*	SPI-2	SPI-2 T3SS effector protein	[6]
SseF	*sseF*	SPI-2	SPI-2 T3SS effector protein: involved in SCV formation and positioning	[96, 154]
SseG	*sseG*	SPI-2	SPI-2 T3SS effector protein: involved in SCV formation and positioning	[95, 96, 154]
SseI	*sseI*	Prophage ΦSE10 (Similar to Gifsy-2)	SPI-2 effector protein: involved in actin remodeling, inhibits host cell migration	[6, 137, 155]
SseJ	*sseJ*	Outside SPI-2	SPI-2 T3SS effector protein: cholesterol acetyltransferase, involved in maintenance of the SCV membrane and sif formation	[95, 133, 138]
SseK1	*sseK1*	Outside SP1-2	SPI-2 T3SS effector protein: localizes to host cell cytoplasm	[156]
SseK2	*sseK2*	Outside SPI-2	SPI-2 T3SS effector protein: unknown function, localizes to host cell cytoplasm	[95, 156]
SseK3	*sseK3*	Bacteriophage	SPI-2 T3SS effector protein	[6, 156]
SseL	*sseL*	Outside SPI-2	SPI-2 T3SS effector protein: deubiquitinates IκBα, which results in inhibition of NF-κB activity and is associated with host cell death	[95, 157, 158]
SspH1	*sspH1*	Gifsy-3 prophage in *S.* Typhimurium Remnant on ΦSE20 in *S.* Enteritidis	SPI-2 T3SS effector: E3 ubiquitin ligase, downregulates inflammation by inhibition of NF-κB expression Also secreted by SPI-1, not present in *S.* Enteritidis	[6, 126, 127]
SspH2	*sspH2*	Bacteriophage	SPI-2 T3SS effector protein: E3 ubiquitin ligase, Involved in inhibition of actin polymerization associated with the SCV	[95, 127, 159]
SsrA	*ssrA*	SPI-2	SPI-2 encoded 2-component regulatory system: sensor kinase	[6, 160, 161]
SsrB	*ssrB*	SPI-2	SPI-2 encoded 2-component regulatory system: transcriptional activator	[6, 160, 161]
SteC	*steC*	Outside SPI-2	SPI-2 T3SS effector protein: serine/threonine kinase, involved in actin formation surrounding the SCV	[95]

SEN1635	*sen1635*	SPI-2	Putative cytoplasmic protein	[6]
TtrA	*ttrA*	SPI-2	Tetrathionate reductase complex: subunit A	[6]
TtrB	*ttrB*	SPI-2	Tetrathionate reductase complex: subunit B	[6]
TtrC	*ttrC*	SPI-2	Tetrathionate reductase complex: subunit C	[6]
TtrR	*ttrR*	SPI-2	Response regulator	[6]
TtrS	*ttrS*	SPI-2	Sensory histidine kinase	[6]

Like the SPI-1 T3SS, expression of the SPI-2 T3SS is regulated by many environmental and genetic signals. Environmental signals that mimic the environment of the SCV (low Mg^{2+} concentration and acidic pH between 4 and 5) are SPI-2-inducing. The preferred replication site of *Salmonella* is within the SCV of macrophages [61, 70, 94, 162].

As with the SPI-1 T3SS, expression of SPI-2 genes is affected by the global two-component regulatory systems PhoP/PhoQ and EnvZ/OmpR. Under conditions of low Mg^{2+} and calcium (Ca^{2+}) PhoP induces SPI-2 gene expression by direct interaction with the *ssrB* gene, and post-transcriptional action on SsrA. In the presence of low osmolarity and acidic pH, OmpR can directly bind both the *ssrA* and *ssrB* promoters, activating transcription. OmpR can also act in conjunction with SsrB to activate transcription of the non-SPI-2-encoded effector SseI [5, 91, 94, 163, 164].

SPI-2 encodes its own two-component regulatory system, SsrA/SsrB. SsrB is able to bind to all SPI-2 promoters, including those of *ssrA*, *ssrB*, and many effectors located outside of SPI-2 [5, 94]. As with SPI-1, H-NS silences the expression of SPI-2 genes by binding directly to many SPI-2 promoters. This binding can be relieved by the SPI-1 protein HilD under certain conditions, such as stationary phase growth in LB, and may also be relieved by SsrB and/or SlyA [5, 94, 165]. The transcription of SPI-2 genes can also be repressed by the NAPs Hha and YdgT. Fis, a NAP that is able to bind the promoter regions of *ssr* and *ssa* operons, is also important for expression of SPI-2 as well as SPI-1 genes. Proper levels of Fis are important for activation of *ssrA*. Fis may also induce SPI-2 gene expression indirectly through controlling expression of PhoP. IHF, another NAP, is also important for expression of both SPI-2 and SPI-1 genes [94]. Some of the mechanisms controlling regulation of SPI-2 are outlined in Figure 1.6.

1.3.4 Cross-talk between the *Salmonella* flagellar and pathogenicity islands 1 and 2 type III secretion systems

The complex regulation of the T3SSs ensures that each system is only expressed under the correct conditions. Expression of multiple versions of each T3SS simultaneously would be energetically expensive, so coordinated expression of the three systems under specific conditions where they are required is desirable. Global regulation by two-component regulatory systems that sense divalent cation concentrations, osmolarity and pH are, in part, responsible for the changes in expression between the flagellar, the SPI-1, and the SPI-2 T3SSs. The SPI-1 T3SS is

preferentially within the distal small intestine, which has low oxygen, high osmolarity, a pH of 8, divalent cations, and is rich in nutrients. The environment of the SCV is much different having low osmolarity, low divalent cation concentration, a pH between 4 and 5, and is nutrient poor. In these conditions, the SPI-2 T3SS is preferentially expressed [75].

The BarA/SirA system positively regulates expression of SPI-1 genes, but negatively regulates expression of flagellar genes. Therefore, in environmental conditions that activate BarA/SirA, the SPI-1 T3SS will be expressed while the flagellar system is downregulated. RtsA and RtsB have also been proposed to be involved in the switch from expression of flagella to expression of the SPI-1 T3SS. RtsA is important for SPI-1 expression, while RtsB represses expression of flagellar genes by interfering with the *flhDC* promoter [166]. In conditions of low divalent cation concentration, PhoP suppresses expression of SPI-1 genes while activating expression of SPI-2 genes. This ensures that once in the SCV, when the SPI-1 T3SS is no longer needed for invasion of non-phagocytic cells, the SPI-2 T3SS expression is induced while the SPI-1 T3SS is downregulated [70].

Interspecies and interkingdom quorum sensing may also be involved in regulating expression of these three systems. In the presence of host norepinephrine, there is an upregulation of flagellar genes in *S.* Typhimurium. *S.* Typhimurium encodes a putative regulatory protein, YhcS, which has high amino acid similarity to QseA of *E. coli*. QseA activates expression of the LEE T3SS by *E. coli* in response to AI-3 quorum sensing molecules produced by intestinal flora, as well as epinephrine and norepinephrine produced by the host. YhcS may act similarly to QseA in *E. coli* by activating expression of either (or both of) the SPI-1 or SPI-2 T3SSs [167-169]. Under certain growth conditions, HilD can relieve H-NS-mediated repression of SPI-2 genes [165]. This may account for the fact that SPI-2 is expressed to some extent along with SPI-1 in the intestinal lumen, and that SPI-1 is expressed for a short time in macrophages before the complete switch to SPI-2 expression. The expression of the SPI-2 T3SS before invasion of intestinal epithelial cells would allow the bacteria to ready itself for the SCV environment. Furthermore, the expression of the SPI-1 T3SS is important for inducing macrophage apoptosis during the initial stage of infection while the bacteria is replicating, and before spread to the rest of the body. Interplay between regulation of SPI-1 and SPI-2 can be visualized in Figure 1.6.

1.3.5 Evolution of the type III secretion system

The flagellar systems of Prokarya are completely different from those of Archaea and Eukarya, suggesting that they evolved convergently in parallel to a structure serving the same function [31, 80]. However, prokaryotic flagellar systems that have a chemotaxis apparatus for sensing environmental signals that lead to changes in the direction of motion share their chemotaxis system with archaeal flagellar systems [80, 82]. As some members of Prokarya do not have this chemotaxis system, it may have been acquired by horizontal transfer from a member of Archaea or may have been present for sensing environmental signals before the diversification of Prokarya and Archaea, and has since been lost in some prokaryotic families.

While the flagellar systems of Prokarya maintain many of the same genes and proteins among members, they can be quite diverse in their function. For instance, the flagella of *Spirochaetes* are located in the periplasm, between the cell membrane and outer membrane sheath, while *Vibrio* species express both polar and lateral flagellar systems that share a chemotaxis transduction system but use different motive forces (Na^+ or H^+). Flagella can also serve in either swarming or swimming type motility, and can rotate either only clockwise or counterclockwise, or be able to switch direction depending on environmental signals. Furthermore, some flagella are always expressed while others are expressed only under certain environmental conditions [73, 80, 82, 170]. Many of the flagellar proteins are homologous, however, not all flagellar system proteins are conserved among all bacterial species. For example, the flagellar structures of Gram-positive bacteria do not have the L and P rings (which would be located in the outer membrane of Gram-negative bacteria). *Spirochaetes* do not have the L and P ring either, as their flagella are located in the periplasm. Some of the structural genes (*flgH, flgI, fliD, fliE* and *fliH*) are missing in some bacteria; this could indicate a later evolution of these genes combined with limited horizontal transfer, or be an example of sporadic loss of genes from some bacterial families. The latter explanation seems more likely in this case as there are many families of bacteria that contain these genes, and only a few who are lacking [170].

The flagella phylogenetic tree is directly related to that of the bacterial speciation genetic tree based on 16S ribosomal RNA. This suggests that flagella have been in existence since before the diversification of bacteria, and have been maintained throughout vertical evolution [81]. Liu and Ochman propose that the entire flagellar system is actually evolved from a single gene. They suggest, based on sequence similarities, that all of the flagellar genes arose from

random duplications and reassortments of a single precursor gene in the ancestor of modern bacteria [80, 170]. This seems quite unlikely; although there may be sequence similarities between an inner membrane component and an outer membrane component, this does not mean that they are related on an evolutionary scale. Convergent evolution is a more likely explanation for this, in which two different proteins have evolved to serve a similar function – in this case to be embedded in the bacterial membrane.

Unlike flagellar systems, the T3SS phylogenetic tree is not related to that of 16S ribosomal RNA, suggesting that T3SSs were acquired at some point after the diversification of bacteria, and evolved via horizontal transfer events [31, 76, 80, 171]. T3SSs are encoded on large pathogenicity islands, while flagellar genes are encoded on the chromosome [32, 52, 77]. It is thought that SPI-2 may have arrived in two separate events, with the *ttr* operon arriving first, followed by the rest of SPI-2 [9].

The effectors of T3SSs are highly variable between species of bacteria, and are quite often encoded on different regions of the chromosome than the pathogenicity island-encoded T3SSs. The effectors and their evolution will not be discussed here, but information on this topic can be found in a review by Stavrinides *et al.* [88]. In general, there are about 10 core proteins of the flagellar T3SS apparatus and the injectisome T3SSs that are highly similar in gene sequence, amino acid sequence, and function (Figures 1.4 and 1.5). For the purposes of this discussion, the flagellar system will be compared only with the two *Salmonella* T3SSs, with homologous proteins given in the order flagella/SPI-1/SPI-2. These homologous proteins are: the cytoplasmic ATPase (FliI/InvC/SsaN), the T3S apparatus (FliH/PrgH/SsaK, FliN/SpaO/SsaQ, FliP/SpaP/SsaR, FliQ/SpaQ/SsaS, FliR/SpaR/SsaT, FlhB/SpaS/SsaU and FlhA/InvA/SsaV), part of the connecting rod (FliF/PrgK/SsaJ), and the needle/hook 'molecular ruler' (FliK/InvJ/SsaP) [1, 75, 78, 130, 172, 173].

The structure of the flagellar apparatus and T3SSs begin to differ more markedly starting at the outer membrane (besides the motor-stator which is only present in the basal body of the flagellar system). The MS ring of the flagellar system is larger than that of the outer membrane secretin of the T3SS [79]. The secretin of the T3SS belongs to the same family of proteins that make up the T2SS and T4SS secretins, and the pore used by filamentous phages, suggesting that filamentous phages either introduced this type of protein to bacteria, or acquired it from them [171]. The T3SS needle is straight and thin, as is its filament, although the filament is slightly

larger, and notably rigid. The flagellar hook apparatus is larger and curved, and its filament is quite long and flexible. These structures lack significant amino acid and genetic homology, but do share helical symmetry, and overall assembly mechanisms. They are both assembled by stepwise polymerization of individual small monomers. The flagellum contains approximately 5.6 subunits of flagellin per turn, with an axial rise of 4.7 Å. To compare, the filament of the LEE T3SS in *E. coli* contains 5.5 subunits of EspA per turn, and has an axial rise 4.6 Å [73, 78, 79, 174]. The inner diameter of the T3SS filament is between 2 and 3 nm, similar to the inner channel of the flagellum [73, 78, 172]. The action of the 'molecular rulers' is likely different as well. It has been proposed that the method for measuring hook length in flagella is more 'measuring cup'-like than 'molecular ruler'-like. Journet suggests that the motor-stator switch area of the flagellum acts as a measuring cup, filling with FliK. FliK acts as an accessory to the hook protein, and is secreted at the same time. Once the 'cup' empties of FliK, the apparatus switches its secretion preference from the hook protein (FlgE) to the flagellin protein (FliC or FljB), completing assembly of the flagellum. In contrast, the InvJ and SsaP proteins (similar to the 'molecular rulers' of other bacterial T3SSs) act more like a ruler. It has been suggested that dimers of these proteins are located outside the cell, with one attached to the outer membrane, and the second extending from that. Once the needle complex (PrgI/SsaG) reaches the same height as the InvJ/SsaP dimer, the T3SS switches to secretion and assembly of the translocon of SPI-1 (SipB, C and D) or filament of SPI-2 (SseB) [73].

Another key area in which the T3SSs and flagellar systems differ is in their chaperones. Although both systems tend to have specific chaperones for specific proteins, the T3SS proteins are recognized by their chaperones at an N-terminal region, while flagellar system chaperones bind at the C-terminal region [170]. Although the flagellar and T3SS chaperones are different, in some cases the three systems can secrete each other's proteins. For example, both the SPI-1 and SPI-2 T3SSs can secrete the flagellar protein FliC, while the flagellar system can secrete the SPI-1 T3SS effector proteins SptP and SopE (if the SptP and SopE chaperones are absent), and in some instances effectors from T3SSs of other bacterial species [73, 78].

1.4 Pathogenesis and host response

1.4.1 Pathogenesis of *Salmonella*

Salmonella can enter host cells in at least two ways. The first involves uptake into phagocytic cells (macrophages), while the second is more complicated and involves the action of the SPI-1 T3SS on non-phagocytic cells. After attachment to epithelial cells, the SPI-1 T3SS induces membrane ruffling by secreting effectors into the host cell to trigger cytoskeleton rearrangement. Once inside the epithelial cell, some of these same effectors 'switch off' the membrane ruffling, returning the host cell membrane to its original state [7, 96, 137, 175]. Entry into the host cell (epithelial or macrophage) results in the bacteria being encased within an SCV. While the goal of many intracellular pathogens would be to escape this vacuolar space into the cell cytoplasm, *Salmonella* takes advantage of this space and remains in the SCV [7, 96, 176].

Once inside the SCV, the SPI-2 T3SS is expressed and begins secreting effector proteins, which are used to manipulate the intracellular environment [61, 96, 177]. Approximately one hour after entry into the host cell, the SCV switches from early endosomal markers, such as early endosome marker 1 (EE-1), to late endosomal/lysosomal markers, such as lysosomal-associated membrane protein (LAMP-1) and lysosomal glycoproteins (lgps). One important factor that the SCV acquires during this switch is the V-ATPase, which facilitates the acidification of the SCV. This acidification is an important factor for the induction of *Salmonella* virulence/survival genes [7, 87, 96, 176-178]. Another important factor for *Salmonella* survival within host cells is iron acquisition. *Salmonella* releases two siderophores for sequestering Fe^{2+} from the host cell, enterobactin and salmochelin [96]. As the SCV matures, it moves along host cell microtubules towards the Golgi apparatus. This process is dependent on many effectors, including SifA, SifB, SopD2, SseF, SseG, SseI, SseJ, SseL, PipB, and PipB2 [87, 96, 177]. SsaB is also important in blocking the fusion of the SCV with lysosomes during this process, which would result in bacterial killing [178]. Movement along the microtubules involves recruitment of a dynein-dynactin motor complex by SifA, SseF, SseG and PipB2. PipB2 interacts with the motor protein kinesin, while the other three proteins have also been shown to be responsible for keeping the SCV localized to the Golgi apparatus in an unknown manner. These proteins are also very important in *Salmonella*-induced filament (sif) formation, which will be discussed in the following paragraph. SCV membrane integrity is important, and is controlled by a number of

SPI-2 T3SS effectors, including SspH2, SseI, SteC, and the *Salmonella* virulence plasmid-encoded protein SpvB. The interaction of these proteins with host filamen and actin causes the formation of an actin-mesh around the SCV [87, 177, 178]. Another function of the SPI-2 T3SS may be to stop the formation of the NADPH phagocytic oxidase (phox) and inducible nitric oxide synthase (iNOS) on the SCV membrane, ultimately resulting in protection of *Salmonella* from reactive oxygen and nitrogen species (ROS and RNS, respectively) [7, 87, 179]. A superoxide dismutase encoded by the Gifsy-2 lysogenic phage helps *Salmonella* survive the oxidative burst, which involves production of ROS and RNS by phagocytic cells that can damage bacteria, and is therefore important for bacterial survival within the SCV [7, 96].

The maturation/movement process of the SCV can take around 4 to 6 hours. At this point, when the SCV has been altered to suit the bacteria, *Salmonella* begin to replicate [87, 180]. Replication of *Salmonella* is associated with the formation of sifs. Sifs have similar markers to the SCV, and many of the same proteins are responsible for their formation/membrane integrity (SifA, SifB, SseF, SseG, SseJ, SseL, SspH2, SpvB, PipB and PipB2). The SPI-1 effector SipA has also been shown to be important in sif formation. These sifs extend from the SCV towards the host cell membrane, and other SCVs, if there are multiple SCVs in one cell [96, 177, 178].

The AvrA effector secreted by the SPI-1 T3SS deubiquitinates both IκB-α and β-catenin, which stabilizes the proteins and results in the continued repression of NFκB-mediated gene transcription. This delays apoptosis of intestinal epithelial cells, thereby allowing *Salmonella* to survive within them for longer [86, 96, 176, 181]. SlrP also mediates ubiquitination of certain host proteins including Thioredoxin-1 (Trx1). Trx1 can activate the NFκB transcription factor, and has functions among other host cell proteins as well. Binding of SlrP to Trx1 stops its action, which under some conditions can lead to apoptotic cell death, although the exact mechanisms of this need to be studied further [86, 176, 177]. SspH1 can also inhibit NFκB transcription [96, 177, 178].

1.4.2 Innate immune response to *Salmonella*

The innate immune system is the first line of defense between the host and microbes from the environment, including parasites, fungi, bacteria, and viruses. The most complex portion of the innate immune system is that of the intestines. The intestinal surface consists of a single layer of columnar epithelial cells that form tight junctions with each other, and is covered by a thick

layer of mucous, and acts as a physical barrier preventing the access of microbes to the underlying tissue [182, 183]. Furthermore, the large amount of commensal bacteria in the intestine may act to prevent *Salmonella* from invading intestinal epithelial cells by competing for nutrients and/or the production of antimicrobial peptides [183, 184]. In fact, recent studies have shown that the inflammatory process is necessary for *Salmonella* to outcompete commensal bacteria and colonize the intestinal tract, and that the SPI-1 and SPI-2 T3SSs are very important in this process [185-188].

Within the intestine there are areas referred to as gut-associated lymphoid tissue (GALT), which consist of Peyer's patches (PPs) and mesenteric lymph nodes (MLNs). The PPs contain B cell follicles and T cell areas, and are infiltrated by large numbers of macrophages and dendritic cells. The cells of the follicular associated epithelium overlying the PPs have a less pronounced brush border and are covered by a lighter layer of mucous than the other intestinal epithelial cells. Microfold (M) cells that reside within the follicular associated epithelium have no surface villi, and do not produce mucous. These cells can pass on antigens to underlying dendritic cells and macrophages within the subepithelial dome [183, 189, 190].

Intestinal epithelial cells and paneth cells are capable of secreting antimicrobial peptides such as defensins, cathelicidins, and calprotectins. These antimicrobial peptides can act on a broad spectrum of bacteria (including *Salmonella*) by creating pores in the bacterial cell wall, ultimately leading to the destruction of the bacteria [182, 183]. It has been shown that these antimicrobial peptides are dependent on bacterial contact to act, and are generally restricted to within the layer of mucous. Pathogenic bacteria, like *Salmonella*, come into contact with these antimicrobial peptides when penetrating the mucous layer [191, 192].

Intestinal epithelial cells, dendritic cells and macrophages possess special pattern recognition receptors (PRRs) that are capable of recognizing microbes. These are the Toll-like receptors (TLRs) and nucleotide-binding oligomerization domain-like receptors (NLRs). TLRs can be found on the plasma membrane of cells and endosomal membranes while NLRs are found within the cytoplasm [181, 184]. Of particular importance in the recognition and clearance of pathogens is TLR5, which binds to the core-conserved portion of bacterial flagellin [193, 194]. *Salmonella* secretes two forms of flagellin, FliC and FljB, both of which are recognized by TLR5 and the NLR Ipaf. *Salmonella* can trigger translocation of secreted flagellin across the epithelium to the basolateral side where TLR5 is expressed. This translocation process is dependent on both

the SPI-1 and SPI-2 T3SSs. This suggests that *Salmonella* has evolved to take advantage of TLR5 signaling and the subsequent inflammatory reaction produced [181, 193, 195, 196]. SPI-2 T3SS dependent transport of flagellin through the epithelial cell, and subsequent binding of flagellin to TLR5, activates myeloid differentiation primary response gene 88 (MyD88), which goes on to activate interleukin (IL) receptor associated kinase 4 (IRAK-4). IRAK-4 activates the mitogen activated protein (MAP) kinases p38 and ERK, as well as the IκB kinase, which results in the activation of NFκB-mediated expression of pro-inflammatory factors such as IL-8, IL-1β, IL-12, IL-18, and tumour necrosis factor α (TNFα). Anti-apoptotic factors are also induced via this pathway, resulting in the delayed cell death of intestinal epithelial cells harbouring *Salmonella* [181, 184, 197]. SPI-1 T3SS-mediated binding of flagellin by the NLR Ipaf results in activation of caspase-1, which in turn results in pyroptosis of macrophages and the release of pro-inflammatory cytokines such as IL-1β, IL-12, and IL-18 [184, 196, 197]. *Salmonella* mutants that do not produce FliC and FljB are inhibited in their ability to cause inflammation in the host, indicating the importance of flagellin in the infection outcome [184, 196]. Furthermore, mice that do not express TLR5 are resistant to colonization by *Salmonella*, another indication that the induction of an inflammatory response by flagellin binding to TLR5 is important in colonization by *Salmonella* [194].

dendritic cells that reside within the lamina propria can extend dendrites out through the tight junctions of intestinal epithelial cells in order to sample the content of the intestinal lumen, including pathogenic bacteria such as *Salmonella*. This process is dependent on CX3 chemokine receptor 1 (CX3CR1), and if the gene coding for this receptor is knocked out in mice, they show a higher susceptibility to typhoid-like infection by *Salmonella* [183, 194]. This indicates that a functional innate immune system is essential for control of *Salmonella* before the infection becomes systemic. Alternately, dendritic cells may also capture bacteria, and serve as another mechanism for *Salmonella* to traverse the intestinal epithelial cell barrier [198].

Although *Salmonella* can invade any intestinal epithelial cells, an ideal spot for entry is through the M cells, as the mucous layer coating these cells is thinner, and there are fewer antimicrobial peptides for the bacteria to come into contact with [194, 196, 198]. Once through the epithelial layer, *Salmonella* comes into contact with resident or recruited macrophages and dendritic cells. Macrophages and dendritic cells phagocytize the bacteria, and then begin to migrate. The ability of *Salmonella* to survive within the SCV in these cells is important for

dissemination of the bacteria to the liver and spleen in the case of systemic infection [194, 196]. However, systemic infection caused by *S.* Typhimurium and *S.* Enteritidis does not normally occur in healthy human adults, who will experience a self-limiting gastroenteritis instead. An effective innate immune response is necessary to clear *Salmonella* from the intestinal tract and stop systemic spread. Recruited macrophages, natural killer (NK) cells, and dendritic cells are major mediators of this response [184, 185, 194]. When dendritic cells that reside in the lamina propria are stimulated with flagellin produced by *Salmonella* they stop producing IL-10 and TNFα, and instead switch to producing IL-6 and IL-12, which induces an inflammatory response. *Salmonella* produces effectors that can both induce and inhibit the production of pro-inflammatory cytokines, depending on the stage of infection [184, 194].

Intestinal epithelial cells can regulate the function of dendritic cells and macrophages by secreting cytokines such as thymic stromal lymphopoeitin (TSLP), prostaglandin E_2, and transforming growth factor β (TGFβ). TGFβ suppresses NFκB activation in macrophages and dendritic cells, thereby limiting expression of pro-inflammatory cytokines. In the presence of TSLP, macrophages and dendritic cells fail to produce IL-12 when they come into contact with bacteria, and instead produce IL-10 [182, 189, 199]. IL-12 can activate NK cells to produce interferon γ (IFNγ), which in turn activates macrophages to kill intracellular bacteria. In contrast, IL-10 is a suppressive cytokine that acts on both macrophages and dendritic cells to inhibit their production of IL-12, resulting in a feedback loop that limits inflammation. Therefore, when secretion of IL-12 is inhibited, *Salmonella* can survive longer within macrophages and dendritic cells. Both IL-10 and IL-12 can also regulate the type of immune response by the adaptive immune system (discussed further in section 1.4.3). Production of TNFα, IL-1, and other chemokines by intestinal epithelial cells and macrophages also act to recruit leukocytes to the site of infection, which are important in limiting systemic spread of *Salmonella* [182, 189, 194, 198].

Salmonella is able to both induce and inhibit the innate inflammatory response in the intestinal mucosa of its host. This is beneficial not only to regulating the infection process as described above, but can benefit the bacteria on a population-wide scale. Inflammation leading to diarrhea facilitates the spread of the bacteria to new hosts. Induction of autophagy and/or pyroptosis in some phagocytic cells may help in systemic spread if intact bacteria located within membrane compartments are then taken up by other macrophages and dendritic cells [184, 194, 195]. The SPI-1 T3SS effector SopE is important in activation of NFκB signaling and therefore

pro-inflammatory cytokine production. A second SPI-1 T3SS effector, SptP, is secreted later and acts to stop this effect. This further suggests that *Salmonella* initially takes advantage of the innate inflammatory response, but must dampen that response before it succeeds in clearing the bacteria [195]. *Salmonella* can induce macrophage cell death, termed pyroptosis, in both a SPI-1- and SPI-2-dependent manner. The SPI-1 T3SS effector SipB, flagellin, and the host cell protein caspase-1 mediate rapid macrophage death shortly after entry of *Salmonella* into macrophages. The active form of caspase-1 acts to process the pro-inflammatory cytokines IL-1β and IL-19 into their mature form, and induces formation of membrane pores in the macrophage. Delayed macrophage death by pyroptosis can also be induced by the SPI-2 T3SS and caspase-1 in the same manner [198, 200-202]. Death of macrophages early on in infection allows replicating *Salmonella* to spread further to uninfected macrophages and dendritic cells, while delayed macrophage death enables *Salmonella* to disseminate to, and invade, cells at systemic sites like the liver and spleen. Proper regulation and cross-talk between the flagellar, SPI-1, and SPI-2 T3SSs are essential for successful coordination of these events. A recent study showed that caspase-1-deficient mice were more resistant to colonization by *Salmonella*, indicating that activation of caspase-1, and thereby the innate inflammatory response, is important for *Salmonella* to spread within the host [184, 194].

A side effect of this inflammatory process is the disruption of tight junctions between intestinal epithelial cells, providing *Salmonella* with easier access to underlying tissue, while subsequent recruitment of macrophages and dendritic cells gives *Salmonella* a place in which to replicate (within the SCV) and disseminate throughout the host [187]. The SPI-2 T3SS effector SseI interacts with the host cell protein TRIP6 to stimulate motility of macrophages and dendritic cells, facilitating the systemic spread of the bacteria [195]. As well, the release of antimicrobial peptides into the intestinal lumen may disrupt the growth of normal commensal bacteria, clearing the way for *Salmonella* to invade intestinal epithelial cells. Inflammation also causes the release of certain nutrients from mucous that *Salmonella* can take advantage of to speed bacterial growth [187].

The innate immune response to *Salmonella* is very complex, and not completely understood. There is contradictory evidence about whether the mucosal inflammatory response facilitates or limits the spread and invasion of *Salmonella*. It is likely that the answer is not black-and-white, and that *Salmonella* needs to strike a balance between induction of

inflammation and control of this response. The inflammatory response allows *Salmonella* to more effectively compete with the resident commensal bacteria, cause diarrhea facilitating its spread to other hosts, and bring in macrophages and dendritic cells. *Salmonella* can then enter macrophages and dendritic cells and disseminate throughout the host. However, the bacteria must also ensure that the inflammatory response subsides before it can act to clear the infection and activate an adaptive immune response. The level of innate immune response induced by *Salmonella* likely determines the differing disease outcomes caused by different serovars in different hosts; be it an asymptomatic infection, gastroenteritis, or systemic disease.

1.4.3 Adaptive immune response to *Salmonella*

In comparison to the innate immune response to *Salmonella*, Much less is known about the adaptive immune response and its role in disease outcome. Most studies regarding the adaptive immune response to *Salmonella* have been done in mice [203, 204]. Early in infection, dendritic cells can respond to LPS and flagellin of *Salmonella* by increasing their expression of major histocompatibility complex (MHC) class II and other co-stimulatory molecules. These dendritic cells are a major link between the innate and adaptive immune response, and can stimulate the proliferation of Th1 $CD4^+$ T cells [190, 198]. Macrophages (and dendritic cells) are also important for production of IL-18 and IL-1β in response to SPI-1-/caspase-1-mediated pyroptosis early on in the infection process. IL-18 stimulates naïve and memory Th1 $CD8^+$ α/β T cells, and NK cells, to produce IFNγ, which in turn stimulates macrophages to become more efficient killers [188, 205]. Macrophages and dendritic cells can also produce IL-15 and IL-23, which stimulates proliferation of γ/δ T cells, NK like T cells, and Th17 $CD4^+$ α/β memory T cells. Th17 T cells produce IL-17 and IL-22; dendritic cells and NK-like T cells can also produce IL-22 [188, 206]. IL-17 activates neutrophils, and IL-22 is important in stimulating the production of antimicrobial peptides by epithelial cells [188, 207]. $CD4^+$ α/β memory type T cells are important in the later stages of infection, during systemic disease. Without these T cells, persistent infection can develop and lead to eventual fatality [206].

In addition to T cells, B cells have also been shown to be important in protection against infection with *Salmonella* after vaccination in mice. This is likely through their interaction with Th1 memory T cells as they are not important in clearance of primary infection with *Salmonella*. In fact, a protective Th1 response does not take place in mice depleted of B cells, and humans

with immunodeficiencies in B cells or antibody production are much more susceptible to severe infections by *Salmonella* [198, 204, 206]. Early after infection, *Salmonella*-specific IgM is produced, followed by IgG in the serum and at the intestinal mucosa. Interestingly, IgA appears only to be produced in the intestine if *Salmonella* specifically enters the PP through M-cells [206, 208]. There is evidence that *Salmonella*-specific antibodies contribute to disease resolution and protection in secondary infection in both humans and mice. However, passive transfer of these antibodies to naïve mice does not confer full protection against infection with *Salmonella*. These antibodies may aid in controlling extracellular bacteria through opsinization, leading to faster uptake and bacterial killing by professional phagocytes [204, 209-211]. However, it is unclear whether all *Salmonella* actually go through an extracellular phase in systemic disease [198]. It may be that after initial entry into professional phagocytes in the PP, *Salmonella* stays protected within these cells. After inducing SPI-1- or SPI-2-mediated pyroptosis of macrophages and dendritic cells, *Salmonella* may stay contained within host cell membrane fragments, which would be taken up by other professional phagocytes. In this case, *Salmonella* would not need to enter an extracellular state until it had reached the liver or spleen, and only then if entry into non-phagocytic cells was beneficial.

1.4.4 Disease outcome

The outcome of disease is dependent on the serovar, host, and host immune response [13, 198, 204]. For instance, the host-adapted serovars *S.* Typhi and *S.* Paratyphi cause typhoid fever in humans and some primates, while other host-adapted serovars like *S.* Choleraesuis (pigs) and *S.* Dublin (cattle) are capable of causing bacteremia in humans, as well as typhoid-like disease in their respective hosts. The non-host-adapted strains *S.* Typhimurium and *S.* Enteritidis can cause a variety of diseases in a variety of hosts. *S.* Typhimurium and *S.* Enteritidis are capable of causing gastroenteritis in cows and pigs. In humans, *S.* Typhimurium and *S.* Enteritidis generally produce self-limiting gastroenteritis, but in rare occasions can instead cause typhoid-like systemic disease. In susceptible mice, *S.* Typhimurium and *S.* Enteritidis cause a lethal typhoid-like disease while resistant mice can develop chronic infection. In streptomycin pre-treated or gnotobiotic mice, *S.* Typhimurium and *S.* Enteritidis cause symptoms resembling gastroenteritis. In contrast, infection with *S.* Typhimurium and *S.* Enteritidis results in an asymptomatic carrier state in most healthy adult chickens [13, 181, 204].

Typhoid fever results when *Salmonella* are able to traverse the intestinal epithelial cell barrier without causing a major inflammatory response, and disseminate to systemic sites via macrophages, dendritic cells, and other professional phagocytes. In humans, typhoid disease occurs one to two weeks after ingestion of *Salmonella*, and results in fever, malaise and abdominal pain. Disease typically resolves after a period of weeks, but can result in carriage and spread of the bacteria for months and even years. In chickens, *S.* Typhimurium and *S.* Enteritidis infections are asymptomatic, and often result in a carrier state with animals shedding bacteria for long periods of time. Systemic disease in chickens follows infection by *S.* Gallinarum (fowl typhoid) and SP (Pullorum disease). *S.* Typhi and *S.* Paratyphi cause typhoid fever in humans, while a typhoid-like systemic disease resulting from *S.* Typhimurium and *S.* Enteritidis occurs only rarely, usually in the immunocompromised individual. Typhoid-like disease in mice is caused by both *S.* Typhimurium and *S.* Enteritidis infection, and is the primary model for understand typhoid in humans [15, 198, 203, 204, 212].

The progression of systemic disease can be roughly broken down into four phases. Phase 1 is the initial stage of systemic infection that occurs after invasion of *Salmonella* across the intestinal epithelium and into dendritic cells and macrophages in the PP. Clearance of bacteria during phase 1 is usually rapid, and occurs in the first day of infection. An appropriate effective innate immune response (one that doesn't result in symptoms of gastroenteritis) is required for clearance of the bacteria at this point. Phase 2 occurs between days 2 and 7, and involves growth of the bacteria in phagocytes, and dissemination to, and growth at, systemic sites such as the liver and spleen. Recruitment of macrophages, dendritic cells, neutrophils, and NK cells is required for control of infection at this stage. In immunocompromised individuals, failure to control the disease at this stage leads to massive bacterial replication and death of the host. Phase 3 can last up to a month, and involves a plateau in bacterial numbers. Without proper control at this point, the host can become a prolonged carrier, instead of clearing the bacteria, and relapse of disease and fatality can also occur. Phase 4 involves clearance of bacteria that requires the action of T cells. Control at this point requires an appropriate adaptive immune response mainly involving Th1 $CD4^+$ α/β T cells [203, 204, 210, 212, 213].

Gastroenteritis occurs as a result of an innate immune inflammatory response at the intestinal mucosa and very rarely results in systemic spread. In humans, gastroenteritis occurs 6 to 72 hours post-ingestion of large amounts of bacteria and is marked by abdominal pain and

cramps, diarrhea and vomiting. Disease is self-limiting, and typically resolves within a week in healthy adults, but can progress to systemic disease in the immunocompromised [13, 198, 203, 204]. Controlling disease before onset of systemic infection requires an appropriate innate immune response. People with innate immune system defects, including IFNγ, IL-12, or phox deficiencies, are prone to severe systemic infection with *S.* Typhimurium and *S.* Enteritidis [13, 206].

When infected by *Salmonella*, chickens produce both a cell-mediated and humoral immune response when infected with *Salmonella.* In response to *S.* Typhimurium and *S.* Enteritidis, *Salmonella*-specific antibodies (IgY, IgA and IgM) are produced, and proliferation of $CD4^+$ T cells and B cells in both the spleen and cecal tonsils is induced. As well, macrophages and *Salmonella*-specific T cells can be found in the ovaries, and correspond with a decline in bacterial numbers [214-216]. Both a strong *Salmonella*-specific T cell response and production of IFNγ have been found to be important for clearance of *S.* Typhimurium and *S.* Enteritidis in resistant chickens [217, 218]. In mice, *S.* Typhimurium and *S.* Enteritidis cause a systemic typhoid-like infection; however, in the streptomycin pre-treated mouse model, mice develop disease more reminiscent of gastroenteritis. In this model, $CD3^+$ T cells are in part responsible for the outcome of disease, enhancing inflammation and therefore symptoms of enteritis [198, 219].

It is clear that a balanced immune response involving both the innate and adaptive immune system are required to control the outcomes of *Salmonella* infection. Better understanding of how different serovars manipulate the host's immune system, and how resistant hosts differ from susceptible hosts, will improve our ability to intervene in both human and animal disease caused by *Salmonella*.

1.5 Control and treatment of *Salmonella*

1.5.1 Treatment of infections caused by serovars Typhimurium and Enteritidis

In humans, gastroenteritis caused by *S.* Typhimurium and *S.* Enteritidis is usually self-limiting and antibiotic intervention is not needed, although fluid replacement to combat dehydration is recommended. When systemic spread of the disease is a concern, such as in the immunocompromised, antibiotics are warranted. *S.* Typhimurium and *S.* Enteritidis can be

treated with chloramphenicol or antibiotics from the cephalosporin (ceftriaxone), fluoroquinilone (ciprofloxacin), penicillin (ampicillin), sulfonamide (trimethoprim-sulfamethoxazole), or cephalosporin (broad spectrum like ceftriaxone) groups [13, 220]. However, antibiotic resistance in *Salmonella* is becoming a major concern. Multiple-drug-resistant strains of both *S.* Typhimurium and *S.* Enteritidis, like *S.* Typhimurium phage-type DT104 (which is resistant to ampicillin, chloramphenicol, streptomycin, sulfamethoxazole, and tetracycline), are being found with increasing frequency. Recently, an *S.* Enteritidis strain was isolated from poultry in Korea that was resistant to 15 out of 21 antimicrobials tested, including antibiotics from the aminoglycoside, cephalosporin, fluoroquinilone, penicillin, sulfonamide, and tetracycline groups [221, 222].

1.5.2 Prevention of infection

In the past, there was widespread antibiotic use in feed animals at sub-therapeutic levels as growth enhancers, and to reduce levels of enteric pathogens. Recently, with the development of more multi-drug-resistant strains, it has been recognized that this antibiotic 'abuse' is a large factor in multi-drug-resistant strain development and has resulted in bans on antibiotic use in food animals in some regions. As more multi-drug-resistant strains of *Salmonella* are developing, the need for an effective strategy to control the spread of *Salmonella*, without reliance on antibiotics, needs to be developed [223-226].

1.5.2.1 Producer practices and consumer education

At the producer level there are many methods that can be employed to reduce levels of *Salmonella* in poultry flocks. Disinfectants like iodophore, peroxygen and formaldehyde/glutaraldehyde have been shown to reduce levels of *Salmonella* in the environment, but rodent and pest carriage of *Salmonella* remains a problem as they can reintroduce *Salmonella* to disinfected areas [227, 228]. Proper housing to ensure animals are in a clean and reduced stress environment are also important factors. Choice of chicken line can also be important, as some chicken lines are genetically resistant to colonization by *Salmonella* [229, 230]. Vaccination of flocks can also be an effective way to control *Salmonella* in poultry flocks (see section 1.5.2.4). *Salmonella* can be introduced into the food supply during packaging. Contamination of eggs and egg packing plants, as well as poultry meat and poultry meat packing

plants, can often be high, however, proper disinfection of surfaces and sterilization of products can reduce these levels. Passing products briefly through high temperature water (80 - 100°C), immersion in baths containing chlorine, iodine, hydrogen peroxide, ozonated water, electrolyzed water, organic acids, and UV light treatment have been somewhat successful in reducing levels of *Salmonella* on poultry products. [226, 231, 232]. As well, the type of equipment used can play a role in contamination; certain types of conveyor belts used in egg facilities harbour more *Salmonella* than others, even disinfection [233]. Organizations like the USDA and Canadian Food Inspection Agency (CFIA) have introduced Hazard Analysis of Critical Control Points (HACCP) programs. These programs outline a series of regulations that require monitoring of the production process at a series of critical control points, including controls at the farm level, transport, packaging and processing of poultry products, to distribution. The generic HACCP model for poultry slaughter in Canada can be found on the CFIA website [234]. For fairly comprehensive reviews on the types of disinfectant techniques and management practices that can be successful for reducing *Salmonella* levels in poultry flocks, and in packing plants, see Ricke *et al*. [226], Dinçer and Baysal [235], and Doyle and Erickson [236]. In order to maintain proper management practices, regulations and standards should be in place, as well as regular testing of poultry farms and packing plants [29, 229, 237-239].

Proper food handling and completely cooking poultry and eggs is essential to prevent the spread of *Salmonella* in humans. Humans can contract salmonellosis from products subject to improper handling or environmental contamination, as well as through direct interaction with animals carrying *Salmonella*. *Salmonella* has been isolated from numerous raw fruits and vegetables, juices, sauces, unpasteurized dairy products, seafood, chocolate, peanut butter, and even pet treats, among other products. Agencies like the CFIA, Center for Disease Control (CDC), World Health Organization (WHO), and the USDA, are trying to increase public education about food safety. One of their strategies includes a '4-point plan', with the four points being CLEAN (wash hands and surfaces), SEPARATE (keep raw and cooked foods separate), COOK (cook foods to the appropriate and safe internal temperature) and CHILL (keep opened jars and cooked foods refrigerated after use and during transport). Implementation of these proper hygiene techniques in the general public can be difficult as HACCP models can not be enforced on the general public. Therefore, public education remains the only option for implementing proper food handling and hygiene [220, 238, 240-244].

1.5.2.2 Feed additives, probiotics, prebiotics, and competitive exclusion

In response to the development of multi-drug-resistant strains, research is being carried out on methods to stop the spread of *Salmonella* that do not involve antibiotics. The area of prebiotics and probiotics, for both humans and animals, is a popular area of research, along with feed additives to boost the host's immune system [224, 225]. For example, β 1-4 mannobiose has been shown to enhance the killing activity of chicken macrophages against *S.* Enteritidis, and so has potential as a feed additive [224]. Mannan oligosaccharides (MOS), β-glucan, and butyric acid-based feed additives also have promise as they have been shown to reduce the colonization levels of young chicks by *Salmonella* [245-247]. MOS bind to a mannose-specific lectin in type 1 fimbriae-producing Gram-negative bacteria. This interaction results in expulsion of bacteria like *Salmonella* from the gastrointestinal tract [225, 247]. Short-chain fatty acids, like butyric acid, have a bacteriostatic effect on Gram-negative bacteria. When added to feed, this results in a reduction of shedding of *Salmonella* by colonized chickens [225, 246].

The gastrointestinal environment is home to around 10^{14} commensal bacteria termed the host microbiota [183, 225, 248]. In order for proper development of the PPs and MLNs, commensal bacteria must be present shortly after birth; as illustrated by the poorly developed PPs and immune deficiencies of gnotobiotic mice [183, 187, 248]. The method of discrimination used by the intestinal innate immune system is just beginning to be understood, and improper distinction between harmful and commensal pathogens by the immune system is thought to be responsible for chronic inflammatory disorders such as irritable bowel syndrome and Crohn's disease [183, 192, 199]. In feed animals, the levels of beneficial bacteria in gastrointestinal tract have been shown to change during periods of stress such as feed withdrawal, transport, or temperature change. Therefore, proper balance of the host's microbiota must be maintained for optimal gastrointestinal health, and the use of probiotics can help to maintain this balance [225].

Prebiotics and probiotics are easy to produce and are an inexpensive method to help combat the spread of *Salmonella* from feed animals to humans. Probiotics involve supplementation of a host with live bacteria that have a positive health benefit to that host. Bacteria of the *Lactobacillus* and *Bifidobacterium* genera have been shown to be protective against gastrointestinal infections, and are common food additives in yoghurt for human consumption [225, 249]. As the microbiota are part of the host's initial line of defense, proper levels of beneficial bacteria in the gastrointestinal tract can help protect chickens against

colonization by enteric pathogens like *S.* Typhimurium and *S.* Enteritidis. The microbiota can limit the growth of harmful bacteria within the host through competition for nutrients and/or the production of antimicrobial peptides and short-chain fatty acids [183, 184, 249]. The main probiotics used in poultry are *Lactobacillus, Enterococcus, Bacillus* and *Saccharomyces* [225]. Prebiotics are food additives that are non-digestible by the host and beneficial to probiotic organisms. Prebiotics can stimulate growth and/or the activity of the host's beneficial bacteria and add to the effectiveness of probiotics [225, 249].

Currently, competitive exclusion is the probiotic method of choice in poultry. Newly hatched chicks, which are most prone to infection by *S.* Typhimurium and *S.* Enteritidis, are given an oral dose of microbiota that has been isolated from a healthy adult chicken. There are several competitive exclusion preparations commercially available for the reduction of *Salmonella* in chickens including Avifree™, Aviguard™, Broilact, MSC™ and Preempt™ [225, 249, 250]. There are a few concerns regarding the use of probiotic strains: mainly, full characterization of strains in the preparation as well as full disclosure of the strains therein, and the possibility of virulence gene transfer between pathogenic bacteria and the introduced probiotic strains [225, 250].

1.5.2.3 Bacteriophages

Bacteriophages, also known as phages, are DNA or RNA viruses that target bacteria. Phages can be either lytic or lysogenic. Lytic phages replicate at a rapid pace within the bacterium, resulting in bacterial lysis, while lysogenic phages can integrate into the host (prophages), or exist within the bacterium as a plasmid. Lysogenic phages are major contributors to bacterial genetic evolution as they are capable of introducing new genes and/or transferring virulence genes between species of bacteria. In fact, many SPI-1 and SPI-2 effectors lie within prophage regions. In contrast to lysogenic phages, lytic phages are an attractive method to treat bacterial infection because of their properties, especially in light of the development of multi-drug-resistant bacterial strains [6, 220, 223, 251].

Despite its potential, there are drawbacks to phage therapy. For instance, phages tend to infect a narrow range of bacteria; so specific treatment regimens would have to be developed on a case-by-case basis. This specificity can be seen as an advantage, however, as treatment with these phages would not likely result in killing of the host microbiota. Alternatively, a preparation

containing multiple phage types could used to target the typical agents of disease producing the observed symptoms. A second drawback is that phages tend to be cleared from the hosts' bloodstream fairly quickly, and oral administration would result in the inactivation of many phages by stomach acid. Some work has consequently been done to isolate phages (specifically λ of *E. coli* and Φ22 of *S.* Typhimurium) that last in circulation much longer than the WT phages, making them better treatment candidates. Administration of stomach acid inhibitors would also improve oral inoculation conditions for the survival of the phage, potentially rendering the treatment more effective [223, 251, 252].

Phages have been used in human medical treatment since the 1930s, primarily in the historical Soviet Union. In fact, it has been reported that phages were used to treat dysentery and wound infections of Russian soldiers during World War II. Phage therapy on humans continues to be practiced in Poland and the Republic of Georgia, and is gaining attention elsewhere as multi-drug-resistant bacterial strains continue to develop [223, 251]. Beyond their potential for treatment of disease in humans, there have been a few studies to date regarding phage therapy in the prevention of *S.* Typhimurium and *S.* Enteritidis in chickens. A study in the early 1990s showed that, after inoculation with a *S.* Typhimurium-specific phage, young chicks were protected from fatal infection by *S.* Typhimurium [253]. A more recent study by Atterbury *et al.* showed that broiler chickens inoculated orally with *S.* Enteritidis-specific Φ151 or *S.* Typhimurium-specific Φ10 shed less bacteria in their feces upon challenge with *S.* Enteritidis or *S.* Typhimurium [254]. The few other studies using phage as treatment of *S.* Enteritidis or *S.* Typhimurium colonization of chickens have had variable results, and, in most cases, bacterial clearance was transient [223]. With so few studies regarding the use of phage therapy in chickens to prevent *S.* Typhimurium or *S.* Enteritidis colonization, it is clear that more research should be done in this area, especially in light of promising results with other enteric bacteria such as *E. coli*, reviewed in Johnson *et al.* [223].

1.5.2.4 Vaccination

1.5.2.4.1 Live vaccines

Effective live vaccines can induce a potent humoral and cell-mediated immune response, including generation of mucosal IgA. These types of vaccines often produce long-lasting

immunity, but fear of reversion to virulence and release of genetically engineered organisms into the environment can be a major concern. Spread of these organisms in the environment can also be a desirable quality, especially when vaccinating wild animal populations where not all animals can be reached for vaccination. In some cases the attenuated strain can cause disease in an immunocompromised host, which is also undesirable. Most live vaccines can be administered orally, allowing for ease of distribution to animals, including poultry, through drinking water. Attenuated strains should be able to survive within the host for long enough to sufficiently stimulate the hosts' immune system, but should not cause any symptoms of disease. Ideally, the vaccine should confer protection against more than one serovar [15, 206, 220].

A number of live attenuated vaccine strains have been tested in chickens. An *S.* Enteritidis *phoP/fliC* mutant was able to reduce colonization of chickens with *S.* Enteritidis. The *phoP* gene encodes the key global regulatory protein PhoP, while *fliC* encodes one of the flagellin subunits of *Salmonella* (FliC) [255]. Using a *S.* Gallinarum strain with deletions of the *cobS* and *cbiA* genes, which are involved in the vitamin B_{12} biosynthetic pathway, colonization of chickens by both *S.* Gallinarum and *S.* Enteritidis was reduced [256]. An *S.* Typhimurium strain with deletions of the adenylate cyclase (*cya*) and cyclic AMP receptor (*crp*), which are involved in carbohydrate utilization and cell surface expression of fimbriae and flagella, has also shown promise in reducing colonization by both *S.* Typhimurium and *S.* Enteritidis [257]. Finally, a *S.* Enteritidis *aroA* mutant was successful in reducing colonization of chickens by *S.* Enteritidis, but not *S.* Typhimurium; AroA being a key component in the aromatic biosynthetic pathway [258].

Live attenuated vaccines are available in North America, but are often not used. However, in light of recent *S.* Typhimurium and *S.* Enteritidis outbreaks and the successes in the EU at controlling the spread of *S.* Typhimurium and *S.* Enteritidis though vaccination, the use of live attenuated vaccines is becoming more attractive to North American producers [19, 259]. Gallivac® Se (also known as Salmovac *S.* Enteritidis) is a live attenuated *S.* Enteritidis strain that is an adenine-histidine auxotroph. Gallivac® confers significant protection of chickens against colonization by *S.* Enteritidis [260]. Megan®Vac 1 is a live attenuated *S.* Typhimurium *cya/crp* mutant that has been successful in reducing colonization levels of chickens by both *S.* Typhimurium and *S.* Enteritidis [261, 262]. TAD Salmonella vac® E (a live attenuated *S.* Enteritidis strain) and TAD Salmonella vac® T (a live attenuated *S.* Typhimurium strain) are both 'metabolic drift mutants' that carry mutations affecting essential enzymes and metabolism

genes and are capable of reducing colonization of chickens (and eggs) by *S.* Enteritidis and *S.* Typhimurium. As well, it has been demonstrated that TAD Salmonella vac® T has potential for cross-protection against *S.* Enteritidis [263].

1.5.2.4.2 Inactivated vaccines

Inactivated bacteria vaccines (also known as bacterins) and subunit protein-based vaccines (discussed in section 1.5.2.4.3) are capable of inducing a strong humoral response, especially when coupled with an appropriate adjuvant. They are attractive compared to live vaccines as there is no chance of reactivated virulence or of live genetically modified bacteria entering the environment. The drawback of these types of vaccines is that they often do not induce production of mucosal IgA, or elicit a potent cell-mediated immune response. Furthermore, higher loads of antigen need to be delivered, often over multiple doses. As well, many inactivated vaccines and most subunit vaccines require needle delivery, making them harder to distribute to poultry [220].

There are a number of inactivated *Salmonella* vaccines available. Gallimune® is an inactivated bacterial vaccine composed of both *S.* Typhimurium and *S.* Enteritidis. When used in combination with Gallivac®, laying hens can be protected against colonization by *S.* Typhimurium and/or *S.* Enteritidis [260]. Layermune *S.* Enteritidis is a bacterin containing multiple *S.* Enteritidis strains that can help reduce colonization of chickens by *S.* Enteritidis, especially when used in combination with one of the live vaccines [25]. Corymune 4K is a multivalent vaccine composed of several inactivated strains: *Avibacterium paragallinarum* serotypes A, B and C, and one *S.* Enteritidis strain. Corymune 7K contains the same strains as Corymune 4K, but also inactivated forms of Newcastle disease virus, infectious bronchitis virus, and egg drop syndrome virus. Vaccination with Corymune 4K and Corymune 7K confers moderate protection against colonization of chickens by *S.* Enteritidis [25]. Salenvac is an *S.* Enteritidis iron-restricted mutant bacterin that has been successful in reducing colonization of chickens by *S.* Enteritidis [264].

1.5.2.4.3 Subunit vaccines

There have been very few studies on the efficacy of subunit protein based vaccines for protection of poultry against colonization with *Salmonella*. One such study demonstrated that

chickens vaccinated with certain outer membrane proteins or FliC produce a strong serum antibody response, and have lower numbers of *S.* Enteritidis in their cecum [265-267]. A study by Kaneshige *et al.* explored the efficacy of the siderophore receptor IroN as a vaccine, and found that vaccinated chickens had a strong serum antibody response to the vaccine, as well as a lower mortality rate compared to non-vaccinates when challenged intravenously with heavy loads of *S.* Enteritidis [268]. Laying hens vaccinated with type 1 fimbriae of *S.* Enteritidis had less colonization of reproductive organs than control hens after challenge with *S.* Enteritidis, and eggs from vaccinates were less often contaminated by *S.* Enteritidis when compared to those of non-vaccinates [269].

1.5.2.4.4 Other immunization methods

Passive immunization involves transfer of serum containing microbe-specific antibodies from an exposed or vaccinated host to a naïve recipient. This transfer can be active (involving drawing of blood from one host and transfer to the recipient) or passive (as in maternal transfer of antibodies to progeny) [220]. Vaccinated hens pass large amounts of antibody to eggs, which can be purified for injection into naïve recipients, or may protect newly hatched chicks against colonization by *Salmonella* [270, 271]. Progeny of hens vaccinated with avirulent *S.* Typhimurium or *S.* Enteritidis strains have lower levels of colonization by *S.* Typhimurium or *S.* Enteritidis than progeny of non-vaccinates [271, 272].

DNA vaccines involve direct injection of a plasmid into the host cells (skin or muscle). These host cells go on to express the antigen of interest, exposing it to the local immune system where a response can be mounted. Defective infectious single-cycle (DISC) virus vaccines involve immunization with a virus that can only undergo one round of replication within the host. This type of system is intended to emulate the first stages of natural infection, resulting in a protective immune response without progression to disease. This type of strategy could be useful for viral diseases as well as for delivery of protein subunit type vaccines using the DISC virus as a delivery method for the antigen [220]. There is almost no research in these areas in regards to the development of a poultry vaccine to combat *Salmonella* colonization. However, one recent study showed that when a DNA vaccine encoding the SopB protein of *S.* Typhimurium was administered to mice in conjunction with a live attenuated vaccine *S.* Typhimurium strain, greater protection of the mice against fatal *S.* Typhimurium infection was achieved than with the live

attenuated strain alone [273].

1.5.2.4.5 *Salmonella* as vaccine vectors

A plethora of studies have recently explored the potential of attenuated *Salmonella* strains as vaccine vectors for other pathogens. Most of these studies involve delivery of plasmid-encoded antigens by *Salmonella* T2SSs and T3SSs to the host. Using attenuated *Salmonella* strains that can be delivered orally and still cross the intestinal epithelial cell barrier and progress to deeper systemic sites can help ensure production of a memory T cell response against the desired antigen. These types of attenuated *Salmonella* strains are excellent at inducing long-lasting protective immunity at both mucosal and systemic sites, but are difficult to develop while still ensuring vaccine strain safety [274, 275]. There are also studies involving integration of the antigen into the *Salmonella* genome by fusion to secreted T3SS effectors of SPI-1 and/or SPI-2. This method has been used to study the delivery of both anti-microbial and anti-tumor antigens, as well as the delivery of anti-inflammatory cytokines for the treatment of inflammatory diseases [276, 277]. There have also been studies involving *Salmonella* delivery of protective *Streptococcus pneumoniae*, *Mycobacterium tuberculosis* and human immunodeficiency virus (HIV) antigens, among others [278-280]. For recent comprehensive reviews of these strategies see Curtiss *et al.*[274], Shahabi *et al.* [275], and Chmekh [276].

Live *Salmonella* strains that can induce protective immunity against the antigen it is delivering as well as immunity to *Salmonella* would be an effective tool for the poultry industry in preventing the spread of food-borne pathogens and promoting the health of immunized animals. For instance, a live attenuated *S.* Typhimurium vaccine strain, harbouring a plasmid coding for a truncated protein of *Clostridium perferingens* that is already known to reduce necrotizing enteritis in chickens, could also protect chickens against colonization with *S.* Typhimurium and *S.* Enteritidis [281]. There have been studies involving *Salmonella* delivery of protective *Streptococcus pneumoniae*, *Mycobacterium tuberculosis* and human immunodeficiency virus (HIV) antigens, among others [278-280].

1.5.3 Comprehensive approach

As with most multi-host pathogens, no one method of control on its own will eliminate *Salmonella*. While certain vaccination regimes and disinfection can reduce *Salmonella* on the

farm, consumer education about proper food handling and storage is still needed. A combination of proper hygiene at the farm and consumer levels, coupled with vaccination regimes, and/or pre- and probiotics, could help to reduce the levels of *S.* Typhimurium and *S.* Enteritidis in poultry, and therefore, prevent the spread of *Salmonella* gastroenteritis to humans. For a comprehensive review on *Salmonella* reduction strategies in the food supply, see Doyle and Erickson [236]

2.0 HYPOTHESIS AND OBJECTIVES

2.1 Hypothesis

The *Salmonella enterica* subspecies *enterica* serovar Enteritidis (*S.* Enteritidis) *Salmonella* pathogenicity island (SPI) 2 type III secretion system (T3SS) is important for colonization of chickens and systemic spread, as well as survival of *S.* Enteritidis in chicken macrophages. Immunization with *S.* Enteritidis SPI-2 T3SS proteins will have a protective effect against *S.* Enteritidis colonization of chickens.

2.2 Rationale

Infections by *Salmonella enterica* subspecies *enterica* serovars Typhimurium (*S.* Typhimurium) and Enteritidis (*S.* Enteritidis) are one of the most common causes of bacterial food borne gastroenteritis (food poisoning) in humans worldwide. *Salmonella* uses two T3SSs, encoded on SPI-1 and SPI-2, to cause disease and spread systemically throughout the host. The SPI-2 T3SS has been shown to be necessary for systemic infection and survival of *Salmonella* in macrophages, however much of this work has been done in mice who develop a typhoid-like illness, and not in chickens who usually become asymptomatic carriers. Because *S.* Typhimurium and *S.* Enteritidis are passed to humans via consumption of contaminated poultry meat, water, and eggs, there is a need for control of *Salmonella* colonization of chickens. Many live vaccines have been shown to confer protection against colonization of chickens by *S.* Typhimurium and *S.* Enteritidis, however, there are many safety concerns associated with live vaccines. Thus, we sought to better understand the role of the SPI-2 T3SS in survival of *Salmonella* in chicken macrophages. Furthermore, we examined the role of the SPI-2 T3SS in cecal colonization and systemic spread of *S.* Enteritidis in chickens. Finally, we examined the potential of various SPI-2 T3SS structural and effector protein vaccines to protect chickens against colonization by *S.* Enteritidis.

2.3 Objectives

1. To construct mutants of *S.* Enteritidis strain Sal18 missing the entirety of SPI-2, and test the ability of these mutants to colonize chickens in comparison to the wild-type (WT) strain.

2. To clone genes encoding potential vaccine candidates, overproduce, and purify said products, and test their ability to protect chickens against colonization by *S.* Enteritidis.
3. To test the ability of the SPI-2 mutant strains to survive in macrophages in comparison to the WT strain.

3.0 THE ROLE OF THE *S.* ENTERITIDIS PATHOGENICITY ISLAND 2 TYPE III SECRETION SYSTEM IN INTESTINAL COLONIZATION OF CHICKENS AND SYSTEMIC SPREAD

The information presented in this chapter has been previously published in Microbiology [282].

3.1 Introduction

Salmonella enterica subspecies *enterica* serovar Enteritidis (*S.* Enteritidis) is an important human pathogen that causes salmonellosis in humans. *S.* Enteritidis is passed to humans mainly via poultry meat contaminated at the time of slaughter and by consumption of contaminated water. In addition, eggs are often heavily colonized by *S.* Enteritidis, and humans can contract salmonellosis via consumption of raw or partially cooked eggs. Chickens colonized with *S.* Enteritidis typically do not show disease symptoms, and whole flocks can become colonized quickly, shedding bacteria in their feces for months [23, 24].

Infection caused by *Salmonella enterica* is the second most common cause of bacterial gastroenteritis (food poisoning) in the developed world, resulting in significant economic loss to the poultry industry and a substantial burden on the health care system [24, 265]. Because there is a need to control the spread of *S.* Enteritidis, the aim of our research was to focus efforts at the beginning of the chain of infection, colonization of the chicken. It has been estimated that there are approximately 1.4 million cases of salmonellosis per year in the USA, resulting in approximately 15,000 hospitalizations, and 400 deaths per year [28]. An estimated 95% of those cases are contracted from contaminated food or food products [29]. Of those 95%, 15% of those cases are caused by *S.* Enteritidis. *S.* Enteritidis is the second most common serovar isolated from poultry in North America, and is the most common serovar isolated from humans in the EU [27, 283]. As *S.* Enteritidis is passed to humans mainly through consumption of contaminated poultry meat and eggs, it is important to better understand the pathogenesis of *S.* Enteritidis in chickens in order to prevent it.

S. Enteritidis is known to use two specialized type III secretion systems (T3SS) that are encoded within *Salmonella* pathogenicity islands 1 and 2 (SPI-1 and SPI-2), and are thought to facilitate invasion and survival within the host cell. These two T3SSs are encoded within

Salmonella pathogenicity islands 1, and 2 (SPI-1 and SPI-2). The T3SSs secrete effectors into the host cell, triggering a number of events in the infected cell that culminate in the symptoms of gastroenteritis in humans: fever, diarrhea, and abdominal pain. It is the current view that the SPI-1 T3SS is mainly involved in invasion of the host cell, while the SPI-2 T3SS plays a role in survival within the host cell and maintenance of the *Salmonella* containing vesicle (SCV) [284, 285].

SPI-2 is a region of approximately 40 kb located at centisome 37 in *S.* Enteritidis, and has been reported to be necessary for systemic infection [55]. The SPI-2 region encodes 44 proteins that are essential for intracellular proliferation, survival, and maintenance of the SCV, as well as its own regulatory system: SsrA/B [286-288]. SPI-2 effectors are secreted across the SCV membrane and stop fusion of lysosomes with the SCV, thereby avoiding reactive oxygen species-(ROS-) and NADPH oxidase-mediated killing of the bacteria [178, 289, 290]. Effectors facilitate systemic spread and the maturation of the SCV, and can act as pro- or anti-inflammatory factors [291, 292]. Once in the mature SCV, *Salmonella* is able to proliferate, can traverse the epithelial cell, and can invade underlying tissue after being released on the basolateral side [293, 294].

Mutations that disrupt the SPI-2 T3SS have been shown to result in highly attenuated virulence, as well as defective growth in macrophages and epithelial cells [55, 178, 295]. Optimal SPI-2 T3SS expression in culture occurs under acidic, low osmolarity, low Ca^{2+}, minimal nutrient conditions which mimic the environment within the SCV [55, 150, 285]. Unlike the SPI-1 T3SS, the SPI-2 needle is extended by a filament composed of SseB subunits that is required for secretion of the translocation proteins SseC and SseD [137, 178, 285, 296, 297]. There are at least 19 effectors secreted by the SPI-2 T3SS, most encoded by genes located outside of the SPI-2 locus [137, 178, 292].

Until recently, it was widely accepted that, of the two T3SSs, SPI-1 was alone in its expression in the intestinal lumen, while SPI-2 expression was only induced after entry of *Salmonella* had entered into the host cells and established it's position within the SCV. However, a study in mice showed that the SPI-2 T3SS is expressed in the intestinal lumen prior to attachment and entry of the bacteria, albeit to a much lower extent than SPI-1. This is probably an attempt by the bacteria to prepare its invasion arsenal [284]. There have also been studies showing that the SPI-1 T3SS continues to be expressed once *Salmonella* is inside the SCV, and that some of the SPI-1 effector proteins work in conjunction with SPI-2 effector

proteins to manipulate the host cell [298-300]. To date, many studies examining the reduction of colonization and shedding of *Salmonella* in chickens have been carried out; however, the majority of these studies involve *Salmonella enterica* subspecies *enterica* serovars Typhimurium (*S.* Typhimurium), Pullorum (*S.* Pullorum), and Gallinarum (*S.* Gallinarum), and not serovar Enteritidis (*S.* Enteritidis) [71, 301-305].

The reduced ability of *S.* Enteritidis SPI-2 knockout mutants produced in our lab to colonize chickens has provided us with knowledge about the importance of the SPI-2 T3SS in the colonization process. Using these mutants, we have observed that in a co-challenge situation the wild-type (WT) *Salmonella* strain out-competes the mutant for systemic spread, as measured by recovering the bacteria from liver and spleen. Using a single-challenge model, we have observed that although the SPI-2 T3SS plays an important role in systemic infection, it is not the only factor involved in this process.

3.2 Materials and methods

3.2.1 Bacterial strains and plasmids

Bacterial strains and plasmids used in this study are described in Tables 3.1 and 3.2, respectively. Standard growth procedures were followed using Luria-Bertani (LB) broth and agar at 37°C. Media was supplemented with 100 or 50 μg/ml ampicillin, 10 or 5 μg/ml tetracycline, 30 or 9 μg/ml chloramphenicol, or 25 μg/ml streptomycin when called for. All antibiotics were obtained from Sigma-Aldritch.

3.2.2 Primers

All primers used in this study (Table 3.3) were designed based on the *S.* Enteritidis PT4 (phage type 4) sequence provided by Wellcome Trust Sanger Institute (UK), GenBank accession number AM933172 [6] and synthesized by Invitrogen.

3.2.3 Polymerase chain reaction

All Polymerase Chain Reactions (PCR) were carried out using New England Biolabs (NEB) Taq Polymerase, applying reaction conditions as suggested by the supplier.

Table 3.1 Bacterial strain list

Bacterial strain	Relevant properties	Source
E. coli BL-21 (DE3)	For protein expression, *relA1*F^-, *dcm, ompT, hsdS*$_B$(r_B^-, m_B^-), *gal,* λ(DE3)	Invitrogen
E. coli BL-21 (DE3) pLysS	For expression of toxic proteins, F^-, *dcm, ompT, hsdS*$_B$(r_B^-, m_B^-), *gal,* λ(DE3), [pLysS Cam^R]	Invitrogen
E. coli DH5α	F^-, φ80dlacZΔM15, Δ(*lacZYA-argF*)U169, *deoR*, *recA1*, *endA1*, *hsdR17*(r_K^-, m_K^+), *phoA*, *supE44*, λ^-, *thi-1*, *gyrA96*, *relA1*	Invitrogen
E. coli M15	For expression of toxic proteins, F^-, *thi, lac, ara, gal*$^+$, *mtl, recA*$^+$, *uvr*$^+$, *lon*$^+$, [pREP4 Kan^R]	Qiagen
E. coli SG13009	For expression of toxic proteins, F^-, *thi, lac, ara, gal*$^+$, *mtl, recA*$^+$, *uvr*$^+$, *lon*$^+$, [pREP4 Kan^R]	Qiagen
WT *S.* Enteritidis Sal8	Virulent for birds. Invades spleen, liver and colonizes gut.	Dr. W. Kay[1]
WT *S.* Enteritidis Sal18	Virulent for birds. Invades spleen, liver and colonizes gut.	Dr. C. Poppe[2]
S. Enteritidis Sal18 (pKD46)	WT *S.* Enteritidis Sal18 harbouring the pKD46 plasmid for use in the λRed system.	This study
Sal18 *att*Tn7::*cat*	WT *S.* Enteritidis Sal18 with a chloramphenicol resistance gene inserted at the *glmS* site using the λRed system.	This study
Sal18 *att*Tn7::*tet*	WT *S.* Enteritidis Sal18 with a tetracycline resistance cassette inserted at the *glmS* site using the λRed system.	This study
Sal18 *att*Tn7::*cat* Δ*spaS*Δ*ssaU*	Sal18 with deletion of the SPI-1 *spaS* and SPI-2 *ssaU* genes as well as a chloramphenicol resistance gene inserted at the *glmS* site using the λRed system.	This study
Sal18 ΔSPI-2::*cat*	Sal18 with the whole SPI-2 region deleted and replaced by a chloramphenicol resistance gene using the λRed system.	This study
Sal18 ΔSPI-2	Derivative of Sal18 ΔSPI-2::*cat* with the chloramphenicol resistance gene deleted using the λRed system.	This study
Sal18 ΔSPI-1ΔSPI-2::*cat*	Sal18 with the whole SPI-1 region deleted and the entire SPI-2 region deleted and replaced by a chloramphenicol resistance gene using the λRed system.	This study
WT *S.* Typhimurium SL1344	WT *S.* Typhimurium, streptomycin resistant.	Dr. B. Finlay[3]

[1] Dr. W. Kay, Department of Biochemistry and Microbiology, University of Victoria, Victoria, British Columbia
[2] Dr. C. Poppe, Laboratory for Foodborne Zoonoses, Health Canada, Guelph, Ontario
[3] Dr. B. Finlay, University of British Columbia, Vancouver, British Colombia

Table 3.2 Plasmid list

Plasmid	Relevant properties	Source
pBR322	Cloning vector, ampicillin and tetracycline resistance.	NEB
pET-15b	His-tag protein expression vector, ampicillin resistance.	Novagen
pGEM-T	Single 3´-T overhangs at the insertion site that greatly improve the efficiency of ligation of a PCR product into the plasmid by preventing recircularization of the vector and providing a compatible overhang for ligation of PCR products generated by certain thermostable polymerases. Can be used for Blue/White screening. Ampicillin resistance	Promega
pHSG415	Temperature sensitive. Ampicillin, chloramphenicol and kanamycin resistance.	Dr. A. White[4] and Dr. M. Surette[5]
pKD3	Used in the λRed system, chloramphenicol resistance.	Dr. A. White[4] and Dr. M. Surette[5]
pKD46	Used in the λRed system.	Dr. A. White[4] and Dr. M. Surette[5]
pQE-30-UA	His-tag protein expression vector, has single 3´-T overhangs at the insertion site for improved efficiency of direct ligation of PCR products into the plasmid.	Qiagen

[4] Dr. A. White, formerly: University of Calgary, Calgary, Alberta; currently: VIDO, University of Saskatchewan, Saskatoon, Saskatchewan

[5] Dr. M. Surette, formerly: University of Calgary, Calgary, Alberta; currently: McMaster University, Hamilton, Ontario

Table 3.3 Primer list

Primer name	Primer sequence (5' → 3')	Restriction site
λRed SPI-1 Fwd	GCTGTCGCGTATGAAGCGATTGGGTATTGATA AAGACGCGTTAGCGTAAGTGTAGGCTGGAGCT GCTTC	None
λRed SPI-1 Rev	ATATGGTCTTAATTATATCATGATGAGTTCAG CCAACGGTGATATGGCCCATATGAATATCCTC CTTA	None
λRed SPI-2 Fwd	TCCAGGACGCGTGGTATTGGCATATCGGTGGG ATGATAGCCAAGACAAACGTGTAGGCTGGAG CTGCTTC	None
λRed SPI-2 Rev	TGCCTCGCTCTAAGGATAGGTGACATCGAAAG AGCGTGCAGAGGAATGTGCATATGAATATCCA CCTTAG	None
glmSABC Fwd	AGCGCAGGTAGGCGTAGCACCTCTTAGTCGCT CTTCAGCCACCATAGAGAGTGTAGGCTGGAGC TGCTTC	None
glmSABC Rev	GGCCGTCGATAGACGGCCTTTTTTTGTGCGCC GTGACAGGCGCTGTTCTTATATGAATATCCTC CTTA	None
pBR322 *tet* Fwd	**AGATCT**GTTTGACAGCTTATCATCGATAGGC	*Bgl*II
pBR322 *tet* Rev	**GGTACC**AATTCTTGGAGTGGTGAATC	*Kpn*I
sifA Fwd	ATGCCGATTACTATAGGGAATGG	None
sifA Rev	TTGAGAAAGCGTCGTCTG	None
sifB Fwd	ATGCCAATTACTATCGGGAGAGG	None
sifB Rev	GTGATCAACTCTGGTGATGAG	None
sipD Fwd	AAGGGAGATT**GGATCC**TTAATTAGTAATGTG GG	*Bam*HI
sipD Rev	TATTTTGG**AAGCTT**TTATGCGCGACTCTG	*Hin*dIII
SPI-2 Flank 1 Fwd	**AAGCTT**GGACATGGCTGCCGTCGCTATC	*Hin*dIII
SPI-2 Flank 1 Rev	**GGTACC**TCTGTTGCGGTAGTGCGTAATC	*Kpn*I
SPI-2 Flank 2 Fwd	**AGATCT**GCGACGGCGATTTC	*Bgl*II
SPI-2 Flank 2 Rev	**GAATTC**CGCGCATTATACGCTG	*Eco*RI
spaS Flank 1 Fwd	TAT**AAGCTT**GCCTCAGCGAGGCGCGG	*Hin*dIII
spaS Flank 1 Rev	GC**TCTAGA**GGCAGTAGCGATGTATTC	*Xba*I
spaS Flank 2 Fwd	TG**TCTAGA**TTATTCGAGGACATGCGTC	*Xba*I
spaS Flank 2 Rev	GT**GAATTC**ATGCTGAACAGGCATCT	*Eco*RI
ssaC Fwd	GGGTGGGGTAT**CATATG**GTA	*Nde*I
ssaC Rev	CA**GGATCC**CTTTGGATTAACC	*Bam*HI
ssaG Fwd	AAGGACAAGC**CATATG**GATATTGC	*Nde*I
ssaG Rev	**GGATCC**TTCAGATTTTAGCAATGA	*Bam*HI
ssaU Flank 1 Fwd	GT**AAGCTT**CTACAACGTCAGCCGTCC	*Hin*dIII
ssaU Flank 1 Rev	CG**TCTAGA**TGCTTTTGGTATGCTTC	*Xba*I
ssaU Flank 2 Fwd	TC**TCTAGA**CAGATGGAAACCAGTC	*Xba*I

ssaU Flank 2 Rev	TC**GAATTC**AGCAGCAACAGG	*Eco*RI
sseBCD Fwd	AACCGCAGCGTACACGTAGT	None
sseBCD Rev	CCTCCTGCCATGAGGCGTAA	None
sseB Fwd	**CATATG**TCTTCAGGAAACATCTT	*Nde*I
sseB Rev	**GGATCC**TCATGAGTACGTTTTC	*Bam*HI
sseD Fwd	**CATATG**GCGAGTAACGTA	*Nde*I
sseD Rev	**GGATCC**TTACCTCGTTAATG	*Bam*HI
sseI Fwd	ATGCCCTTTCATATTGG	None
sseI Rev	TGCGCTTACATTTTACC	None
sseL Fwd	GACAGGAGGGTACCATGAGCGATGAGGCGCT TGCGTTGTTG	None
sseL Rev	TTACGCATAAGCTTTTACTGGAGACTGTATTC ATATATTTG	None

3.2.4 Cloning

Flanking regions of *spaS* and *ssaU* were amplified using the following primers: *spaS* Flank 1 Fwd and Rev; *spaS* Flank 2 Fwd and Rev; *ssaU* flank 1 Fwd and Rev; and *ssaU* flank 2 Fwd and Rev. Restriction sites were added to these flanking regions during PCR, resulting in the products *Hind*III-Flank 1-*Xba*I and *Xba*I-Flank 2-*Eco*RI. These products were inserted into the pGEM-T vector (Promega) for confirmation of the correct sequence (data not shown). The resulting two plasmids, one harboring the left flanking region and one with the right flanking region of the gene of interest, were digested with the restriction enzymes *Xba*I and *Sca*I. The fragments of the plasmids containing the flanking regions were then ligated together to create a plasmid containing both the right and left flanking regions of the gene of interest. All regions of the pGEM-T vector remained intact, including the ampicillin resistance gene and the origin of replication. The combined right/left flanking regions were excised from the pGEM-T vector and placed into the temperature sensitive plasmid pHSG415 using the *Hin*dIII and *Eco*RI restriction sites. The resulting plasmid was electroporated into WT Sal18 competent cells with an Electro Cell Manipulator 630 (BTX Harvard Apparatus), using standard techniques. Recombination of the pHSG415 plasmid harboring the flanking regions of the genes of interest was induced as described previously [306], and the correct recombinants were selected (Figure 3.1).

The flanking regions of SPI-2 were amplified by PCR (using primers: SPI-2 Flank 1 Fwd and Rev, and SPI-2 Flank 2 Fwd and Rev) along with the tetracycline resistance gene (*tet*) from plasmid pBR322 (using primers: pBR322 *tet* Fwd and Rev). The SPI-2 flanking regions and *tet* gene were then assembled into the pGEM-T vector as above. The resulting plasmid contained the amplified products in the following order: SPI-2 Flank 1 – tetracycline resistance cassette – SPI-2 Flank 2. The tetracycline resistance cassette including the flanking regions was then amplified via PCR, using primers SPI-2 Flank 1 Fwd and SPI-2 Flank 2 Rev, and the resulting PCR product of 4286 bp used in the Lambda Red (λRed) system [307].

For protein expression purposes, the *sseB* gene was amplified by PCR, using primers *sseBCD* Fwd and Rev, and *sseB* Fwd and Rev, and cloned into the pGEM-T vector for sequencing. Once the presence of the genes was confirmed by sequencing and restriction gel analysis (data not shown), the genes were excised and inserted into the pET-15b vector (Novagen) using the *Bam*HI and *Nco*I restriction sites.

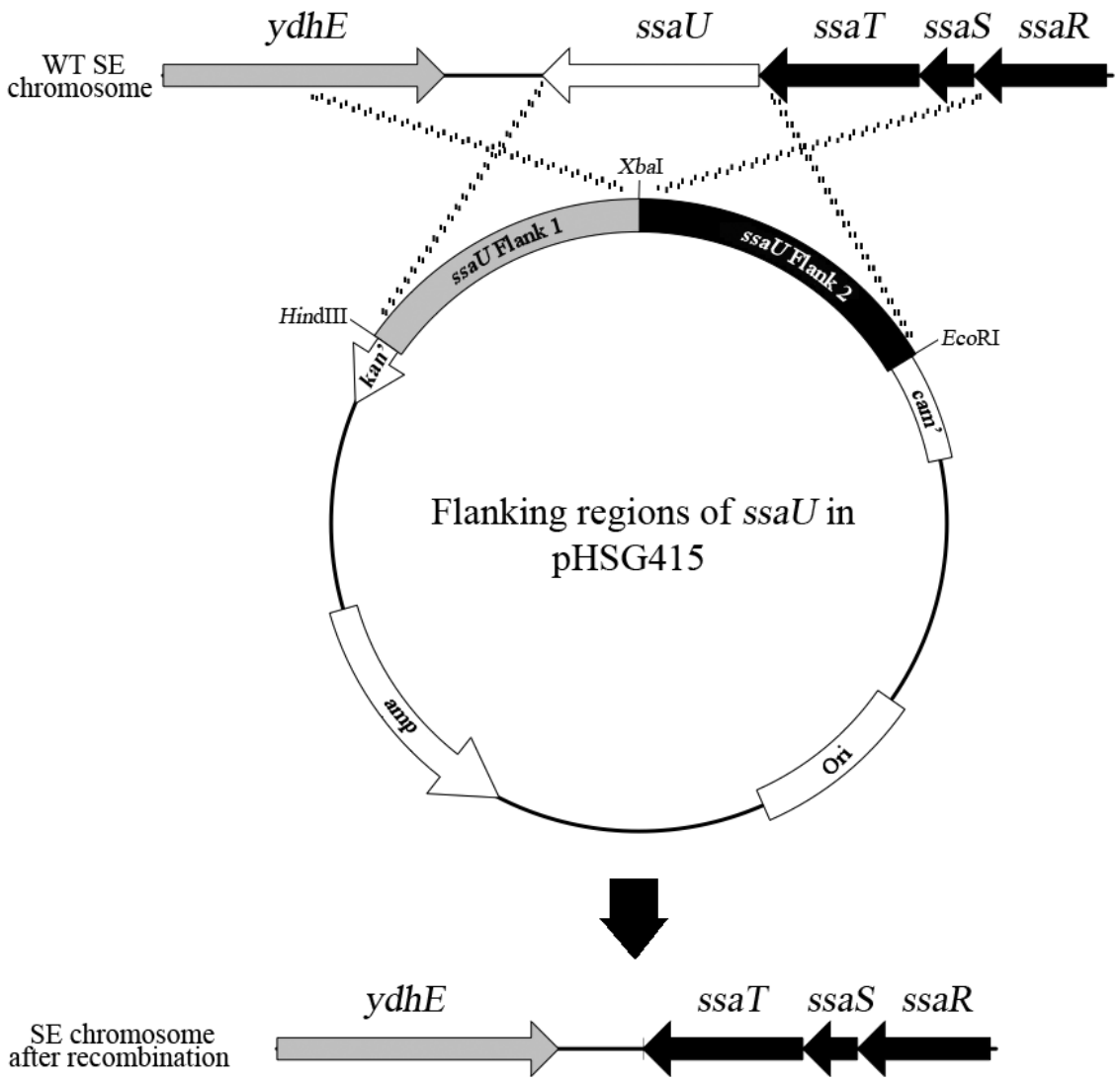

Figure 3.1 Schematic representation of the pHSG415 method for gene replacement

Flanking regions of genes of interest were amplified via PCR. The resulting products were inserted into the pGEM-T vector (Promega) for confirmation of the correct sequence (data not shown). The plasmids harboring both the right and left flanking regions were digested with *Xba*I and *Sca*I prior to ligation. This created a single plasmid containing both the right and left flanking regions of the gene of interest. The combined right/left flanking regions were excised from the pGEM-T vector and placed into the temperature-sensitive plasmid pHSG415 using the *Hin*dIII and *Eco*RI restriction sites. The resulting plasmid was then electroporated into WT *S.* Enteritidis Sal18, and successful recombinants were selected.

3.2.5 Construction of *Salmonella* pathogenicity island 2 mutants using the λRed system

The λRed system is an efficient and widely used method for the inactivation of chromosomal genes in *E. coli* and *S.* Typhimurium [307-310]. This system was used to produce two SPI-2 knockout mutants. The SPI-2 knockout mutant (Sal18 ΔSPI-2::*cat*) was produced using primers (λRed SPI-2 Fwd and Rev) designed based on the protocol originally described by Datsenko and Wanner [307]. A SPI-1 knockout mutant (Sal18 ΔSPI-1::*cat*) and SPI-1/SPI-2 knockout mutant (Sal18 ΔSPI-1ΔSPI-2::*cat*) were also produced using the same method (SPI-1 primers: λRed SPI-1 Fwd and Rev). A chloramphenicol resistance marker was also added to WT Sal18 (Sal18 *att*Tn7::*cat*) and the Δ*spaS*Δ*ssaU* strain (Sal18 *att*Tn7::*cat* Δ*spaS*Δ*ssaU*) by inserting the chloramphenicol resistance gene into an intergenic region downstream of the glucosamine-6-phosphate synthetase (*glmS*) gene using the λRed method. This site was chosen because it represents the Tn7 insertion site, and insertions at this position are unlikely to interfere with cellular functions [311]. Briefly, PCR primers were designed that recognized the intergenic region downstream of the *glmS* gene (*glmSABC* Fwd and Rev) based on the *S.* Enteritidis PT4 sequence provided by Wellcome Trust Sanger Institute (UK), GenBank accession number AM933172 [6], and the chloramphenicol resistance gene encoded on the pKD3 plasmid [307]. A tetracycline resistance gene from pBR322 was also added to WT Sal18 (Sal18 *att*Tn7::*tet*) in the same manner.

3.2.6 Strain and construct confirmation

DNA sequencing was carried out at on all constructs and PCR products at the Plant Biotechnology Institute (PBI) using a 3730 XL DNA analyzer (Applied Biosystems).

3.2.7 Animal handling and treatment

This work was approved by the University of Saskatchewan's Animal Research Ethics Board, and adhered to the Canadian Council on Animal Care guidelines for humane animal use.

3.2.8 Protein expression and antibody production

The SseB protein was expressed and purified as an N-terminal His-tag fusion protein using standard protocols as described [312] and antisera raised in New Zealand white rabbits obtained from Charles River Canada. Rabbits were immunized subcutaneously on days 0 (with

100 μg protein in Freund's complete adjuvant), 21, and 42 (with 50 μg protein in Freund's incomplete adjuvant). Blood was collected from the rabbits on day 52 and serum IgG antibody titres were determined using enzyme linked immunosorbent assay (ELISA).

3.2.9 Induction of *Salmonella* pathogenicity island 2 type III secretion system expression, and Western blot

Strains were subjected to SPI-2 inducing conditions as previously described [150] with minor modifications. Briefly, Strains were grown overnight in a rotation shaker (220 rpm) at 37°C in LB medium. The next day, 25 ml of LB was inoculated with 1/50 of the overnight culture. Cultures were grown to an OD_{600} of approximately 0.600. Cells were sedimented by centrifugation for 10 minutes at 6000 *g*. Cells were washed by gentle resuspension in low phosphate, low magnesium media (LPM) and subject to centrifugation for 5 minutes at 1000 *g*. LPM is made up of 5 mM KCl, 7.5 mM $(NH_4)_2SO_4$, 0.5 mM K_2SO_4, 8 μM $MgCl_2$, 337 μM PO_4^{3-}, 38 mM glycerol, 80 mM 2-(N-morpholino)ethanesulfonic acid (MES) and brought to a pH of 5.8. LPM was supplemented with 1% casamino acids. After growth in LPM for 6 hours, the cells were pelleted by centrifugation at 3800 x *g* for 10 minutes at 4°C, the supernatants were filtered and secreted proteins were precipitated with 20% trichloroacetic acid (TCA) overnight. The precipitated proteins were subjected to centrifugation at 21000 *g* for 30 minutes at 4°C and the TCA supernatant was subsequently removed. A mixture of 5 ml 1X phosphate buffered saline A (PBSA) pH 7.3 (8.0 g/L NaCl, 0.2 g/L KCl, 1.15 g/L Na_2HPO_4, 0.2 g/L KH_2PO_4), 0.2 ml 1.5 M TRIS pH 8.8, and 5 ml ice cold acetone were added to wash the sediment. This mixture was centrifuged at 21000 x *g* for 15 minutes at 4°C and the supernatant was subsequently discarded. The resulting sediment was washed with 2 ml ice cold acetone, and centrifuged at 21000 x *g* for 5 minutes at 4°C. After removing the acetone supernatant, the sedimented proteins were dried at which point a visible pellet was seen. The cell lysate (pellet), total membrane, and culture supernatant (secreted) fractions were isolated using standard techniques and separated by sodium dodecyl sulphate (SDS) – polyacrylamide gel electrophoresis (PAGE) prior to being visualized by staining with Coomassie Brilliant Blue G-250 (Bio-Rad). Proteins of the pellet and secreted fractions were transferred to nitrocellulose membranes (Bio-Rad) using a Semi-Dry Transfer Cell (Bio-Rad) according to instructions provided by the supplier. Western blot analysis was carried out as suggested for the Odyssey Infrared Imaging System (LI-COR Biosciences).

Primary antibodies used were rabbit polyclonal anti-SseB (raised in rabbits in-house) and mouse monoclonal anti-DnaK supplied by Kirkegaard and Perry Laboratories (KPL). Secondary antibodies used were IRDye 680CW-conjugated goat polyclonal anti-rabbit IgG and IRDye 800CW-conjugated goat polyclonal anti-mouse IgG supplied by LI-COR Biosciences. Once dry, membranes were scanned with an Odyssey infrared imaging system.

3.2.10 Passage of *Salmonella* strains

Strains of interest were streaked for single colonies and grown overnight in LB broth supplemented with antibiotics to an OD_{600} (optical density at 600 nm) of 0.7. The bacterial cell culture was centrifuged at 3500 x *g* for 10 minutes, and the resulting cell pellet was resuspended in saline for a final count of 2 x 10^{10} Colony forming units (CFU)/ml in 1 ml total volume. Chickens (two birds per strain) were orally challenged with 0.5 ml of the above bacterial culture. On days 2 and 4 after challenge, birds were euthanized and tested for the presence of the bacterial strain of interest by swabbing the insides of sampled organs (liver, spleen, and cecum) and plating on Brilliant Green (BG) agar. After incubation of the agar plates for 24 hours, resulting *Salmonella* colonies were tested by colony PCR to confirm the identity of each challenge strain. Strains were stocked in 50% glycerol (v/v) and stored at -80°C for use in following trials.

3.2.11 Recovery of *Salmonella* on Brilliant Green agar

All strains used in the trials below were first subject to plating on BG and LB agar, with and without antibiotics, to determine plating efficiency and strain fitness. Recovery of *Salmonella* on BG agar by direct plating is approximately 40% compared to recovery on LB agar. As the addition of antibiotics to BG agar further reduces the recovery rate by an additional 10% to 20%, it was decided not to use antibiotics with BG agar for recovery of *Salmonella* from chickens used in the following trials. Addition of antibiotics to LB agar did not affect recovery. Further, there was no difference in the recovery and plating efficiency between any of the strains used on either BG or LB agar, or in their growth kinetics in LB broth (data not shown). In order to determine total numbers of chickens positive for *Salmonella*, samples of liver, spleen, and cecum were taken, placed in saline, and weighed. Liver and spleen samples were homogenized using a hand homogenizer and cecum samples were vortexed to mix. Portions (1 ml) of all samples (liver, spleen, and cecum) were enriched in selenite broth and incubated overnight at

37°C in order to determine the total numbers of birds positive for *Salmonella*.

3.2.12 Co-challenge trial

Specific Pathogen Free (SPF) chickens were obtained from Charles River Laboratories, Inc. (USA) and placed in isolation rooms at the Vaccine and Infectious Disease Organization (VIDO). At 35 days of age, birds were separated into three groups of 21 birds each and co-challenged orally with two strains equaling 1 x 10^{10} bacteria total (0.5 x 10^{10} bacteria per strain) in 0.5 ml. Previous trials with SPF chickens were used to determine the appropriate dose (data not shown). Birds received either Sal18 *att*Tn7::*tet* + Sal18 *att*Tn7::*cat*, Sal18 *att*Tn7::*tet* + Sal18 *att*Tn7::*cat* Δ*spaS*Δ*ssaU*, or Sal18 *att*Tn7::*tet* + Sal18 ΔSPI-1ΔSPI-2::*cat*. The challenge was subject to plating on BG agar with subsequent re-streaking of colonies onto LB agar containing tetracycline and LB agar containing chloramphenicol in order to confirm that the challenge was, in fact, a 50/50% (CFU/CFU) mixture of the two strains in question (data not shown). Birds were euthanized on days 1, 2, and 4 after challenge and their liver, spleen, and cecum tested for the presence of the strains of interest. Samples of liver and spleen were weighed and homogenized in 10 ml of phosphate buffered saline (PBS). Portions (100 µl) of homogenized liver and spleen were plated on BG agar, and CFU/g were calculated for each strain. Cecal contents were weighed, vortexed in 5 ml of PBS, and 25 µl of 10^{0}, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5} serial dilutions plated on BG agar. Colonies recovered by direct plating on BG agar were re-streaked onto both LB agar plates containing tetracycline and LB containing chloramphenicol in order to differentiate the strains recovered. CFU/g were calculated for each strain. As the recovery of *Salmonella* on BG agar is only 40%, samples from the liver, spleen, and cecum of each bird were enriched in selenite broth, and incubated at 37°C overnight. The next day a loop of each culture was plated on BG agar. This data was used to determine the total number of samples and birds positive for *Salmonella*.

3.2.13 Single-challenge trial

SPF chicken eggs were obtained from Charles River Laboratories (USA), and were incubated for 21 days until hatch at the Department of Poultry Science (University of Saskatchewan). On the day of hatching, birds were transferred to isolation rooms at VIDO. One week after transfer, 20 birds were euthanized, and their ceca collected and tested for the presence

of *Salmonella* by plating on BG agar. At this time, five swabs per room were taken, again to test for the presence of contaminating *Salmonella* as identified by plating on BG agar. Groups of 40 birds were challenged orally at 7 days of age (Day 0) with one of the following strains: Sal18 *att*Tn7::*cat*, Sal18 ΔSPI-1::*cat*, Sal18 ΔSPI-2::*cat* or Sal18 ΔSPI-1ΔSPI-2::*cat*, at a dose of 10^{10} CFU in 0.5 ml. On days 1, 2, 3, and 4 after challenge ten birds per group were euthanized and their liver, spleen, and cecum sampled, enriched, and plated as described above. Results pertaining to the group that received the total SPI-1 mutant (Sal18 ΔSPI-1::*cat*) were reported previously [312].

3.2.14 Statistical analysis

All statistical analyses were performed using GraphPad Prism® 5.0. For the co-infection trial, Wilcoxon signed rank test was used to determine the difference between colonization levels of each strain within the groups. For the single-infection trial, the one-way analysis of variance (ANOVA) Kruskal-Wallis test was performed on the CFU/g data followed by Dunn's multiple comparison test when significance was observed. Enrichment data was subjected to the chi-square test followed by Fisher's exact test when significance was observed. p-values < 0.05 were considered significant.

3.3 Results

3.3.1 Detection of *Salmonella* pathogenicity island 2 type III secretion system proteins by Western blot

As expected, WT *S.* Enteritidis Sal18 and its derivatives (Sal18 *att*Tn7::*cat* and Sal18 *att*Tn7::*tet*) were positive for SseB in both the pellet fraction and the total membrane fraction (Figure 3.2, lanes 1-3). The *S.* Enteritidis strains missing the entire SPI-2 region were found to be negative for production of SseB (Figure 3.2, lanes 4 and 7). These results, along with those of DNA sequencing of PCR products, confirmed that Sal18 ΔSPI-2::*cat* and Sal18 ΔSPI-1ΔSPI-2::*cat* were devoid of the SPI-2 region. The strain with the *ssaU* gene deleted within the SPI-2 region was found to be positive for production of SseB; SseB was found in both the pellet and total membrane fractions of this strain (Figure. 3.2, lane 6). Since only the SsaU protein is absent in this strain, it is likely that the rest of the basal components (structural Ssa proteins and

the ATPase [adenosine triphosphatase] SsaN) are assembled at the inner membrane. It is possible that SseB is still directed to the base of the incomplete SPI-2 T3SS by its chaperone, as in the WT strain, and is therefore associated with the total membrane fraction, as well as found free in the cytoplasm. Alternatively, SseB that is not secreted might form aggregates that co-purify with the total membrane fraction. Despite its presence in the pellet and total membrane fractions, SseB was not found in the secreted fraction of any strains. This is expected, as SseB is a part of the apparatus and not a true secreted protein. It has been previously reported that strains missing the *ssaU* gene do not secrete SPI-2 effectors [303]. DnaK was used as a loading control and was found in the pellet fraction, but not the secreted fraction, as expected. Although DnaK is not normally associated with the membrane, except under certain shock conditions [313], it was also found in the total membrane fraction in our study. However, as the bacteria were under a certain amount of stress when they were switched from LB broth to SPI-2 inducing LPM, which has a low pH (5.6) and a different salt concentration compared to LB, it is not surprising that DnaK was found associated with the total membrane fraction in this case.

3.3.2 Co-challenge trial

The co-challenge trial was carried out as described in materials and methods, and the CFU/g calculated for *Salmonella* recovered from the liver, spleen, and cecal contents of each bird (Figure 3.3). Colonies were re-streaked on antibiotic-containing plates to determine the percentage of WT strain versus mutant strain in all organs sampled. On days 1, 3, and 4 post-challenge (PC) there was no significant difference observed between the colonization levels of the two WT strains in the liver, spleen, and cecum, as determined by Wilcoxon signed rank test. This indicates that the antibiotic resistance markers inserted into the WT genome downstream of the *glmS* gene had no effect on the strains' ability to colonize the cecum and to spread systemically. There were also no significant differences observed between the WT colonization levels and the mutant strain levels on day 1 PC. Although there was still no difference in cecal colonization on day 2 PC, a significant difference (p-value = 0.01562) between the WT strain and the Δ*spaS*Δ*ssaU* strain was observed, with the WT strain outnumbering the mutant. Results of previous trials undertaken by our group established that insertion of an antibiotic marker alone in the chromosome did not affect the performance of strains compared to the WT (data not shown). Therefore, the results seen in this trial are due only to the introduced mutations, and not the

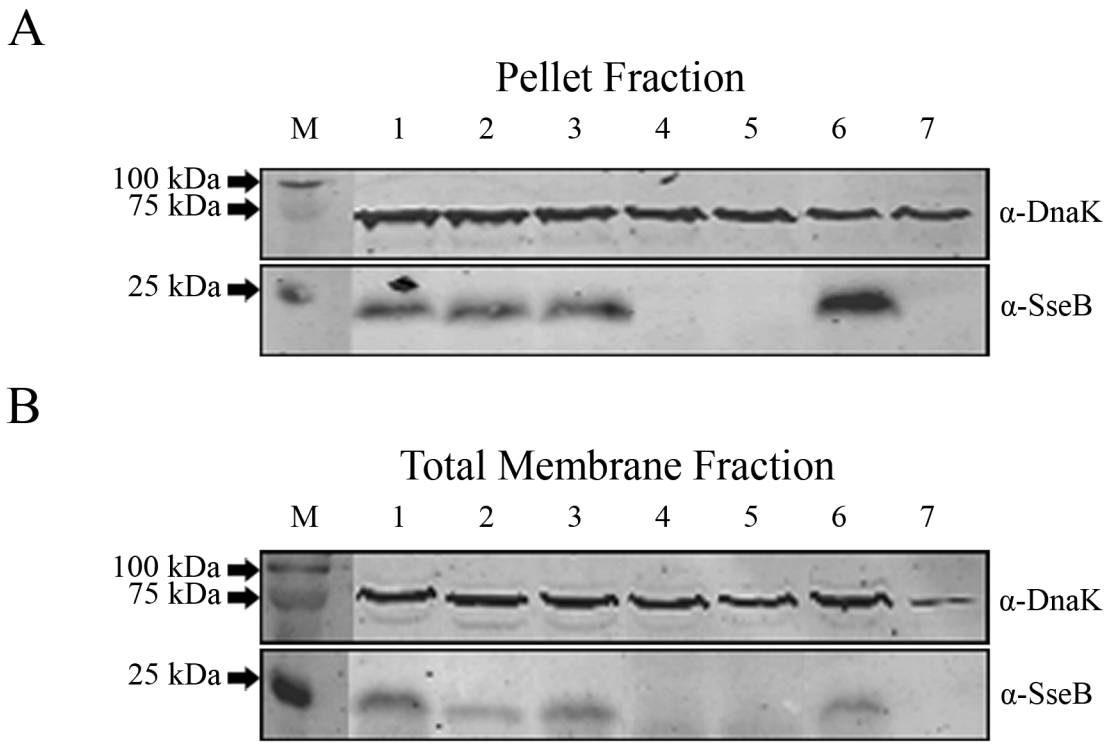

Figure 3.2 Detection of SseB in the pellet and total membrane fraction

The pellet and total membrane fractions of all strains shown in this figure were subjected to Western blot analysis to detect SseB and DnaK using specific antibodies. **Panel A** shows both DnaK and SseB detected in the pellet fraction and **panel B** displays the same proteins detected in the total membrane fraction. Lanes are as follows: M – Marker, 1 – WT Sal18, 2 – WT Sal18 *att*Tn7::*cat,* 3 – WT Sal18 *att*Tn7::*tet*, 4 – Sal18 ΔSPI-2, 5 – Sal18 ΔSPI-2::*cat*, 6 – Sal18 *att*Tn7::*cat* Δ*spaS*Δ*ssaU*, and 7 – Sal18 ΔSPI-1ΔSPI-2::*cat.*

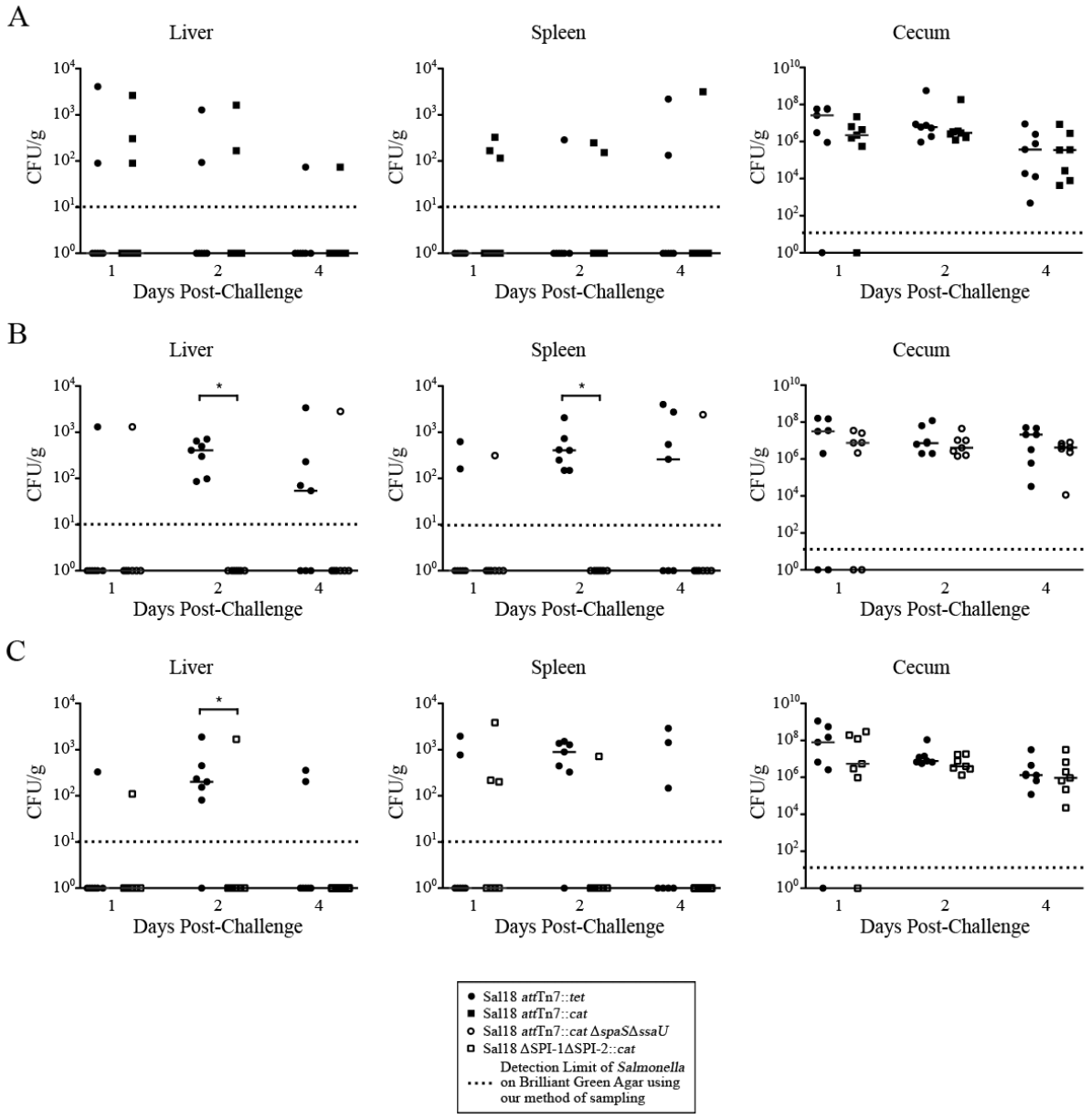

Figure 3.3 Co-challenge trial: Recovery of *Salmonella* from liver, spleen, and cecum

Panel A shows the calculated CFU/g for recovered bacteria in the liver, spleen and cecum of birds challenged with WT Sal18 *att*Tn7::*cat* and WT Sal18 *att*Tn7::*tet* on days 1, 2, and 4 PC. **Panel B** shows the calculated CFU/g in the liver, spleen and cecum of birds challenged with WT Sal18 *att*Tn7::*tet* and Sal18 *att*Tn7::*cat* Δ*spaS*Δ*ssaU* on days 1, 2, and 4 PC and **Panel C** shows the calculated CFU/g in the liver, spleen and cecum of birds challenged with WT Sal18 *att*Tn7::*tet* and Sal18 ΔSPI-1ΔSPI-2::*cat* on days 1, 2, and 4 PC. We cannot detect *Salmonella* counts below 10^1 CFU/g by direct plating with our sampling method. A dotted line has been placed indicating this, and birds negative for countable CFU after direct plating on BG agar were assigned a value of 1 for graphical purposes. *, p-value < 0.05; **, p-value < 0.01; ***, p-value < 0.001.

introduction of different antibiotic markers. A significant difference was also observed (*p*-value = 0.03125) on day 2 PC between the WT strain and the ΔSPI-1ΔSPI-2 strain, again with the WT strain higher in colonization levels. By day 4 we saw a trend towards clearance of the mutant strain in the liver and spleen, with 100% of the recovered colonies at this point belonging to the WT strain (Figure 3.4). Although strains were mixed equally before challenge, only one strain was recovered from spleens of the group that received the WT strain and the Δ*spaS*Δ*ssaU* strains after enrichment. This is likely a result of the poor recovery rate of *Salmonella* on BG agar as well as the small amount of sample used for enrichment (1 ml out of 10 ml total), as prior to enrichment both strains were found in the spleen (Figure 3.3, panel C).

3.3.3 Single-challenge trial

The single-challenge trial was carried out as described in materials and methods. CFU/g were determined for *Salmonella* isolated from liver, spleen and cecum of all birds (Figure 3.5). Samples were also enriched in selenite broth to identify samples with numbers of *Salmonella* too low to be detected by direct plating (Figure 3.6). One-way ANOVA Kruskal-Wallis tests were performed on the CFU/g data of the liver, spleen, and cecum. Chi-square Fisher's exact test was performed on the positive/negative results in order to determine if there were significant differences between the colonization levels of each strain on days 1, 2, 3, and 4 PC. On day 1 PC, no significant difference was observed between the CFU/g of the WT strain and either of the mutant strains. There was, however, a statistically significant difference in the number of birds that tested positive for *Salmonella* in the liver between the WT strain and the ΔSPI-2 strain (*p*-value = 0.0198), as well as between the WT strain and the ΔSPI-1ΔSPI-2 strain (*p*-value = 0.0011). In both cases, the number of birds positive for *Salmonella* was higher in the group challenged with the WT strain as opposed to the groups challenged with the mutant strains. On day 2 PC, we saw a significant difference in the cecal CFU/g between the group challenged with the WT strain and the group challenged with the ΔSPI-2 strain (*p*-value = 0.0111). The same was observed in the liver (*p*-value = 0.0001) and the spleen (*p*-value = 0.0108). The number of birds positive for *Salmonella* after enrichment coincides with this data, showing a higher number of birds with livers testing positive in the WT group versus the ΔSPI-2 group (*p*-value = 0.0001). The same was observed in the spleen (*p*-value = 0.0198). There was also a significant difference between the CFU/g of the WT group versus the ΔSPI-1ΔSPI-2 group in both the liver (*p*-value =

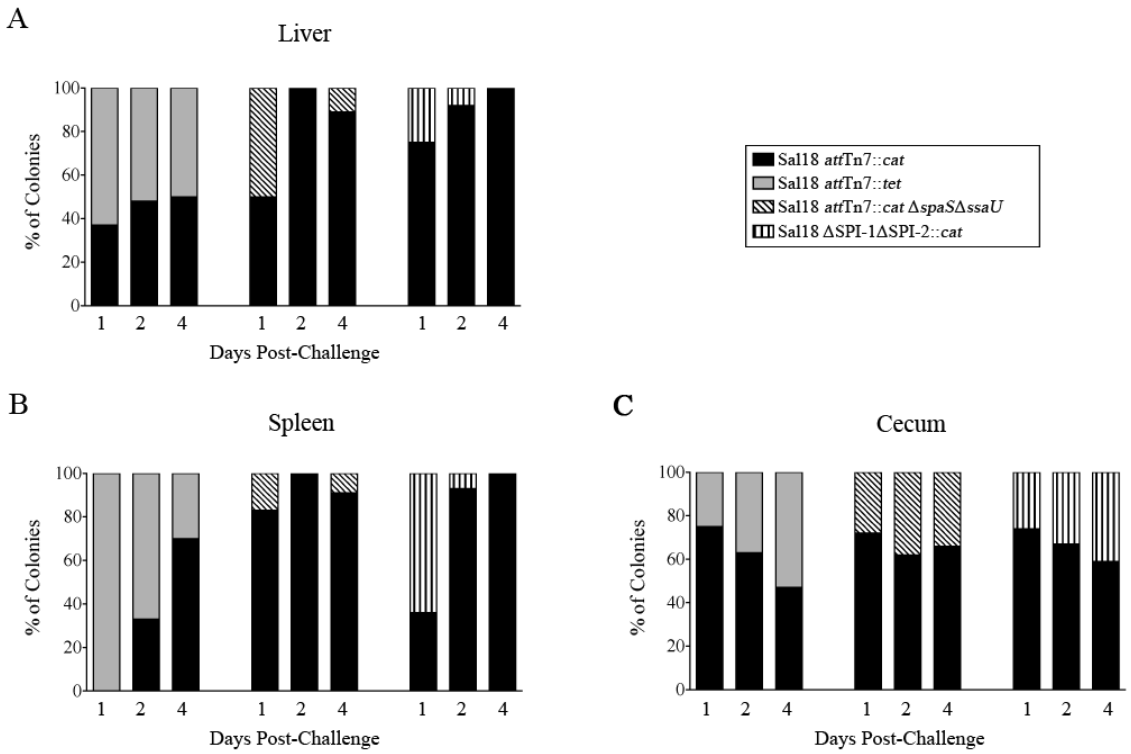

Figure 3.4 Co-challenge trial: Strain distribution in liver, spleen, and cecum

Panel A shows the type of *Salmonella* colonies found in the liver on Days 1, 2, and 4 PC with equal amounts of either: WT Sal18 *att*Tn7::*tet* and WT Sal18 *att*Tn7::*cat* (left 3 bars), WT Sal18 *att*Tn7::*tet* and Sal18 *att*Tn7::*cat* Δ*spaS*Δ*ssaU* (middle 3 bars) or WT Sal18 *att*Tn7::*tet* and Sal18 ΔSPI-1ΔSPI-2::*cat* (right 3 bars). **Panels B** and **C** have the same data arrangement, but depict the colonization profiles of the spleen and cecum, respectively.

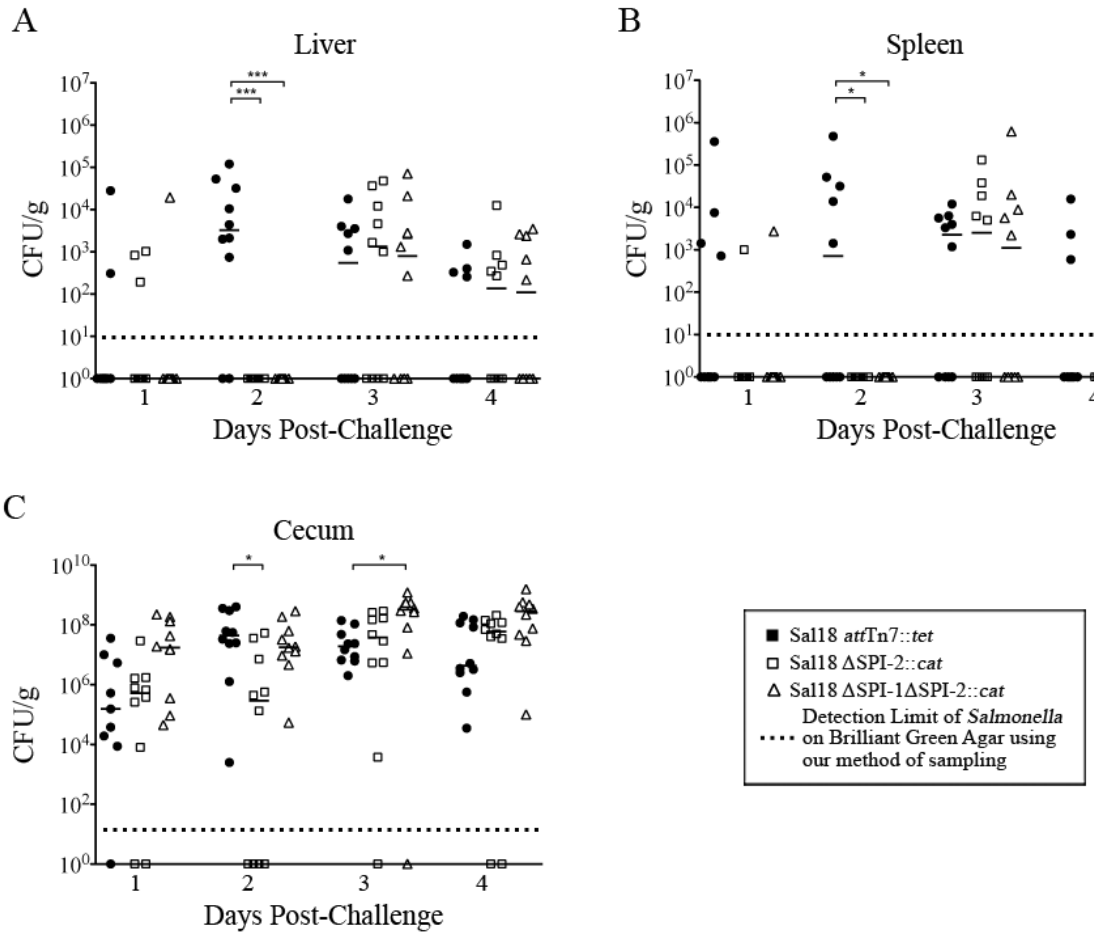

Figure 3.5 Single-challenge trial: Recovery of *Salmonella* from liver, spleen, and cecum

Panel A shows the calculated CFU/g in the livers of birds challenged with either WT Sal18 *att*Tn7::*tet*, Sal18 ΔSPI-2::*cat* or Sal18 ΔSPI-1ΔSPI-2::*cat* on days 1, 2, 3, and 4 PC. **Panels B** and **C** show the CFU/g in the spleen and cecum of birds. We cannot detect *Salmonella* counts below 10^1 CFU/g by direct plating with our sampling method and a dotted line has been placed indicating this. Birds negative for countable CFU after direct plating on BG agar were assigned a value of 1 for graphical purposes. *, p-value < 0.05; **, p-value < 0.01; ***, p-value < 0.001.

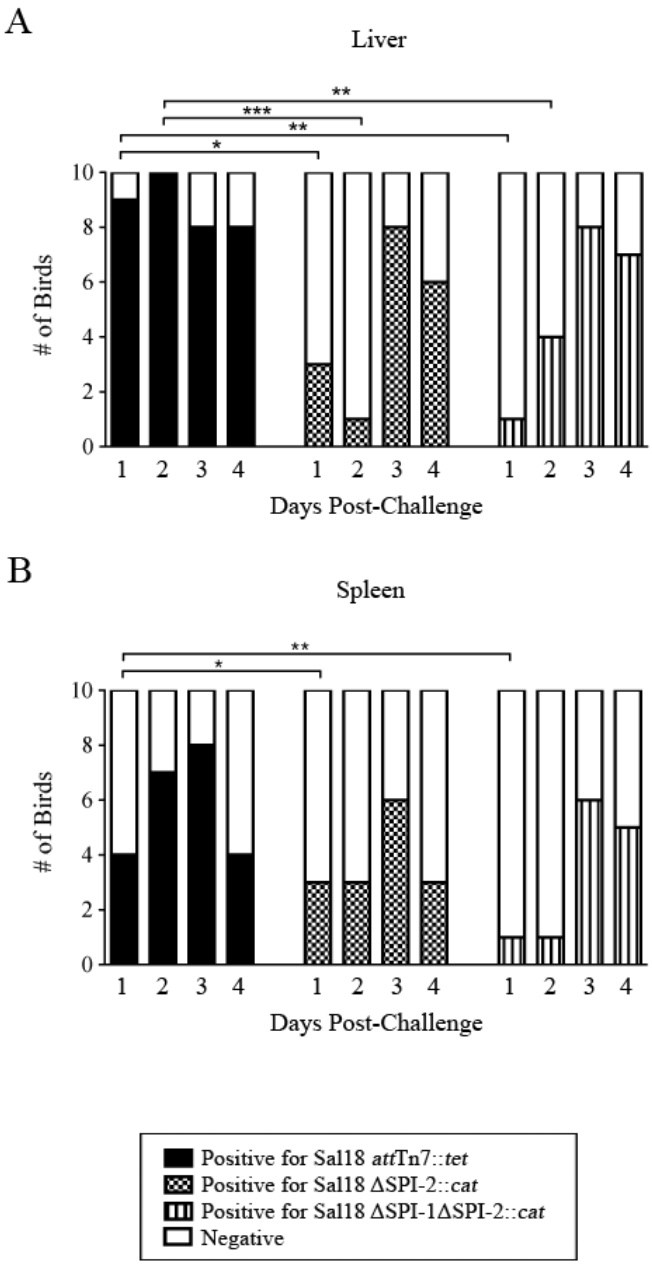

Figure 3.6 Single-challenge trial: Colonization levels after enrichment

Panel A depicts the number of birds positive or negative for the challenge strain in the liver on days 1, 2, 3, and 4 PC, while **Panel B** depicts the number of birds positive or negative for the challenge strain in the spleen. *, p-value < 0.05; **, p-value < 0.01; ***, p-value < 0.001.

0.0001) and the spleen (*p*-value = 0.0108). Again, enrichment data supports this observation, showing a higher number of birds with livers and spleens testing positive in the WT group than those in the ΔSPI-1ΔSPI-2 group (*p*-value = 0.0011 and 0.0198, respectively). Based on either CFU/g or enrichment data, no significant differences between the strains were observed on days 3 and 4 PC.

3.4 Discussion

Prior to this study, no knockout mutants of the complete SPI-2 region in *S.* Enteritidis had been reported. However, some recent work by Rychlik *et al*. [314], published after completion of our animal trials, describes the complete knock out of all the SPI regions, both together and separately, and their effect on *S.* Enteritidis colonization of 1-day-old chicks. In addition, at least partial deletions of SPI-2 have been produced in *S.* Typhimurium, as well as full deletions of SPI-1 [308, 315, 316]. Using the λRed system, developed by Datsenko and Wanner [307], derivative strains of WT Sal18 missing either one or both of the entire SPI-1 and SPI-2 regions (Sal18 ΔSPI-2::*cat* and Sal18 ΔSPI-1ΔSPI-2::*cat*) were constructed.

A previous study testing colonization levels of one-week-old chickens challenged with *S.* Enteritidis strain 1009 on days 7, 14, 28, 35, and 42 PC showed maximal colonization levels of spleen and cecum on day 7 [217]. Sadeyen *et al.* [317] observed similar results when working with 30-week-old chickens challenged with the same bacterial strain. Another study testing layer hens challenged with *S.* Enteritidis Y-24 PT4 showed that the highest colonization in cecum was achieved on days 3 and 4 PC [318]. As the maximum colonization in these studies happened within the first week, we believed that it was important to monitor the window between days 1 and 7 PC. In preliminary trials (data not shown), it was found that by days 3 and 4 PC the WT strain Sal18 was beginning to clear from the liver, spleen, and cecum of the chickens. Therefore, days 1, 2, 3, and 4 PC were chosen for further trials. The challenge dose used in these studies is quite high, at 10^{10} CFU, compared to similar studies, which tend to range from 10^4 CFU to 10^8 CFU [213, 217, 317, 319]. However, preliminary experiments done by our group (data not shown) involving the challenge of 21-day-old Broiler chickens and challenge of 1-day-old SPF chicks with 10^6, 10^8, and 10^{10} CFU of WT *S.* Enteritidis strain Sal18 indicated that oral challenge with 10^{10} CFU of our strain yields the best colonization levels of liver and spleen, while cecal colonization levels remain the same at all doses.

The results of the co-challenge trial show that when 35-day-old SPF chickens are challenged with both a WT strain and a strain impaired in both the SPI-1 and SPI-2 T3SSs, the WT strain begins to out-compete the mutant strain in the liver and spleen by day 2 PC (Figure 3.3, panels B and C). However, there is no detectable difference in the level of WT versus mutant strain in the cecal contents (Figure 3.3, panels B and C). Dieye *et al*. [315] used a similar experimental design in a recent study comparing colonization levels of WT *S.* Typhimurium UK-1 and either a ΔSPI-1 strain missing the entire SPI-1 region, a ΔSPI-2 strain missing a portion of the SPI-2 region encoding structural genes, or a combination ΔSPI-1ΔSPI-2 strain. In this study, 1-week-old SPF chickens were co-challenged with different combinations of the above strains, and chickens were euthanized at days 3, 7, and 14 PC to test colonization levels in the spleen and cecum. Similar to our findings, this group recovered a greater amount of the WT *S.* Typhimurium strain from the spleen than the ΔSPI-2 and ΔSPI-1ΔSPI-2 strains. They also observed that colonization levels of the WT versus the mutant strains in the cecum were not different, again supporting our results.

The *spaS* and *ssaU* genes encode structural proteins of the SPI-1 and SPI-2 T3SSs, respectively. The *spaS* gene is last in the *inv-spa* operon of the SPI-1 while the *ssaU* gene is likewise positioned at the end of the *ssaK-U* operon of SPI-2. Deletion of the *spaS* and *ssaU* genes should stop the secretion of both SPI-1 and SPI-2 secreted proteins, but should not affect transcription of other genes in the SPI-1 or SPI-2 regions, or elsewhere in the chromosome [1, 52, 70, 303, 304]. Our study comparing the Δ*spaS*Δ*ssaU* mutant strain with the ΔSPI-1ΔSPI-2 strain *in vivo* shows that the strain with only the two genes deleted behaves similarly to the strain missing the entirety of both islands when given as a co-challenge with a WT strain.

The results of the single-challenge trial indicate that strains missing either the SPI-2 or both the SPI-1 and SPI-2 regions are impaired in their ability to infect the liver and spleen of SPF chickens. On day 1 PC, both the WT and mutant strains begin to spread systemically to organs (liver and spleen), but while the WT strain reaches peak colonization of the liver and spleen by day 2 PC, and is beginning to clear by day 3 PC, the mutant strains do not reach their peak until day 3 PC. By day 4, both the WT and mutant strains are clearing from the birds (Figures 4.5 and 4.6). Bohez *et al.* [319] observed that peak presence of *Salmonella* in liver and spleen of SPF birds challenged with 10^8 CFU *S.* Enteritidis on the day of hatching occurred on day 2, supporting our observations. A further study by Jones *et al.* [304] found that when SPF chickens

were challenged at 1 week of age with *S.* Typhimurium F98, there was a peak colonization of the liver and cecum at 3 days PC. When challenged with a derivative strain of *S.* Typhimurium F98, impaired in the SPI-1 region (Δ*spaS*), peak colonization was again observed 3 days PC, but seemed to have cleared faster in the liver and spleen. In chickens receiving a strain impaired in the SPI-2 region (Δ*ssaU*), systemic infection was not observed and clearance of the strain from the cecum was evident by day 7 PC. This is partly in accordance with our results presented here. Our observations indicate that mutant strains are cleared faster from the liver and spleen than the WT strain. However, we did not see the difference in cecal colonization between WT and mutant strains that was observed by Jones *et al*. [304]. This may be attributed to differences in strain, as their studies used an isolate of *S.* Typhimurium while our studies used *S.* Enteritidis, or differences in challenge dose.

As mentioned, we have indicated that the SPI-2 T3SS is not important for cecal colonization, and while the SPI-2 T3SS does appear to be important for efficient systemic infection, it is obviously not the only factor involved. These observations are in line with the results of another group that found that neither the SPI-1 nor the SPI-2 T3SS of a *S.* Typhimurium strain were essential to the invasion of M cells *in vitro.* Their studies used an *in vitro* model involving the co-culture of Caco-2 cells with Raji B cells (which causes the Caco-2 cells to exhibit traits of M cells), in order to better mimic the intestinal environment that *Salmonella* encounters in the human host [320]. Another group using an *in vitro* model found that while the SPI-2 T3SS was expressed at all times within the SCV of mouse macrophage cells, the expression level in the SCV of human epithelial cells were reduced compared to those in the macrophages [300]. This further supports our conclusion that SPI-2 is important for systemic infection in chickens, but not essential for cecal colonization.

A recent study by Rychlik *et al.* [314] used *S.* Enteritidis strain 147 SPI mutants missing one of the islands (SPI-1, 2, 3, 4, or 5), missing both SPI-1 and SPI-2, missing all islands (SPI-1 to 5), retaining one of the islands only (SPI-1, 2, 3, 4, or 5) or retaining both SPI-1 and SPI-2 only, to test the ability of these mutants, in comparison to the WT strain, to colonize the liver and spleen of 1-day-old chicks. They found that after oral challenge with 5 X 10^7 CFU, the strains missing either SPI-1 or SPI-2 and the strains containing only SPI-1 or SPI-2 maintained a medium level of virulence and were still found in the liver and spleen of challenged chicks both 5 and 7 days PC, although in less abundance than the WT strain. However, the strain missing all of

the pathogenicity islands (SPI-1 to 5) and those containing only one of SPI-3, SPI-4, or SPI-5 were avirulent and only very small amounts were isolated from the liver and spleen. Those strains missing SPI-3, SPI-4, or SPI-5 were isolated from the liver and spleen at the same levels as the WT strain. They also found that the strain missing both SPI-1 and SPI-2 was isolated from the liver and spleen at lower levels than the WT strain, or the strain containing only the SPI-1 and SPI-2 regions. Their study not only demonstrates the importance of both SPI-1 and SPI-2 in colonization of the liver and spleen, but also shows that mutants missing one of or both SPI-1 and SPI-2 can still be isolated from the liver and spleen, although at much lower levels than WT, further confirming our results. They found no difference in cecal colonization by any of their strains, also in accordance with our results.

A study by Morgan *et al.* in 2004 [42] involved screening a large number of *S.* Typhimurium mutants, including several single gene knockouts in the SPI-1 and SPI-2 regions. They found that their SPI-1 and SPI-2 mutants were not effective colonizers of bovine ileum between 3 and 5 days PC. At first glance, these results seem contrary to our findings. However, this group also found that the majority of the SPI-1 and SPI-2 mutants tested were able to successfully colonize the cecum of 14-day-old chickens. They also tested a number of other mutants including single-gene knockouts of certain surface structures and found that while many of these mutants were equivalent to the WT strain in their ability to colonize cattle, they were attenuated in chickens. In addition, they observed that mutations of the SPI-4 region caused attenuation in cattle, but not in chickens, further demonstrating that *Salmonella* must use different approaches for colonization or infection of different hosts. This is not surprising as *S.* Typhimurium causes systemic disease in cattle, but does not cause disease in chickens older than 1 week of age. Although *S.* Typhimurium and *S.* Enteritidis are considered to be distinct serovars, they are very close genetically and can cause similar disease in humans. The fact that Morgan's group found no difference in the ability of their WT strain and their SPI-1 and SPI-2 mutant strains to colonize chicken ceca corresponds to our results with *S.* Enteritidis in both 7-day-old and 35-day-old chickens, and validates our finding that the SPI-2 T3SS is not essential for colonization of chickens. As well, their observations that deletions of certain fimbrial and other structural genes caused attenuation of their *S.* Typhimurium strain in chickens may be the answer to how *S.* Typhimurium and *S.* Enteritidis colonize chickens without the use of the SPI-1 and SPI-2 T3SSs. Further work must be done in this area to determine the exact mechanisms by

which *S.* Typhimurium and *S.* Enteritidis colonize the chicken cecum and spread systemically in these animals.

In summary, the results of our studies indicate that in a co-challenge situation in 35-day-old chickens, the WT strains of *S.* Enteritidis are more competitive than strains impaired in the SPI-1 and SPI-2 regions. As well, we have shown that in a single-challenge model in 1-week-old chicks, mutants missing either the SPI-2 region or both the SPI-1 and SPI-2 regions are initially impaired in their ability to invade the liver and spleen compared to the WT strain, but remain present in the cecum at similar levels to the WT strain.

4.0 IMMUNIZATION OF CHICKENS WITH *S.* ENTERITIDIS PATHOGENICITY ISLAND 2 TYPE III SECRETION SYSTEM STRUCTURAL AND EFFECTOR COMPONENTS

The information presented in this chapter is currently 'in press' for publication in Veterinary Microbiology [321].

4.1 Introduction

Infections by *Salmonella* are one of the leading causes of food poisoning in humans [21]. *Salmonella* is transmitted to humans via consumption of contaminated poultry meat, water or eggs. *Salmonella enterica* subspecies *enterica* serovar Enteritidis (*S.* Enteritidis) is the most commonly isolated serovar in the European Union (EU), and second most common in North America. *S.* Enteritidis consequently places a significant burden on the health care system and the poultry industry [18, 19, 27-29].

Because chickens do not generally exhibit symptoms of disease, flocks can become colonized quickly, resulting in bacteria being shed in their feces for extended periods of time [23, 25, 26]. Thus, the demand for a vaccine protecting poultry against *S.* Enteritidis and *S.* Typhimurium is high. Although there have been previous successes in this area using live attenuated strains of host-adapted *Salmonella*, there has been far less success in achieving a protective vaccine against the non host-adapted strains of *Salmonella*, such as *S.* Enteritidis and serovar Typhimurium (*S.* Typhimurium) [15, 16, 25]. The use of probiotics and competitive exclusion to reduce initial colonization of chicks by *S.* Enteritidis and *S.* Typhimurium has also had limited success [322, 323]. To date, only a small number of studies have investigated the suitability of subunit vaccines for protection of poultry against *Salmonella* [265, 266, 269].

The focus of this study was to determine if vaccination of chickens with *Salmonella* pathogenicity island 2 (SPI-2) type III secretion system (T3SS) components could elicit a humoral immune response while conferring protection against experimental challenge with *S.* Enteritidis [285]. The SPI-2 T3SS is one of two T3SSs that *Salmonella* uses to invade and survive within the host. Both T3SSs are expressed prior to entry into the host cell, although the SPI-1 T3SS is expressed in greater amounts [284], and, as they are exposed to the hosts immune system, are potential vaccine candidates. This study also sought to determine whether vaccinated

hens could transfer protective antibodies to progeny. The ability of SPI-2 T3SS components to elicit a strong humoral response in chickens that can be transferred to progeny, as well as their ability to slightly lower overall bacterial loads per bird in certain situations, may make these components a valuable addition to a combination subunit vaccine.

4.2 Materials and methods

4.2.1 Bacterial strains

Bacterial strains used in this study were *S.* Enteritidis strains Sal18 and Sal8 (Table 3.1). Sal18 was chosen as the challenge strain in vaccine trial 1 as it has been shown to be virulent for birds, and invades the liver and spleen as well as colonizes the gut. Sal8 was chosen for the subsequent vaccine trials (2a, 2b, 2c, and 3) as our group found that Sal8 reaches the liver and spleen of more birds than Sal18 (100% of birds positive for Sal8 in the liver and/or spleen compared to 60% positive with Sal18) (unpublished data).

With respect to culturing bacteria, standard procedures were followed using Luria-Bertani (LB) broth and agar, and Brilliant Green (BG) agar at 37°C. Media was supplemented with 100 or 50 μg/ml ampicillin, 10 or 5 μg/ml tetracycline, or 30 or 9 μg/ml chloramphenicol when called for. All antibiotics were obtained from Sigma-Aldritch.

4.2.2 Passage of *Salmonella* strains

Strains of interest were passaged in chickens as previously described in section 4.2.10.

4.2.3 Recovery of *Salmonella* on Brilliant Green agar

All strains used in the trials below were first subjected to plating on BG agar, as well as LB agar with and without antibiotics to determine plating efficiency and strain fitness as described in section 4.2.11.

4.2.4 Protein expression and purification

Genes of potential vaccine candidates were cloned according to standard procedures. Proteins were expressed and purified as described previously [312]. Bacterial strains, plasmids, and primers used for this purpose are listed in Tables 3.1, 3.2, and 3.3, respectively.

4.2.5 Animal handling and treatment

This work was approved by the University of Saskatchewan's Animal Research Ethics Board, and adhered to the Canadian Council on Animal Care guidelines for humane animal use.

4.2.6 Vaccination of chickens

Specific pathogen free (SPF) chicken eggs were obtained from Charles River Laboratories, Inc (US). Eggs were incubated for 21 days until hatch at the Department of Poultry Science (University of Saskatchewan). After hatching, the chicks remained at the Department of Poultry Science, but were transferred to off-the-ground wire cages. Swabs were taken from the birds and around their environment throughout the trial and were plated on BG agar to test for the presence of *Salmonella.* Shortly before challenge, the birds were transferred to an isolation room at the Vaccine and Infectious Disease Organization (VIDO). These chickens were used for vaccine trial 1.

4.2.6.1 Vaccine trial 1

Chickens were separated into three groups of 30 birds each, and injected with phosphate buffered saline (PBS), SPI-1 proteins (PrgI and SipD), or SPI-2 proteins (SseB and SseD). Components used for vaccination were formulated to be 100 μg total protein in 0.5 ml PBS with 30% emulsigen D, and delivered subcutaneously. Groups were vaccinated on days 14 (primary vaccination) and 27 (secondary vaccination/boost) of age, and challenged orally with 10^{10} wild-type (WT) *S.* Enteritidis strain Sal18 containing a tetracycline resistance cassette [282] on day 34 of age. One third of each group was sacrificed on days 1, 2, and 4 post-challenge (PC). Colony forming units (CFU)/g were calculated for the cecum, liver and spleen of all groups, and portions of the liver and spleen were enriched in order to determine the total number of birds positive for *Salmonella.* Enzyme-linked immunosorbant assay (ELISA) was used to test sera from chickens at trial day 27 and on days of euthanization for IgY antibody titres against vaccine components. Only the data from the control group and SPI-2 group will be presented here. Data concerning the SPI-1 vaccinates was included in the statistical analyses, and has recently been reported [324].

4.2.6.2 Vaccine trial 2

Leghorn layer hens were obtained from, and housed at, the Department of Poultry Science (University of Saskatchewan). Groups of ten hens were divided into five groups and received one of five vaccines: the negative control (PBS), the positive control vaccine (AviPro® – Lohmann Animal Health International), SPI-1 protein vaccine (PrgI, InvG, SopB, SipC, and SipD), SPI-2 structural protein vaccine (SsaC, SsaG, SseB, SseD, and SipD), or SPI-2 effector protein vaccine (SseI, SseL, SifA, SifB, and SipD). A list of SPI-2 proteins and their function can be found in Table 1.3. The SPI-1 T3SS translocon protein SipD was added to each of the multi-protein vaccines. As previously reported by our group, serum containing SipD specific antibodies inhibits the invasion of *Salmonella* into Caco-2 cells, making it a promising vaccine candidate [325]. Three vaccine doses were given subcutaneously at three-week intervals. Each protein component vaccination dose consisted of 100 μg total protein in 0.5 ml of PBS containing 30% emulsigen D. The negative control group received 0.5 ml PBS with 30% emulsigen D per dose, while the positive control group only received 0.25 ml per dose as suggested by the manufacturer. Blood was collected at the time of each vaccination (days 0, 21, and 42), and three weeks after the third vaccination (day 63). In addition, one to three eggs were collected from each bird on days 64, 65, and 71. Serum was also collected at days 105, 116, and 121 of the trial. ELISAs were used to measure serum and egg yolk IgY antibody titres against the vaccine components. Throughout the trial, swabs were taken from the hens and their environment and plated on BG agar to test for the presence of *Salmonella*. Eggs from these hens were used in vaccine trials 2a, 2b, and 2c, while the hens themselves were used in vaccine trial 3. For the second set of trials, 2a, 2b, and 2c, a seeder model of infection was utilized in order to better emulate a natural infection process in a poultry flock. This method has been well established by other groups [326, 327]. Only data pertaining to the negative and positive controls, as well as the SPI-2 structural and effector protein vaccines will be presented here. See figure 4.1 for a timeline of vaccine trials 2 and 3.

4.2.6.2.1 Vaccine trial 2a

A total number of 165 eggs set on day 71 of the trial were hatched at the Department of Poultry Science (University of Saskatchewan), tagged, and placed in an isolation room at VIDO. A total of 75% of the chicks were orally challenged (seeder chicks) with 10^8 CFU of WT *S.*

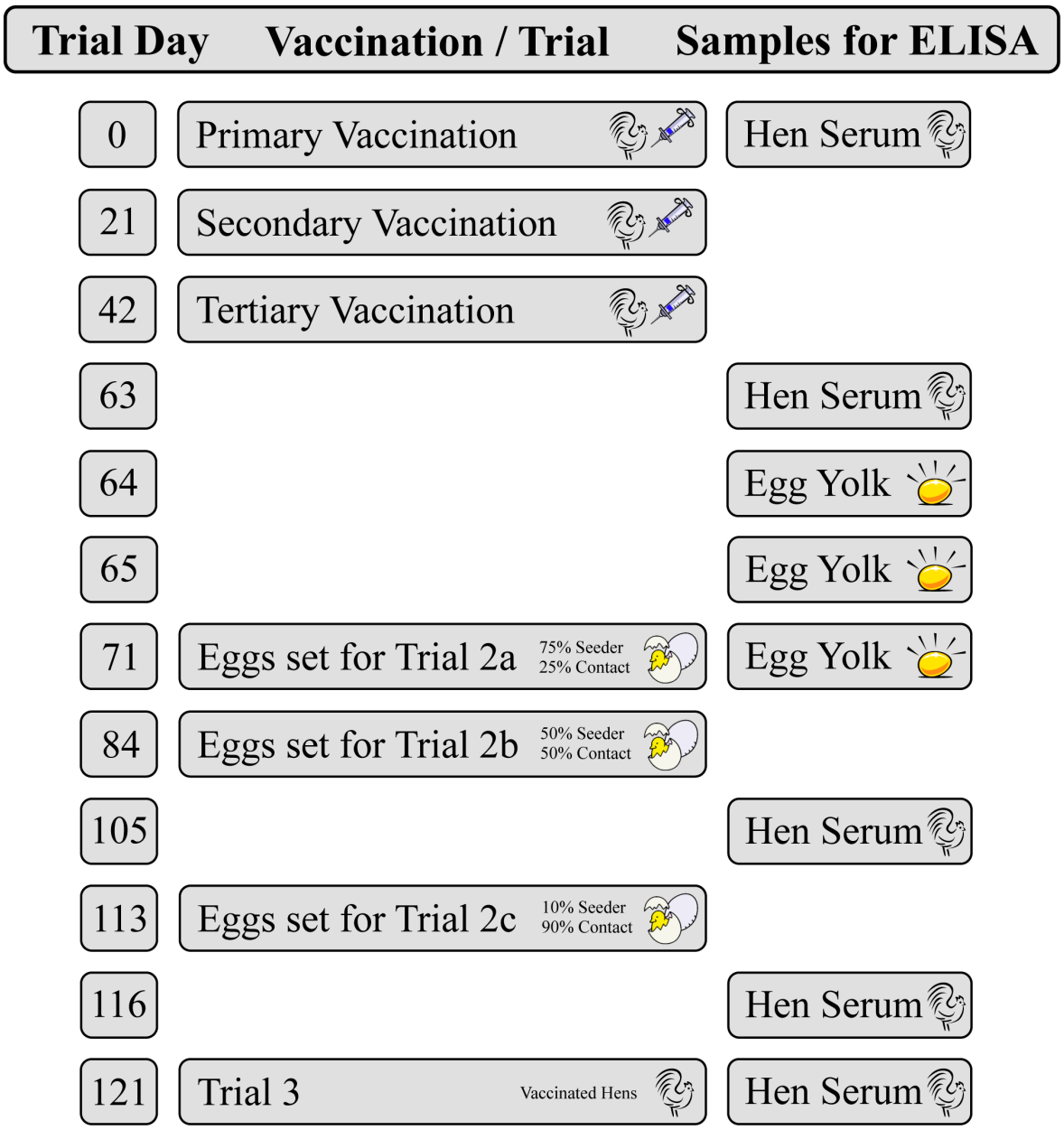

Figure 4.1 Timeline for vaccine trials 2 and 3

The column on the left represents the trial day, the middle column shows the days hens were vaccinated, when eggs from vaccinated hens were set for the seeder-contact model chick trials (trials 2a, 2b, and 2c), and finally the vaccinated hen trial (trial 3). See materials and methods section 4.2.6.2 and 4.2.6.3 for specific details pertaining to vaccine trials 2 and 3.

Enteritidis strain Sal8 in 0.5 ml PBS. The remaining 25% of chicks were not challenged (contact chicks), but allowed to commingle with the challenged chicks. Half of the chicks were euthanized on day 2 PC and their livers, spleens, and ceca processed to assess the amount of challenge strain present. The remaining chicks were euthanized on day 3 PC, and processed identically.

4.2.6.2.2 Vaccine trial 2b

A total of 213 eggs set on trial day 84 were hatched, tagged, and placed in a VIDO isolation room. This time, 60% of the chicks were seeders, while 40% were contact birds. Seeder chicks were orally challenged with 10^8 CFU of WT *S.* Enteritidis strain Sal8 in 0.5 ml PBS. Half of the chicks were euthanized on day 2 PC in order to process the liver, spleen, and cecum to assess the amount of challenge strain present. The remaining chicks were euthanized on day 3 PC, and, again, the liver, spleen, and cecum were processed.

4.2.6.2.3 Vaccine trial 2c

A total of 219 eggs set on day 113 were hatched, tagged, and placed in a VIDO isolation room. Only 10% of the chicks were assigned to be seeder chicks and orally challenged with 10^8 CFU of WT *S.* Enteritidis strain Sal8 in 0.5 ml PBS, while the remaining 90% of chicks were contact chicks. Again, half of the chicks were euthanized on day 2 PC and half euthanized on day 3 PC in order to process the liver, spleen, and cecum to assess the amount of challenge strain present.

4.2.6.3 Vaccine trial 3

Six hens from each vaccine group in vaccine trial 2 were chosen for this trial. All hens were challenged orally with 5 X 10^8 CFU of WT *S.* Enteritidis strain Sal8 in 0.5 ml PBS. The hens were euthanized on day 2 PC and liver, spleen, ovaries, blood, and cecum were processed to assess the amount of challenge strain present.

4.2.7 Antibody isolation from egg yolks

Egg yolk (1 ml) was diluted one in four with acidified water (pH 2.5). The yolk and water mixture was mixed via nutator at room temperature for 15 minutes and frozen at -20°C.

After 48 hours, the mixture was thawed at room temperature and subjected to centrifugation at 3500 x *g* for 30 minutes at 4°C. The resulting supernatant was used in ELISAs to test IgY antibody titres.

4.2.8 Enzyme–linked immunosorbant assay

Immulon® 2HB plates (Nunc) were coated with 100 ng total of SPI-1 or SPI-2 vaccine components (equal parts per protein), or 100 μg of previously prepared Sal 8 bacterin for the AviPro group ELISAs, in 100 μl of coating buffer per well (12.5 mM Na_2CO_3 and 37.5 mM $NaHCO_3$) and incubated overnight at 4°C. The next day, plates were washed 6 times with double distilled water (ddH_2O) using a mechanical plate washer. Plates were then blocked with borate buffered saline containing Tween 20 and bovine serum albumin (BSA) (BBS-TB) (0.17 M H_3BO_3, 0.12 M NaCl, 1 mM EDTA, 0.05% Tween 20, 0.25% BSA) for 30 minutes at room temperature. The plates were washed as previously, and BBS-TB was added at 100 μl per well, except for the first set of wells, which received serum samples in BBS-TB at a dilution of 1/40 in 130 μl total. An amount of 33.3 μl from the first set of wells was transferred to the next set of wells, mixed, and four fold dilutions performed similarly in subsequent wells. Plates were incubated for 30 minutes at room temperature, washed, and blocked with BBS-TB (50 μl per well) for 10 minutes at room temperature prior to another wash step and addition of secondary antibody (horse radish peroxidase labeled goat anti-chicken IgG supplied by KPL) at a dilution of 1/1000, at 50 μl per well. Plates were incubated with the secondary antibody for 30 minutes at room temperature, washed as previous, and blocked again with BBS-TB at 50 μl per well for 30 minutes at room temperature. Plates were washed, and substrate was added at 75 μl per well (ABTS [2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)] peroxidase substrate – KPL), following a final wash step. After a 15 minute incubation at room temperature, the reaction was stopped by adding 75 μl per well of ABTS peroxidase stop solution (KPL) diluted one in five in ddH_2O and absorbance was read using a plate reader (Bio-Rad) at a wavelength of 405 nm (with a reference wavelength of 490 nm).

4.2.9 Statistical analysis

All statistical analyses were performed using GraphPad Prism® 5.0 for Mac OS X. For vaccine trial 1, a one-way ANOVA Kruskal-Wallis test was performed on the ELISA data, as

well as the CFU/g data obtained from liver, spleen, and cecum of birds, followed by Dunn's multiple comparison test if significance was observed. Enrichment data was subject to the chi-square test. For vaccine trial 2, ELISA data were ranked (using Microsoft® Excel™) within day of sample procurement, and two-tailed t-tests were performed. The CFU/g data was also ranked within day of sample acquisition. Ranked data was then subject to one-way ANOVA (including Bartlett's test for equal variance), and followed with Dunnett's multiple comparison test if significance was found. Enrichment data was subject to the chi-square test. A p-value < 0.05 was considered significant.

4.3 Results

4.3.1 Vaccine trial 1

Sera from control animals vaccinated with PBS, and those vaccinated with the SPI-2 protein vaccine (SseB and SseD), were tested on ELISA plates coated with the vaccine components (SseB and SseD). Birds in the SPI-2 vaccine group had significantly higher IgY antibody serum titres than those of the control group on days 1, 2, and 4 PC (p-values < 0.05, 0.001, and 0.01, respectively) (Figure 4.1). Those birds vaccinated with the SPI-2 proteins were found to have less recoverable CFU/g of the challenge strain in their livers than those that received PBS on days 2 and 4 PC; however, a statistically significant difference was only observed on day 4 PC (p-value < 0.01) (Figure 4.2, panel A). No statistically significant difference was found in the spleens and ceca of these birds (Figure 4.2, panels B and C). After enrichment of samples from the liver and spleen, there was no significant difference observed between the numbers of birds positive for presence of the challenge strain; and by day 2 PC nearly 100% of the birds were positive for the challenge strain in their liver and/or spleen (Figure 4.3).

4.3.2 Vaccine trial 2

4.3.2.1 Vaccination of hens

Sera from hens that received PBS, and those vaccinated with the SPI-2 structural protein vaccines were tested by ELISA for SPI-2 structural component specific IgY. Birds in the SPI-2

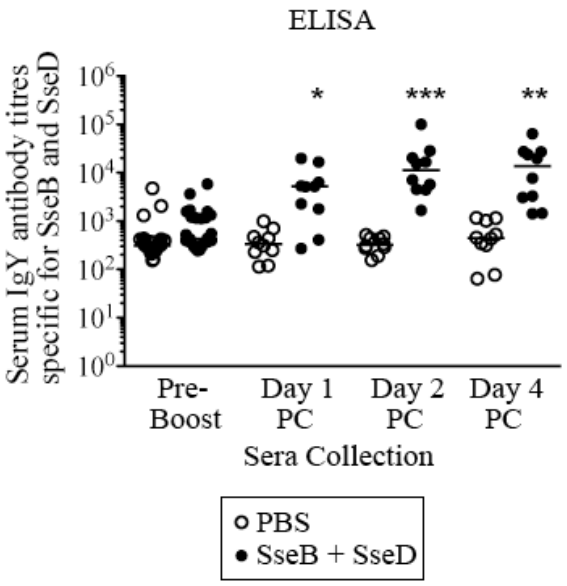

Figure 4.2 Vaccine trial 1: Serum antibody titres specific for vaccine components

IgY titres specific for SseB and SseD in sera from chickens vaccinated with SseB and SseD (●) and control chickens that received PBS (O). Sera were obtained before the secondary vaccination, and on day of euthanization (days 1, 2, and 4 PC). *, p-value < 0.05; **, p-value < 0.01; ***, p-value < 0.001; ****, p-value < 0.0001.

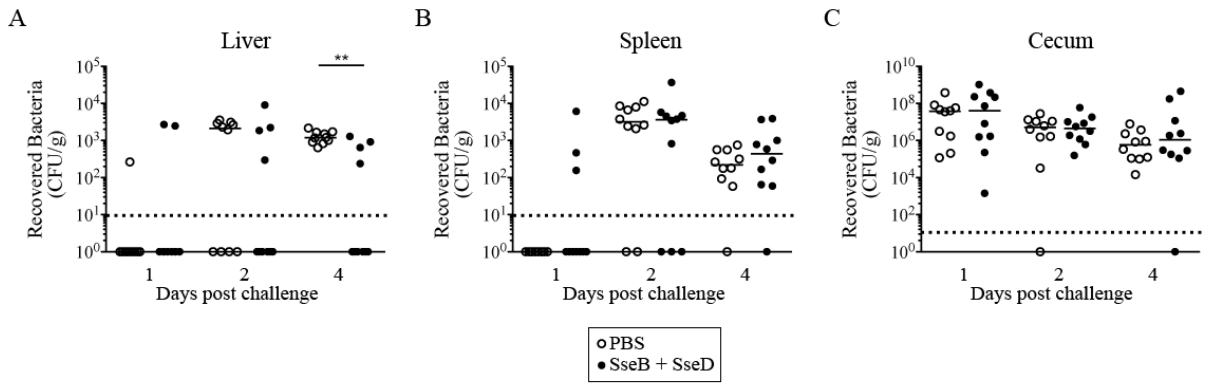

Figure 4.3 Vaccine trial 1: Recovery of Salmonella from liver, spleen, and cecum

Panel A shows the calculated CFU/g for recovered bacteria in the liver of chickens vaccinated with SseB and SseD (●) and control chickens that received PBS (O) on days 1, 2, and 4 PC. **Panels B** and **C** show the same for spleen and cecum. *S.* Enteritidis counts below 10^1 CFU/g cannot be detected by direct plating using the sampling method described here. A dotted line (▪▪▪▪▪▪) has been placed marking this detection level, and birds negative for countable CFU after direct plating on BG agar were assigned a value of 1 for graphical purposes. *, p-value < 0.05; **, p-value < 0.01; ***, p-value < 0.001; ****, p-value < 0.0001.

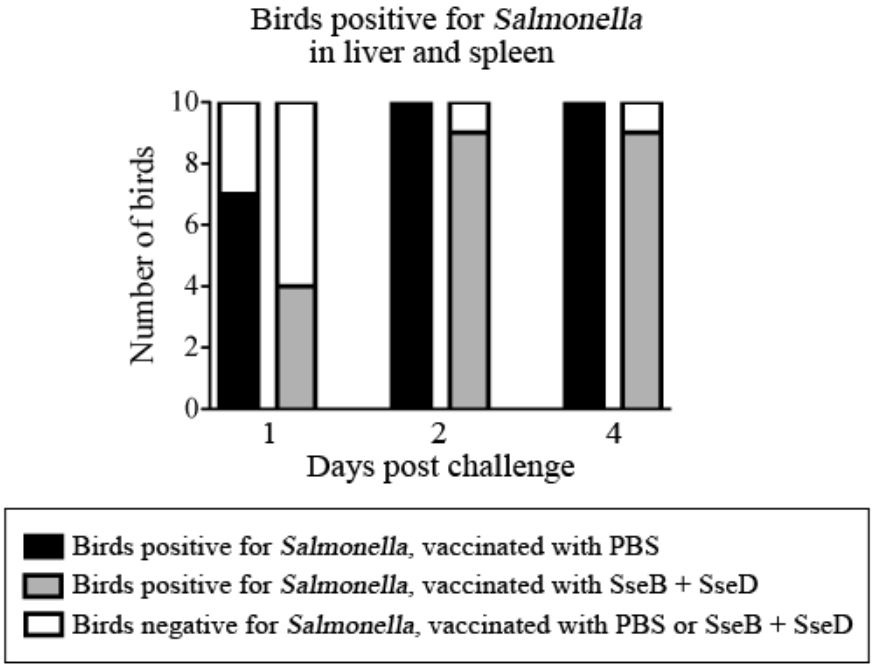

Figure 4.4 Vaccine trial 1: Colonization levels after enrichment

As colonization levels of the liver and spleen can often be quite low, portions of these organs were enriched in selenite broth to increase detection sensitivity. The number of birds vaccinated with SseB and SseD that were found to be positive for presence of the challenge strain after enrichment are represented by black bars. Grey bars represent those positive that received PBS. White bars represent birds that were negative for presence of *Salmonella* in both their liver and spleen after enrichment.

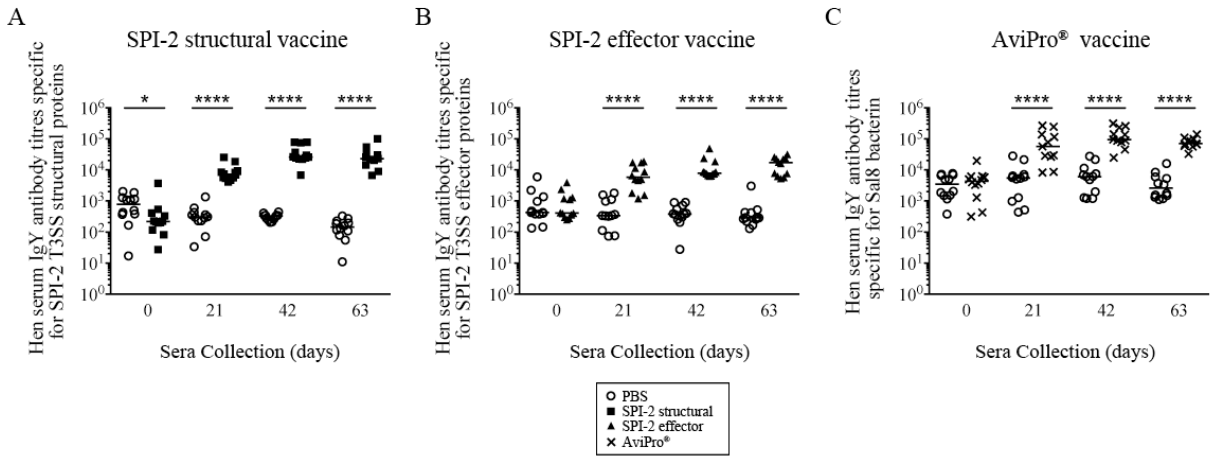

Figure 4.5 Vaccine trial 2: Serum antibody titres of hens specific for vaccine components

Sera were collected before vaccination (day 0), on the days of secondary and tertiary vaccination (days 21 and 42, respectively) and 3 weeks after the tertiary vaccination (day 63). **Panel A** shows the IgY titres specific for vaccine components in sera from chickens vaccinated with SPI-2 T3SS structural proteins (■) compared to control chickens vaccinated with PBS (O). **Panels B** and **C** depict the same, but for birds vaccinated with SPI-2 T3SS effector proteins (▲) or the AviPro® vaccine (X) compared to control chickens vaccinated with PBS (O). *, p-value < 0.05; **, p-value < 0.01; ***, p-value < 0.001; ****, p-value < 0.0001.

structural vaccine group had significantly higher IgY antibody serum titres than those of the control group after vaccination on day 21 (secondary vaccination), day 42 (tertiary vaccination), and day 63 (3 weeks after tertiary vaccination) (p-values < 0.0001) (Figure 4.4, panel A). The same was observed for hens vaccinated with SPI-2 effector proteins compared to those vaccinated with PBS (p-values < 0.0001 on days 21, 42, and 63) (Figure 4.4, panel B). Those receiving the AviPro® vaccine also had higher serum IgY antibody titres than control birds (p-values < 0.0001 on days 21, 42, and 63) (Figure 4.4, panel C). On day 0 (day of first vaccination and start of the trial), all vaccinate groups had similar serum IgY levels to the control group (PBS), however the group that received the SPI-2 structural vaccine had slightly lower serum IgY levels than the control group (p-value = 0.0403) (Figure 4.4).

4.3.2.2 Maternal transfer of antibodies to eggs

Serum in sufficient quantity for analysis by ELISA is quite difficult to obtain from chicks as the process places an extreme amount of stress on them, causing increased mortality. In order to determine whether IgY antibodies specific to the vaccines given to the hens were transferred to the progeny, eggs were collected on trial days 64, 65, and 71. Egg yolks were processed and analyzed for IgY antibodies specific to the vaccine the hen had received. All eggs collected from hens that received the SPI-2 structural protein vaccine had significantly higher egg yolk IgY antibody titres than those from hens that received PBS (p-values < 0.0001 for eggs collected on days 64, 65, and 71) (Figure 4.5, panel A). Similarly, eggs collected from hens vaccinated with the SPI-2 effector vaccine had IgY antibody titres significantly higher than those from hens vaccinated with PBS (p-values < 0.0001 on days 64, 65, and 71) (Figure 4.5, panel B). Eggs collected from hens that had received the AviPro® vaccine had higher IgY antibody titres than the control group on all days as well (p-values < 0.0001 for eggs collected on days 64, 65, and 71) (Figure 4.5, panel C).

4.3.2.3 Vaccine trial 2a

In vaccine trial 2a, 75% of the progeny were orally challenged with Sal8 (seeder birds) while the 25% that were unchallenged remained in contact with the seeder birds (contact birds). Seeder birds whose mothers had been administered the SPI-2 structural or SPI-2 effector vaccine had significantly higher recoverable CFU/g of the challenge strain in their liver on day 3 PC (p-

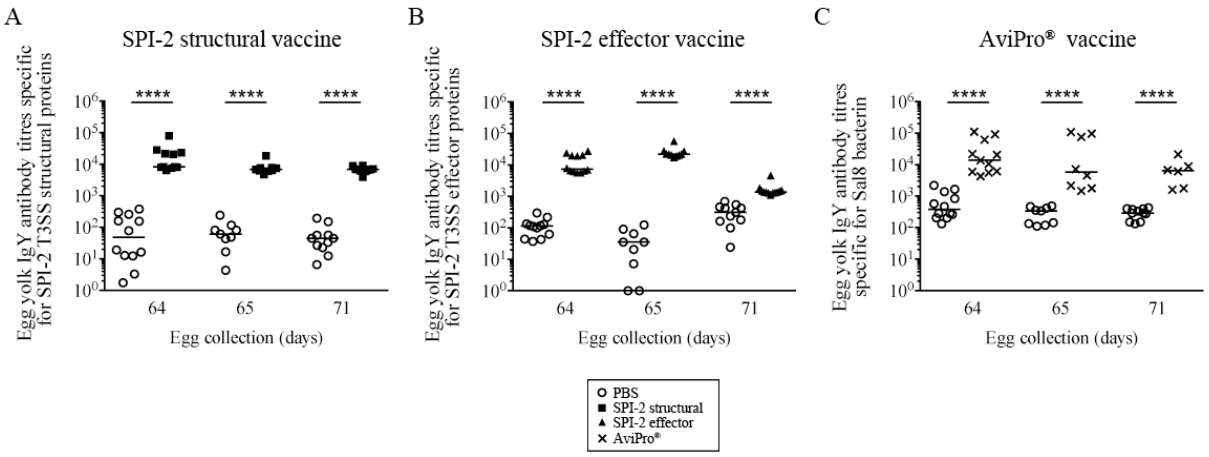

Figure 4.6 Vaccine trial 2: Egg yolk antibody titres specific for vaccine components

Eggs from vaccinated hens were collected on days 64, 65, and 71. **Panel A** shows the IgY titres specific for vaccine components in egg yolks from eggs collected from hens vaccinated with SPI-2 T3SS structural proteins (■) compared to control chickens that received PBS (O). **Panels B** and **C** depict the same, but for eggs from birds vaccinated with SPI-2 T3SS effector proteins (▲) or the AviPro® vaccine (X) compared to control chickens that received PBS (O). *, p-value < 0.05; **, p-value < 0.01; ***, p-value < 0.001; ****, p-value < 0.0001.

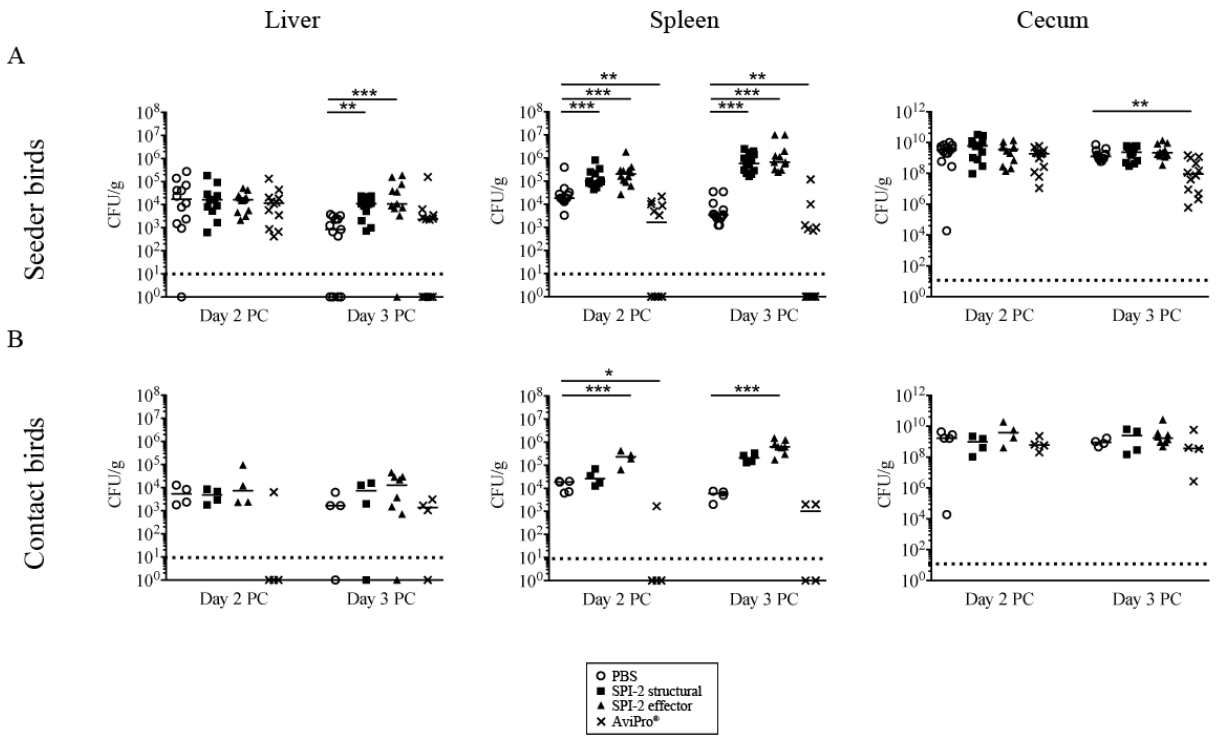

Figure 4.7 Vaccine trial 2a: Recovery of Salmonella from liver, spleen, and cecum of progeny

In vaccine trial 2a, 75% of progeny were challenged on the day after hatch (seeder birds) while 25% of progeny remained unchallenged (contact birds), but were allowed to commingle with the seeder birds. **Panel A** shows the calculated CFU/g for recovered bacteria in the liver, spleen, and cecum of seeder birds on days 2 and 3 PC. O, chicks whose mothers received with PBS; ■, chicks whose mothers were vaccinated with SPI-2 T3SS structural proteins; ▲, chicks whose mothers were vaccinated with SPI-2 T3SS effector proteins; ✕, chicks whose mothers were vaccinated with the AviPro® vaccine. **Panel B** shows the same for contact birds. *S.* Enteritidis counts below 10^1 CFU/g cannot be detected by direct plating using the sampling method described here. A dotted line (▪▪▪▪▪▪) has been placed marking this detection level, and birds negative for countable CFU after direct plating on BG agar were assigned a value of 1 for graphical purposes. *, *p*-value < 0.05; **, *p*-value < 0.01; ***, *p*-value < 0.001; ****, *p*-value < 0.0001.

values < 0.01 and < 0.001 respectively) (Figure 4.6, panel A). A similar trend was also observed in the spleens of these birds (p-value < 0.001 for both groups on both days 2 and 3 PC). There was, however, no difference in bacterial cecal load in any of the seeder birds from these same groups. Seeder birds whose mothers had received the AviPro® vaccine had similar bacterial loads in the liver to the control group, but significantly lower levels in the spleen on day 2 and 3 PC (p-values < 0.01), as well as lower loads in the cecum on day 3 PC (p-value < 0.01) (Figure 4.6, panel A). Birds in the AviPro® group were the only ones who had members of the group negative for recovery of challenge strain in the spleen; however, upon enrichment, all birds were found to be positive for the challenge strain in their liver and/or spleen (data not shown).

Contact birds had similar bacterial loads across all groups in the liver and cecum, but the trend in the spleen was similar to those of the seeder birds (Figure 4.6, panel B). Contact birds whose mothers had received either the SPI-2 structural or SPI-2 effector vaccine had higher levels of challenge strain in their spleens on both days 2 and 3 PC, although the results were only statistically significant for the SPI-2 effector group (p-values < 0.001). Contact birds whose mothers had been given the AviPro® vaccine had similar bacterial loads to the control group in the liver and cecum, but lower loads in the spleen on day 2 PC (p-value < 0.05) (Figure 4.6, panel B). Again, this group was the only one that had birds negative for recovery of the challenge strain. However, upon enrichment of samples, all birds were found to be positive for *Salmonella* in their liver and/or spleen (data not shown).

4.3.2.4 Vaccine trial 2b

For vaccine trial 2b, 60% of the progeny were challenged with Sal8 (seeder birds) while 40% remained unchallenged, but in contact with the seeder birds (contact birds). This time, seeder birds whose mothers had been administered the SPI-2 structural or SPI-2 effector vaccine had significantly lower recoverable CFU/g of the challenge strain in their liver on day 2 PC (p-values < 0.01 and < 0.05 respectively) and day 3 PC (p-values < 0.001 and < 0.01 respectively) (Figure 4.7, panel A). There was no difference between levels of bacteria in the liver between the AviPro® group and the control group. Spleens of the seeder birds in the SPI-2 vaccinate groups showed a similar trend (p-value < 0.001 for the SPI-2 structural group on both days 2 and 3 PC, and p-values of < 0.01 on day 2 PC and < 0.001 on day 3 PC for the SPI-2 effector group). A difference between the levels of bacteria recovered from the spleens of the AviPro® group and

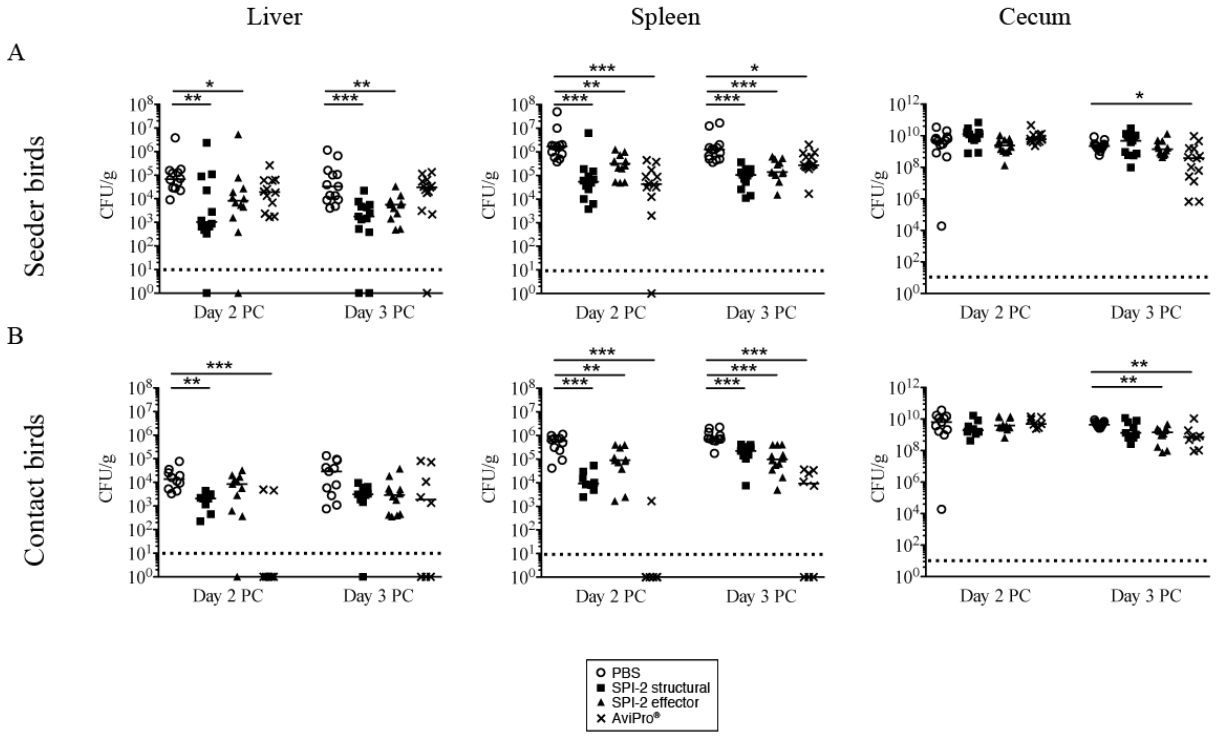

Figure 4.8 Vaccine trial 2b: Recovery of Salmonella from liver, spleen, and cecum of progeny

In vaccine trial 2b, 60% of progeny were challenged on the day after hatch (seeder birds) while 40% of progeny remained unchallenged (contact birds), but were allowed to commingle with the seeder birds. **Panel A** shows the calculated CFU/g for recovered bacteria in the liver, spleen, and cecum of seeder birds on days 2 and 3 PC. O, chicks whose mothers received PBS; ■, chicks whose mothers were vaccinated with SPI-2 T3SS structural proteins; ▲, chicks whose mothers were vaccinated with SPI-2 T3SS effector proteins; ✕, chicks whose mothers were vaccinated with the AviPro® vaccine. **Panel B** shows the same for contact birds. *S.* Enteritidis counts below 10^1 CFU/g cannot be detected by direct plating using the sampling method described here. A dotted line (▪▪▪▪▪▪) has been placed marking this detection level, and birds negative for countable CFU after direct plating on BG agar were assigned a value of 1 for graphical purposes. *, *p*-value < 0.05; **, *p*-value < 0.01; ***, *p*-value < 0.001; ****, *p*-value < 0.0001.

the control group was also observed, with bacterial loads being lower in the AviPro® group on days 2 (p-value < 0.001) and 3 PC (p-value < 0.05). Although there was no difference in bacterial cecal load in any of the seeder birds from the SPI-2 structural or effector groups, those in the AviPro® group had slightly lower cecal loads on day 3 PC (p-value < 0.05) (Figure 4.7, panel A). Upon enrichment, all birds were found to be positive for the challenge strain in their liver and/or spleen (data not shown).

When the contact birds were examined, those whose mothers had received either the SPI-2 structural or AviPro® vaccine had lower levels of challenge strain in their liver on day 2 PC (p-value < 0.01 and < 0.001, respectively) (Figure 4.7, panel B). Results for the spleens match those of the livers. All groups had a statistically significant reduction in bacterial loads on days 2 and 3 PC (SPI-2 structural p-values < 0.001 on both days PC, SPI-2 effector p-values < 0.01 on day 2 PC and < 0.001 on day 3 PC, and AviPro® p-values < 0.001 on both days PC). On day 2 PC, all contact birds had similar bacterial loads in their ceca, while one day 3 PC, birds whose mothers had been given the SPI-2 effector or AviPro® vaccine had lower loads in their ceca than the control group (p-values < 0.01) (Figure 4.7, panel B). Only contact birds whose mothers had received AviPro® were found to be negative for recovery of the challenge strain in the spleen. However, upon enrichment of samples, all birds were again found to be positive for *Salmonella* in their liver and/or spleen (data not shown).

4.3.2.5 Vaccine trial 2c

In order to better simulate the infection situation in the field, in vaccine trial 2c only 10% of the progeny were challenged with Sal8 (seeder birds) while 90% remained unchallenged, but in contact with the seeder birds (contact birds). As such a low number (two per group) of birds were challenged, statistics could not be performed on the seeder group. However, it appears that seeder birds whose mothers had been vaccinated with SPI-2 effector proteins had higher levels of challenge strain in their livers on day 3 PC, while all other groups appeared to have similar loads to the control group on both days 2 and 3 PC (Figure 4.8, panel A). Those whose mothers had been vaccinated with AviPro® were observed to have lower levels in the spleen on both days 2 and 3 PC, while the other two groups appeared to match the control group. There was no difference between any of the groups on either day in cecal bacterial loads (Figure 4.8, panel A). After enrichment, all birds were found to be positive for the challenge strain in their liver and/or

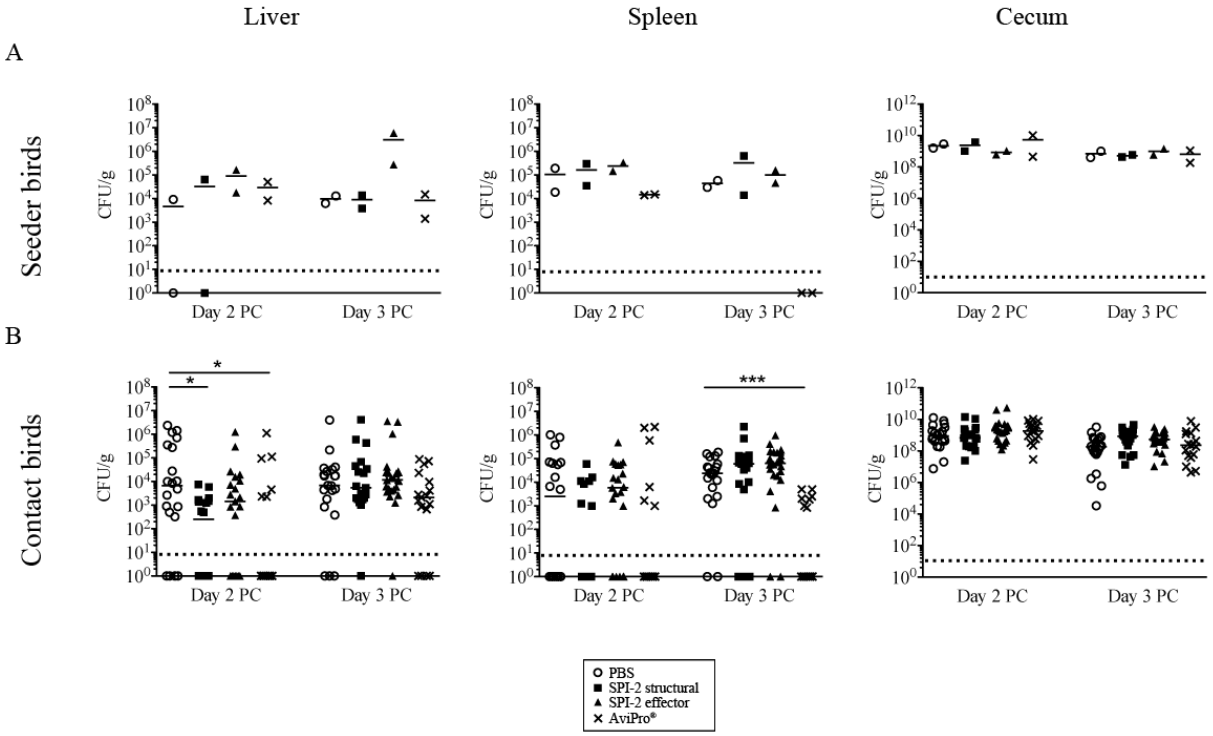

Figure 4.9 Vaccine trial 2c: Recovery of Salmonella from liver, spleen, and cecum of progeny

In vaccine trial 2c, only 10% of progeny were challenged on the day after hatch (seeder birds) while 90% of progeny remained un-challenged (contact birds), but were allowed to comingle with the seeder birds. **Panel A** shows the calculated CFU/g for recovered bacteria in the liver, spleen, and cecum of seeder birds on days 2 and 3 PC. O, chicks whose mothers received PBS; ■, chicks whose mothers were vaccinated with SPI-2 T3SS structural proteins; ▲, chicks whose mothers were vaccinated with SPI-2 T3SS effector proteins; ✕, chicks whose mothers were vaccinated with the AviPro® vaccine. **Panel B** shows the same for contact birds. *S.* Enteritidis counts below 10^1 CFU/g cannot be detected by direct plating using the sampling method described here. A dotted line (▪▪▪▪▪▪) has been placed marking this detection level, and birds negative for countable CFU after direct plating on BG agar were assigned a value of 1 for graphical purposes. *, *p*-value < 0.05; **, *p*-value < 0.01; ***, *p*-value < 0.001; ****, *p*-value < 0.0001.

spleen (data not shown).

Contact birds whose mothers had received either the SPI-2 structural or AviPro® vaccine had lower levels of challenge strain in their liver than the control group on day 2 PC (p-values < 0.05), while those in the SPI-2 structural group were similar to the control group (Figure 4.8, panel B). All groups had similar levels in their livers on day 3 PC. When the spleens of contact birds were examined, no difference in bacterial load between any groups was observed on day 2 PC, while those in the AviPro® group had lower levels of challenge strain present on day 3 PC (p-value < 0.001). There was no difference between any of the groups on either day in cecal loads (Figure 4.8, panel B). Again, upon enrichment of samples, all birds were found to be positive for *Salmonella* in their liver and/or spleen (data not shown).

4.3.3 Vaccine trial 3

Vaccine trial 3 involved challenge of 30 of the hens from vaccine trial 2. Vaccinated hens were found to have maintained higher serum IgY antibody titres specific to vaccine components than those of the hens that received PBS (Figure 4.9). Those that had received the SPI-2 structural vaccine had high titres against those components on days 105, 116, and 121 compared to those that received PBS (p-values = 0.0461, < 0.0001, and = 0.0002 respectively) (Figure 4.9, panel A). Hens in the SPI-2 effector group had significantly higher IgY titres than those of the control on days 105 and 116 (p-values < 0.0001), and day 121 (p-value = 0.0077) (Figure 4.9, panel B). Those hens which received the AviPro® vaccine followed the same trend as the SPI-2 effector vaccine with statistically higher serum IgY titres observed on days 105 and 116 (p-values < 0.0001), and day 121 (p-value = 0.0032) (Figure 4.9, panel C). When the levels of bacteria in the hens were examined, no bacteria could be isolated via direct plating from the ovaries or blood, while only one hen had countable levels of bacteria within its liver and spleen (Figure 4.10, panels A and B, respectively). Within the ceca, only those hens that had been vaccinated with the AviPro® vaccine had significantly lower bacterial loads (p-value < 0.001) (Figure 4.10, panel C). After enrichment of liver, spleen, ovaries, and blood, three (out of six) of the hens in the PBS group were positive for the challenge strain, four of the hens in the SPI-2 structural group, two of the hens in the SPI-2 effector group, and only one of the hens in the AviPro® group were positive for the challenge strain in their liver and/or spleen (Figure 4.11). There was, however, no statistical significance between these groups.

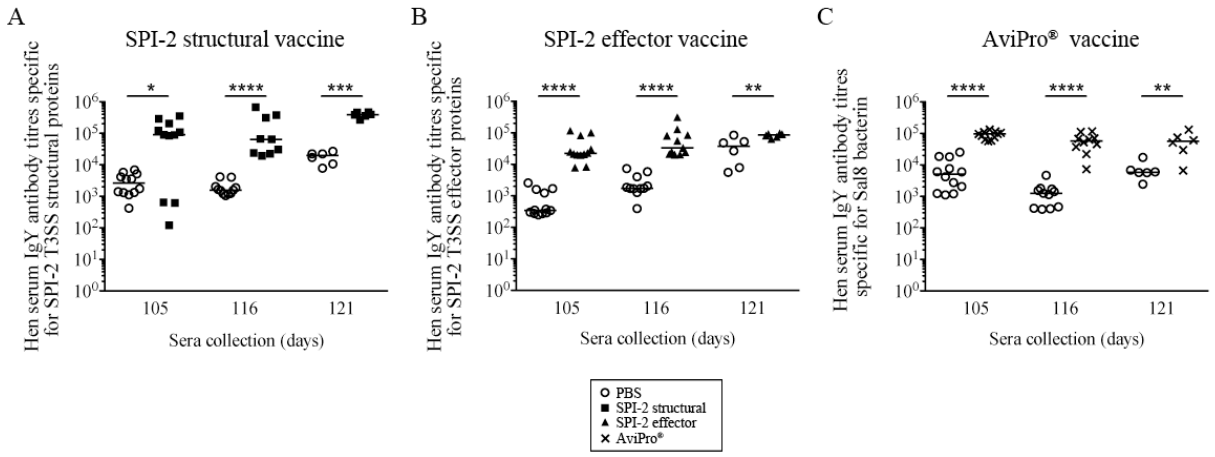

Figure 4.10 Vaccine trial 3: Serum antibody titres of Hens specific for vaccine components

Sera were collected on day 105 (9 weeks after tertiary vaccination), 116, and 121 in order determine if serum IgY titres specific for vaccine components remained high in vaccinated hens. **Panel A** shows the IgY titres specific for vaccine components in sera from chickens vaccinated with SPI-2 T3SS structural proteins (■) compared to control chickens that received PBS (O). **Panels B** and **C** depict the same, but for birds vaccinated with SPI-2 T3SS effector proteins (▲) or the AviPro® vaccine (✕) compared to control chickens that received PBS (O). *, p-value < 0.05; **, p-value < 0.01; ***, p-value < 0.001; ****, p-value < 0.0001.

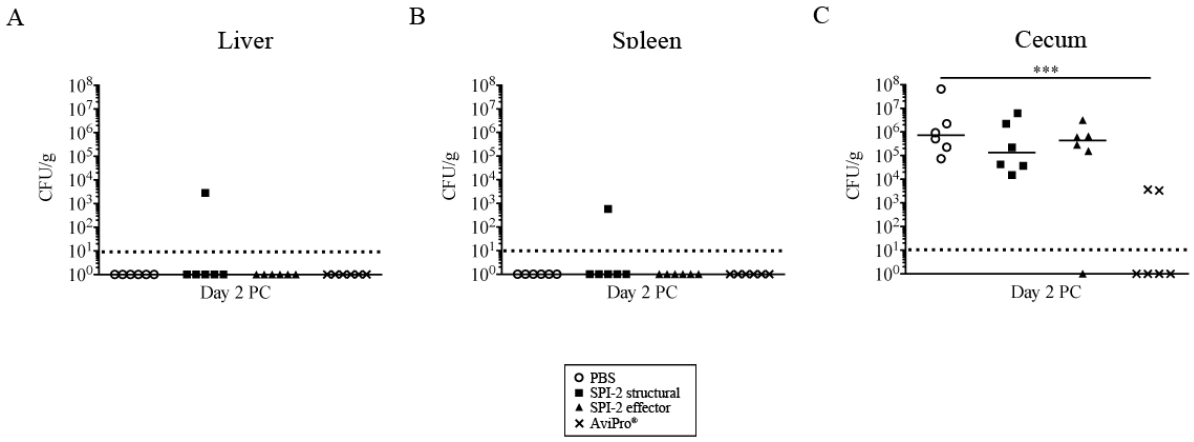

Figure 4.11 Vaccine trial 3: Recovery of Salmonella from liver, spleen, and cecum of hens

In vaccine trial 3, hens were challenged with *S.* Enteritidis 11 weeks after tertiary vaccination. **Panel A** shows the calculated CFU/g for recovered bacteria in the liver on day 2 PC, while **panels B** and **C** show the calculated CFU/g in spleen and cecum on day 2 PC. O, hens that received PBS; ■, hens vaccinated with SPI-2 T3SS structural proteins; ▲, hens vaccinated with SPI-2 T3SS effector proteins; ✕, hens vaccinated with the AviPro® vaccine. *S.* Enteritidis counts below 10^1 CFU/g cannot be detected by direct plating using the sampling method described here. A dotted line (▪▪▪▪▪▪) has been placed marking this detection level, and birds negative for countable CFU after direct plating on BG agar were assigned a value of 1 for graphical purposes. *, *p*-value < 0.05; **, *p*-value < 0.01; ***, *p*-value < 0.001; ****, *p*-value < 0.0001.

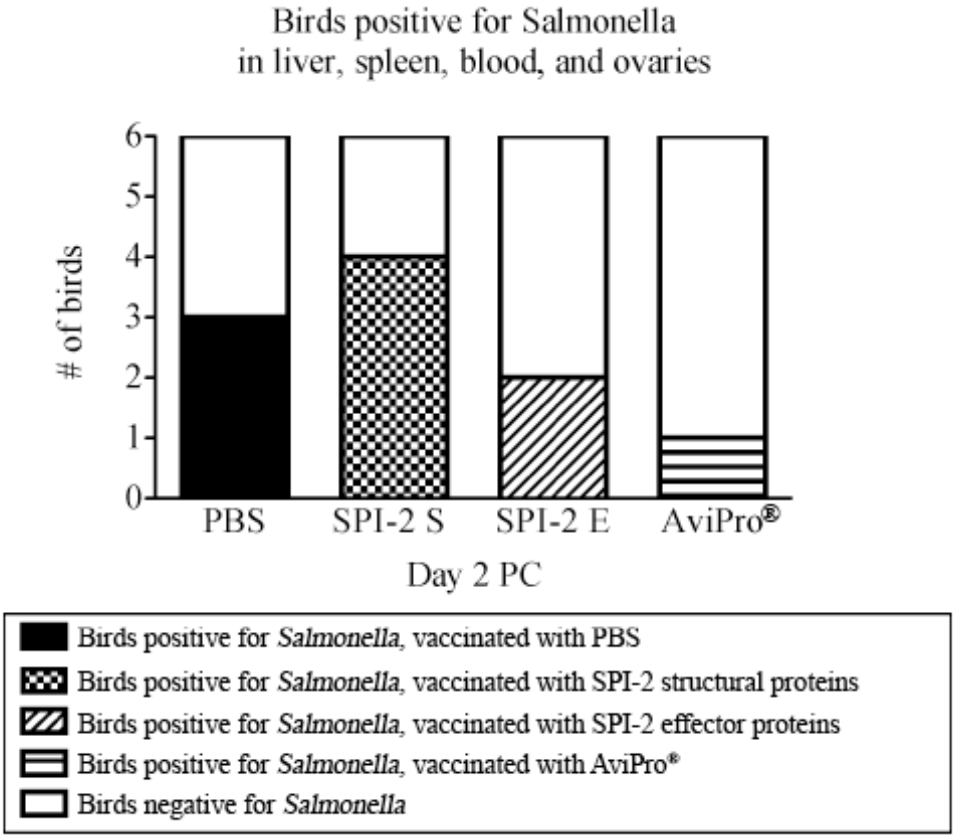

Figure 4.12 Vaccine trial 3: Colonization levels after enrichment of liver, spleen, ovaries, and blood

As colonization levels of the liver, spleen, ovaries, and blood can often be quite low, portions of these samples were enriched in selenite broth to increase detection sensitivity. The number of birds that had received PBS found to be positive for the presence of *S.* Enteritidis on day 2 PC after enrichment is represented by a black bar. A checkered bar represents those positive that were vaccinated with SPI-2 T3SS structural proteins, a diagonally lined bar represents the number of positive hens that had been vaccinated with SPI-2 T3SS effector proteins, and a horizontally lined bar represents those positive who were vaccinated with the AviPro® vaccine. White bars represent birds that were negative for presence of *Salmonella* in their liver, spleen, ovaries, and blood after enrichment.

4.4 Discussion

There is currently a need for a safe and efficacious vaccine that will protect poultry against colonization by *S.* Enteritidis, and thus protect humans against salmonellosis caused by *S.* Enteritidis associated with poultry meat and eggs. This is the first study, to our knowledge, that has examined the potential of a vaccine containing proteins of the SPI-2 T3SS. The focus of the first and third trials was to gauge whether a vaccine containing SPI-2 T3SS components could induce a humoral response in chickens, and confer protection against colonization of those chickens by *S.* Enteritidis. The focus of the second set of trials was to determine if hens immunized with SPI-2 T3SS components could transfer a significant quantity of maternal antibodies to progeny, and to determine whether these transferred antibodies would have a protective effect on the progeny against challenge with *S.* Enteritidis.

Ideally, a good vaccine should confer strong protection against both intestinal and systemic colonization of poultry, while not causing disease symptoms in either poultry or humans [14]. It is generally accepted, based mainly on evidence obtained using the mouse typhoid model, that a strong cell-mediated immune response is more important in clearance of *Salmonella* than a humoral immune response, although some studies have shown that the humoral immune response is still important. Therefore, a good vaccine should induce both a strong cell-mediated and humoral immune response, both systemically and mucosally [15, 26, 206, 209]. Killed bacteria and subunit-based vaccines tend to elicit a good humoral immune response, but lack in their ability to induce a strong cell-mediated immune response. Although live bacterial vaccines have an advantage in this area, as they are able to induce both strong humoral and cell-mediated immune responses, killed bacteria and subunit vaccines tend to be more accepted by the public, and have fewer human and animal health risks associated with them. There is the possibility of live attenuated bacterial vaccine strains spreading from animal to animal, and even to humans, as well as a chance of these strains reacquiring virulence [25, 271, 328]. Therefore, the development of an effective killed bacteria or subunit vaccine that elicits both a strong humoral and cell-mediated immune response is important for reducing the spread of *S.* Enteritidis and *S.* Typhimurium to humans.

While it has been stated that cell-mediated immune response is more important for clearance of *Salmonella* than a humoral immune response, most of these studies have been done in mice [15, 26, 329]. However, recent work has shown that passive immunization with *S.*

Typhimurium-specific antibodies can partially protect mice against virulent challenge, illustrating that humoral immunity can play an important role in protection from *Salmonella* [211]. This group showed that a humoral immune response cooperated with a cell-mediated immune response in mice infected with *S.* Typhimurium, inducing protective immunity against *S.* Typhimurium. Mouse macrophages infected with *S.* Typhimurium that had been treated with *S.* Typhimurium-specific antibodies had similar bacterial loads as those that were not treated with *S.* Typhimurium-specific antibodies, however, mice given *S.* Typhimurium-specific antibodies and then challenged with *S.* Typhimurium had much lower mortality rates than those that were not given *S.* Typhimurium-specific antibodies. This indicates the importance of both humoral and cell-mediated immunity in protecting against fatal infection by *S.* Typhimurium, even though actual bacterial loads may remain similar [211].

To date, very little work has focused on developing a protein subunit vaccine against *Salmonella* for use in poultry, although a few studies involving siderophore protein vaccines, and one involving fimbria have been carried out. In one study by Kaneshige *et al.* [268], 30-day-old SPF chickens were immunized with the siderophore receptor IroN and subsequently challenged with *S.* Enteritidis. Vaccinated chickens showed a strong serum IgY antibody response to the vaccine, and vaccinates had a 10% mortality rate compared to the 80% mortality rate of non-vaccinates. A study by Meenakshi *et al.* [265] in 1999 demonstrated that chickens vaccinated either parenterally or orally with *S.* Enteritidis outer membrane proteins had high serum antibody titres compared to non-vaccinates (similar to our results), and, upon challenge with *S.* Enteritidis, shed significantly less *S.* Enteritidis in their feces. Similarly, when 9-week-old SPF chickens were vaccinated subcutaneously with two *S.* Enteritidis outer membrane proteins and challenged with *S.* Enteritidis, vaccinates had significantly lower numbers of *S.* Enteritidis attached to their intestinal mucosa [266]. Unfortunately, we did not see this decreased shedding effect with our protein subunit vaccines.

To our knowledge, there has been no attempt at developing a protein subunit vaccine based on SPI-2 T3SS components for use in chickens. One group has studied the potential of a SPI-1 T3SS effector-based vaccine in pigs challenged with *S.* Typhimurium [330]. This group found that while vaccinates had lower levels of *S.* Typhimurium in their ileum and colon, bacterial levels in the intestinal lymph nodes and mucosa were similar to non-vaccinates. They also found that the lower levels of *S.* Typhimurium found in vaccinates was not SPI-1 specific,

indicating that a SPI-1 T3SS effector-based vaccine is not a viable option for protecting pigs against colonization by *S.* Typhimurium. There has, however, been some success using T3SS protein based vaccines and passive immunization for protection against other species of pathogenic bacteria including *Chlamydia, Shigella*, *Yersinia, Pseudomonas,* and *E. coli* [195]. Of note is the first licensed vaccine based on the strategy of using T3SS components in a vaccine – Econiche™. Econiche™ is protective against colonization of cattle by *E. coli* O157:H7 [331, 332].

Taken together, the results of these studies suggest that the use of certain SPI-2 T3SS protein components in a subunit vaccine, in combination with other components, may be a viable option. While showing mild protective effects on their own, in certain situations, these proteins elicit a strong humoral immune response in chickens that can be transferred maternally to progeny. These results may be useful to further vaccine development for a protein subunit vaccine that will elicit both cell-mediated and humoral immune responses and induce protection of chickens against colonization by *S.* Enteritidis.

5.0 EFFECT OF THE *SALMOENLLA* PATHOGENICITY ISLAND 2 TYPE III SECRETION SYSTEM ON *SALMONELLA* SURVIVAL IN ACTIVATED CHICKEN MACROPHAGE-LIKE HD11 CELLS

5.1 Introduction

Infections by *Salmonella enterica* subspecies *enterica* are one of the leading causes of food borne gastroenteritis in humans [21]. Among those serovars responsible for food poisoning in humans, serovars Typhimurium (*S.* Typhimurium) and Enteritidis (*S.* Enteritidis) are most commonly isolated serovars from both humans (Figure 1.1) and animals (Figure 1.2) in many regions. In North America, *S.* Typhimurium is the primary serovar isolated from both humans and animals, while *S.* Enteritidis is the second most common serovar isolated from humans. The opposite is true for most of the European Union, with *S.* Enteritidis being the number one isolate from both humans and animals and *S.* Typhimurium being number two [27]. Both *S.* Typhimurium and *S.* Enteritidis are capable of causing systemic disease in humans, although this is not the normal course of infection and only occurs in very young, very old and/or immunocompromised individuals [184].

Salmonella uses two specialized type III secretion systems (T3SS) that facilitate invasion and survival within the host cell. These two T3SSs are encoded within *Salmonella* pathogenicity islands 1 and 2 (SPI-1 and SPI-2) and secrete effectors into the host cell, triggering a number of events in the infected cell. These events ultimately lead to the symptoms of disease. It is the current view that the SPI-1 T3SS is mainly involved in invasion of the host cell, while the SPI-2 T3SS plays a role in survival within the host cell and maintenance of the *Salmonella* containing vesicle (SCV) [59, 284, 285]. SPI-2 is a region of approximately 40 kb located at centisome 31 in *Salmonella* species, and has been reported to be necessary for systemic infection, intracellular proliferation and survival, and maintenance of the SCV. However, the majority of these studies have been performed in mice, where *S.* Typhimurium and *S.* Enteritidis produce a typhoid-like infection rather than gastroenteritis, and therefore may not be indicative of the course of infection in healthy adult humans and chickens [55, 286-288].

The preferred site of invasion for *Salmonella* is through microfold (M) cells of the intestine. M cells reside within the follicular associated epithelium that overlays the Peyer's patches, have a less pronounced brush boarder, and are associated with mucous in less abundance

than other intestinal epithelial cells. Once through the epithelial barrier, *Salmonella* are taken up by resident or recruited macrophages and dendritic cells [183, 189, 190, 194, 196]. An effective innate immune response is necessary to clear *Salmonella* and prevent systemic spread; recruited macrophages, natural killer (NK) cells and dendritic cells are paramount in this process, but in some cases *Salmonella* is able to manipulate and invade the host immune response and spread systemically [184, 185, 194, 333]. However, within phagocytic cells, SPI-2 effectors are secreted across the SCV membrane and stop the fusion of lysosomes with the SCV, thereby avoiding bacterial killing by defensins, cathelicidins, lysozymes, lipases, proteases, and reactive oxygen and nitrogen species (ROS and RNS) [178, 289, 290, 334]. T3SS effectors facilitate the maturation of the SCV, and can act as pro- or anti-inflammatory factors [291, 292].

Previously, our group found that while *S.* Enteritidis SPI-2 mutants were slower to colonize the spleens and livers of chickens, the levels of the mutant and WT were similar by day 4 post-challenge (PC) [282]. A major mode of transport for *Salmonella* to systemic sites like the liver and spleen is likely within macrophages [335]. There is a vast array of conflicting evidence in the literature about the importance of the SPI-2 T3SS to the survival of *S.* Typhimurium and *S.* Enteritidis within macrophages. In this study, we demonstrate that in activated HD11 chicken macrophage-like cell line, the SPI-2 T3SS does not contribute to survival of *S.* Typhimurium and *S.* Enteritidis.

5.2 Materials and methods

5.2.1 Cloning and production of *Salmonella* pathogenicity island 2 mutants

Construction of SPI-2 mutants and cloning are have been described previously in chapter 3 [282].

5.2.2 Bacterial strains and growth conditions

Bacterial strains used in this study are described in Table 3.1. Standard growth procedures were followed using Luria-Bertani (LB) broth and agar at 37°C.

5.2.3 HD11 cell line and growth conditions

HD11 cells were kindly provided to VIDO by Dr. Kirk C. Klasing (currently Department

of Animal Science, University of California – Davis, Davis, CA, USA). HD11 cells are a macrophage-like immortalized cell line derived from chicken bone marrow and transformed with the avian myelocytomatosis type MC29 virus [336]. HD11 cells were maintained at 42°C, in a humidified incubator (5% CO_2), in RPMI 1640 media (Gibco) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine (Gibco), and 10 mM HEPES. For all assays involving bacteria the was changed to RPMI 1640 containing 10% heat-inactivated FBS, 2 mM L-glutamine, and 10 mM HEPES before cells were seeded into 24-well plates. HD11 cells were used for all assays between passages 15 and 25.

5.2.4 Gentamicin protection assay

Approximately 12 hours before infection, HD11 cells were placed in RPMI 1640 (10% heat-inactivated FBS, 2 mM L-glutamine and 10 mM HEPES), treated with 100 ng/ml phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich), and seeded into 24-well cell-bind plates (Corning) at a concentration of 5 x 10^5 cells per well. At this time growth of bacterial strains were started. 12 hours post-activation, HD11 cells were checked to ensure that differentiation was induced by PMA (Figure 5.1) and bacterial overnight cultures were sub-cultured and grown to an OD_{600} corresponding to 1 x 10^8 CFU/ml. The number of viable HD11 cells was determined from three wells via trypan blue exclusion. Media was removed from the cells, and bacteria (in pre-warmed RPMI 1640) were added to each well at a multiplicity of infection (MOI) of 25 (time 0 h). Serial dilutions of the bacteria were made and plated in order to confirm that each strain was added to the HD11 cells at a multiplicity of infection (MOI) of approximately 25. Plates containing HD11 cells and bacteria were subject to centrifugation at 200 x *g* in a Sorvall benchtop centrifuge for 5 minutes at room temperature, and then placed at 42°C. After 0.5 h, media containing bacteria was carefully removed from the HD11 cells, and the cells were washed once with PBS containing 500 μg/ml gentamicin. RPMI 1640 containing 250 μg/ml gentamicin was then added to each well, and the plates were placed back at 42°C. At each time point (0.5, 3, 6, 12 and 24 hours post-infection [PI]), media was removed from three wells per bacterial strain and centrifuged at 20,800 x *g* in an Eppendorf benchtop microfuge for 10 minutes at 4°C. Sediments from this media fraction were resuspended in 0.5 ml 1% Triton X-100, and 100 μl portions from each sample were plated on LB-agar using 3 mm borosilicate glass beads. Cells from three wells per bacterial strain were washed once with PBS, and lysed in 0.5 ml 1% Triton

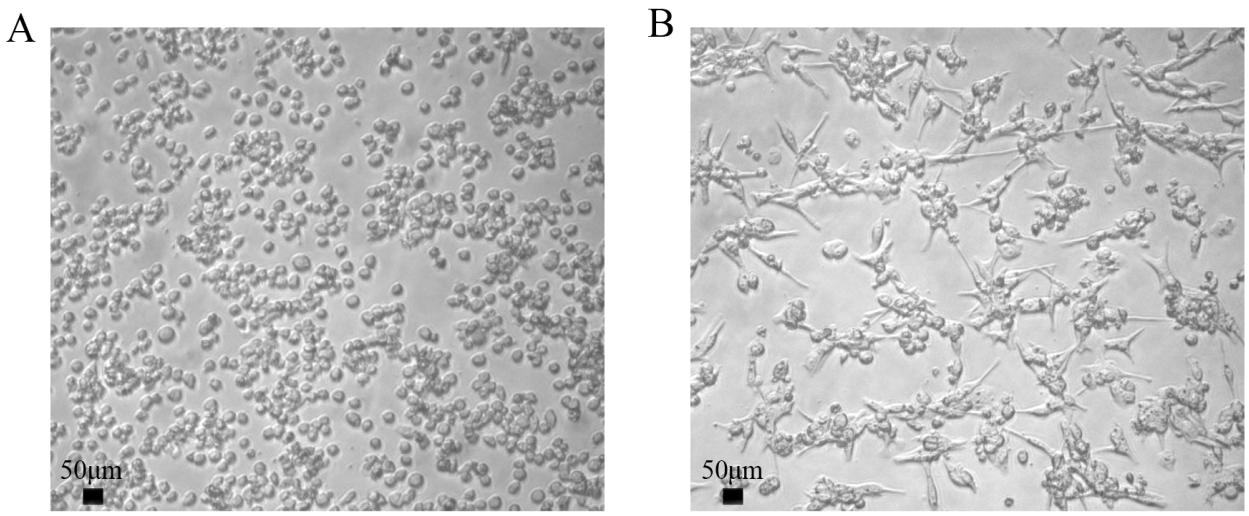

Figure 5.1 HD11 chicken macrophage-like cells

Panel A depicts HD11 cells that have not been stimulated with PMA, while **Panel B** shows HD11 cells 12 hours after stimulation with PMA. Photographs were taken under 10X magnification.

X-100 in PBS. Dilution series of the cell monolayer fractions were made and 100 μl of each dilution (10^{-4}, 10^{-3}, 10^{-2}, 10^{-1}, 10^{0}) was plated on LB agar. In order to confirm the effectiveness of the gentamicin, at the 0 h time point bacteria were also added to a culture tube containing 5 ml of RPMI and 5 ml of RPMI with 250 μg/ml gentamicin added. 100 μl from each tube was plated on LB agar at each time point. This experiment was repeated three times, and data pooled for statistical analysis.

5.2.5 Location of *Salmonella* in the media fraction

To determine if the bacteria in the media fraction were free, or contained within detached HD11 cells or cell portions, 100 μl of the media fraction from the 3 h PI time point, from HD11 cells infected with *S.* Typhimurium strain SL1344, were plated on LB agar for initial CFU/ml values. The media fraction was then divided into two 5 ml portions in 14 ml culture tubes. To one tube, 1% Triton X-100 was added in order to lyse any eukaryotic cell membranes, and the tubes were placed in a shaker at 37°C for 3 hours, following which a further 100 μl from each tube was plated on LB agar. A portion of the initial media fraction was also subject to staining with Giemsa or PKH26 (Sigma-Aldrich) and viewed using a fluorescence microscope (Zeiss Axiovert 200M). PKH26 is a red fluorescent dye linked to long aliphatic tails that can insert into lipid regions of cell membranes. PKH26 and Giemsa staining were carried out as indicated by the manufacturer.

5.2.6 Immunofluorescence

Survival assays were performed in 8 well chamber slides as above; however, bacterial numbers were not enumerated by plating. Instead, at each time point (0, 0.5, 3, 6, 12, and 24 hours PI), the media fraction was collected, sedimented, and resuspended in 100 μl 0.1% EDTA in PBS. The samples from the media fraction were then placed on slides using a CytoSpin 4 centrifuge (Thermo Scientific). Briefly, slides were placed in the CytoSpin centrifuge, and 100 μl of FBS was added and centrifuged onto the slides at 400 x *g* for 3 minutes, followed by the addition of 100 μl of sample under the same conditions. Slides were allowed to dry overnight, and fixed in ice-cold acetone for 10 minutes. After fixation, slides were washed 3 times with PBS, and then incubated for 0.5 hour with fluorescein-conjugated mouse anti-*Salmonella* IgG or fluorescein-conjugated rabbit anti-*E. coli* IgG (1/50 in PBS). Samples were washed as before,

and then treated with rhodamine-conjugated goat anti-mouse IgG or rhodamine-conjugated goat anti-rabbit IgG (1/50 in PBS). Samples were washed, and then treated with 0.5% Triton X-100 for 5 minutes to permeabilize HD11 cell membranes. Following an additional wash, goat anti-*Salmonella* IgG conjugated with fluorescein or rabbit anti-*E. coli* IgG conjugated with fluorescein (1/50 in PBS) were added to the samples. All antibodies were purchased from AbD Serotec. Samples were washed, and then stained with DAPI (10 μg/ml) (Sigma-Aldrich) for 15 minutes at room temperature. Coverslips were added to the slides using FluorSave™ mounting medium (EMD chemicals INC), and all samples were viewed using the Zeiss Axiovert 200M microscope with a mercury vapour short-arc lamp for fluorescence. Photographs were taken using the Zeiss Axiocam and were processed using Adobe© Photoshop© CS 5 for Mac OS X. Manipulations included cropping for space and level adjustment to reduce background noise.

5.2.7 Superoxide assay

The assay to measure superoxide production via luminometry was adapted from Thrasher *et al.* [337]. Briefly, 12 hours before the experiment, 2 x 10^7 HD11 cells were seeded into two 75 cm^2 tissue culture flasks. PMA was added to one of the flasks at a concentration of 100 ng/ml. The next day cells were washed once with PBS and harvested by the addition of 0.5% trypsin. Cells were counted and amounts corresponding to 5.0 x 10^5 cells were added to individual eppendorf tubes prior to being centrifuged at 2700 x *g* for 5 min at 4°C, and resuspended in 100 μl Hanks' buffered saline (HBS) with calcium and magnesium (137 mM NaCl, 5.4 mM KCl, 358 mM $NaHCO_3$, 0.44 mM KH_2PO_4, 0.34 mM Na_2HPO_4, 0.5 mM $CaCl_2$, 1 mM $MgCl_2$). Directly before reading luminescence, 100 μl of 10 μM luminol (Sigma-Aldrich) in HBS and 10 U of horseradish peroxidase (HRP) (Sigma-Aldrich) in 50 μl HBS were added to the cells. Samples were read for 5 seconds at 3 – 5 minute intervals using a GloMax 20/20 luminometer (Promega Biosciences).

5.2.8 Statistical analysis

Data from each survival assay were pooled and ranked using Microsoft® Excel™ for Mac OS X. All statistical analyses were performed using GraphPad Prism® 5.0 for Mac OS X. One-way ANOVAs were performed on each time point. If significance between groups was found, the data was further analyzed using the post-hoc Tukey test. *p*-values ≤ 0.05 were

considered significant. Data from two superoxide assays were ranked and a two-way ANOVA was performed, followed by the post-hoc Bonferroni multiple comparisons test.

5.3 Results

5.3.1 Activation of HD11 cells

HD11 cells are normally loosely adherent to plastic and have an ovoid shape; but can be activated to become more macrophage-like after stimulation with PMA (Figure 5.1, panel A). Twelve hours after exposure to 100 ng/ml PMA, the HD11 cells become more adherent and the morphology becomes further macrophage-like, with a spindle shape (Figure 5.1, panel B). In addition, stimulated cells were found to produce more reactive oxygen species than unstimulated cells, as measured by production of hydrogen peroxide (p-value < 0.0001) (Figure 5.2).

5.3.2 Survival of *Salmonella* pathogenicity island 2 mutants within HD11 cells

At 0.5 hours PI, the non-pathogenic *E. coli* DH5α strain was recovered from the cell monolayer fraction at much higher levels than were any of the *Salmonella* strains (SL1344, Sal18, ΔSPI-2 p-value < 0.001 and Δ*ssaR* p-value < 0.01) (Figure 5.3, panel A). At 3, 6, 12, and 24 h PI, viable *E. coli* DH5α were no longer recoverable from the cell monolayer fraction (all p-value < 0.001, except Δ*ssaR* at 24 h PI: p-value < 0.01) (Figure 5.3, panels B-E). This observation indicates that even though more viable *E. coli* DH5α bacteria were recovered 0.5 h PI compared to the *Salmonella* strains, it is clear that the HD11 cells are able to effectively kill the *E. coli* strain after the initial infection process. Non-viable (or non-recoverable) *E. coli* DH5α are still visible by immunofluorescence at all time points, although in less abundant amounts than the *Salmonella* strains (Figures 5.5 – 5.9, panel E). When the *Salmonella* strains were examined it was found that more of the *S.* Typhimurium wild-type (WT) strain (SL1344) was recovered from the cell monolayer fraction than the WT *S.* Enteritidis strain (Sal18) at all time points, with statistical significance observed at 0.5, 3, 6 and 12 h PI (p-value < 0.001) (Figure 5.3). There was no difference in recovery between the WT *S.* Typhimurium and SPI-2 mutant (Δ*ssaR*) *S.* Typhimurium strains, while greater amounts of the *S.* Enteritidis SPI-2 (ΔSPI-2) mutant were recovered than the WT *S.* Enteritidis strain at 3, 6 (p-values < 0.001) and 12 h PI (p-value < 0.05) (figure 5.3).

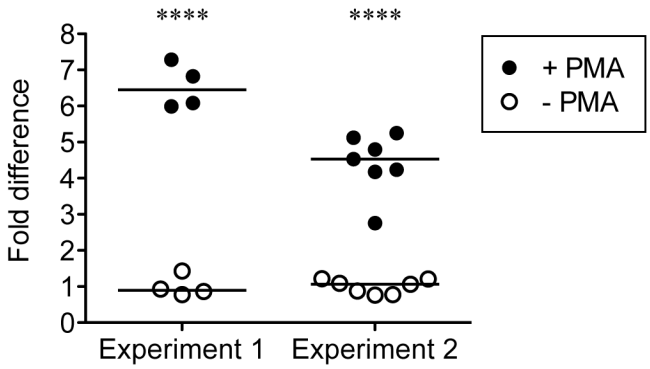

Figure 5.2 Hydrogen peroxide production by activated HD11 cells

Fold difference in hydrogen peroxide production in HD11 cells 12 hours after stimulation with PMA compared to that of unstimulated cells, as measured by luminescence produced by the reaction between hydrogen peroxide, luminol and horseradish peroxidase (HRP).

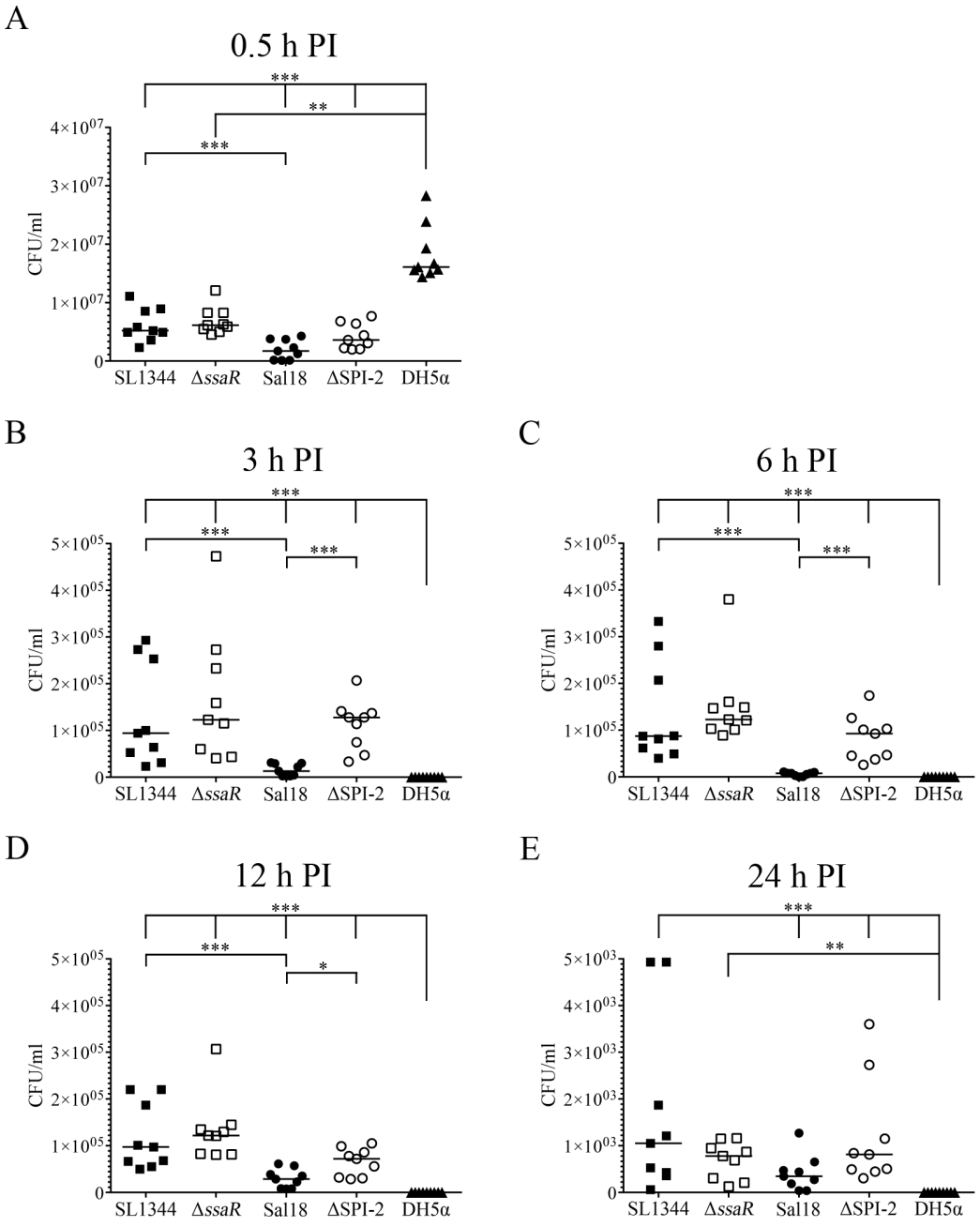

Figure 5.3 Recovery of *Salmonella* from the cell monolayer fraction of HD11 cells over time

The ability of SPI-2 mutants (ST Δ*ssaR* and SE ΔSPI-2) to survive in the chicken macrophage HD11 cell line was compared to their parent WT strains (ST SL1344 and SE Sal18) as well as to the non-pathogenic *E. coli* strain E. coli DH5α. **Panel A** shows the recovered CFU/ml from the cell monolayer fraction at 0.5 h post-infection (PI), prior to addition of gentamicin. **Panels B, C, D and E** show the recovered CFU/ml from the cell monolayer fraction after the addition of gentamicin at 3, 6, 12 and 24 h PI respectively. *, p-value < 0.05; **, p-value < 0.01; ***, p-value < 0.001. Note that the scale of the Y-axis is linear, and differs between time points.

5.3.3 Bacteria in the media fraction

At 3, 6, 12 and 24 h PI (after the addition of gentamicin), *Salmonella* could be recovered from the media taken off the cell monolayer. When the media fraction was separated into two tubes (one containing 1% Triton X-100) and grown for 3 hours, bacteria was only recoverable from the media fraction to which no Triton X-100 had been added. As 1% Triton X-100 is capable of disrupting eukaryotic, but not bacterial, cell membranes, this indicates that the bacteria are contained within eukaryotic cell membranes. Furthermore, PKH and Giemsa staining of the media fraction showed both whole cell and smaller membrane fragments in the media fraction (data not shown) and Immunofluorescence at 0.5, 3, 6, 12 and 24 h PI showed that most *Salmonella* were associated with whole HD11 cells that have detached from the monolayer (Figure 3.10 panels A, B and C), or fragmented cells (Figure 3.10 panels D and E). At 3 and 6 h PI, more of the WT *S.* Typhimurium strain SL1344 was recovered from the media fraction than the WT *S.* Enteritidis strain Sal18 (p-value < 0.05 and < 0.001 respectively) (Figure 5.4, panels A and B). At 6 h PI, both mutant strains (Δ*ssaR* and ΔSPI-2) were recovered in greater amounts than their respective parent strains (SL1344 and Sal18) (Figure 5.4, panel B). Finally, *E. coli* strain DH5α was not recoverable from the media fraction at any time point (Figure 5.4).

5.4 Discussion

Chicken macrophage-like HD11 cells can be induced to be more macrophage-like by stimulation with PMA, as evidenced by morphology and production of reactive oxygen species (Figures 2.1 and 2.2, respectively). At 0.5 h PI, prior to addition of gentamicin, more *E. coli* DH5α were recovered from the cell monolayer fraction than the *Salmonella* strains. It is unclear why this may be, as similar numbers of intracellular and extracellular bacteria were present in all samples when visualized by immunofluorescence microscopy (Figure 2.5). However, this microscopy does not differentiate viable from killed bacteria and it is possible that more live *E. coli* DH5α bacteria are initially phagocytosed by the macrophages, or that more *E. coli* DH5α remain associated with (but not phagocytosed) by the cells after the washing process. Following the addition of gentamicin, activated HD11 cells effectively killed *E. coli* DH5α, as viable *E. coli* DH5α were not recoverable from either the cell monolayer fraction or media fraction at any time point past 0.5 h. In comparison, all *Salmonella* strains were recoverable up to 24 h PI from HD11 cells. At most time points, the *S.* Typhimurium strain appeared to survive better within the

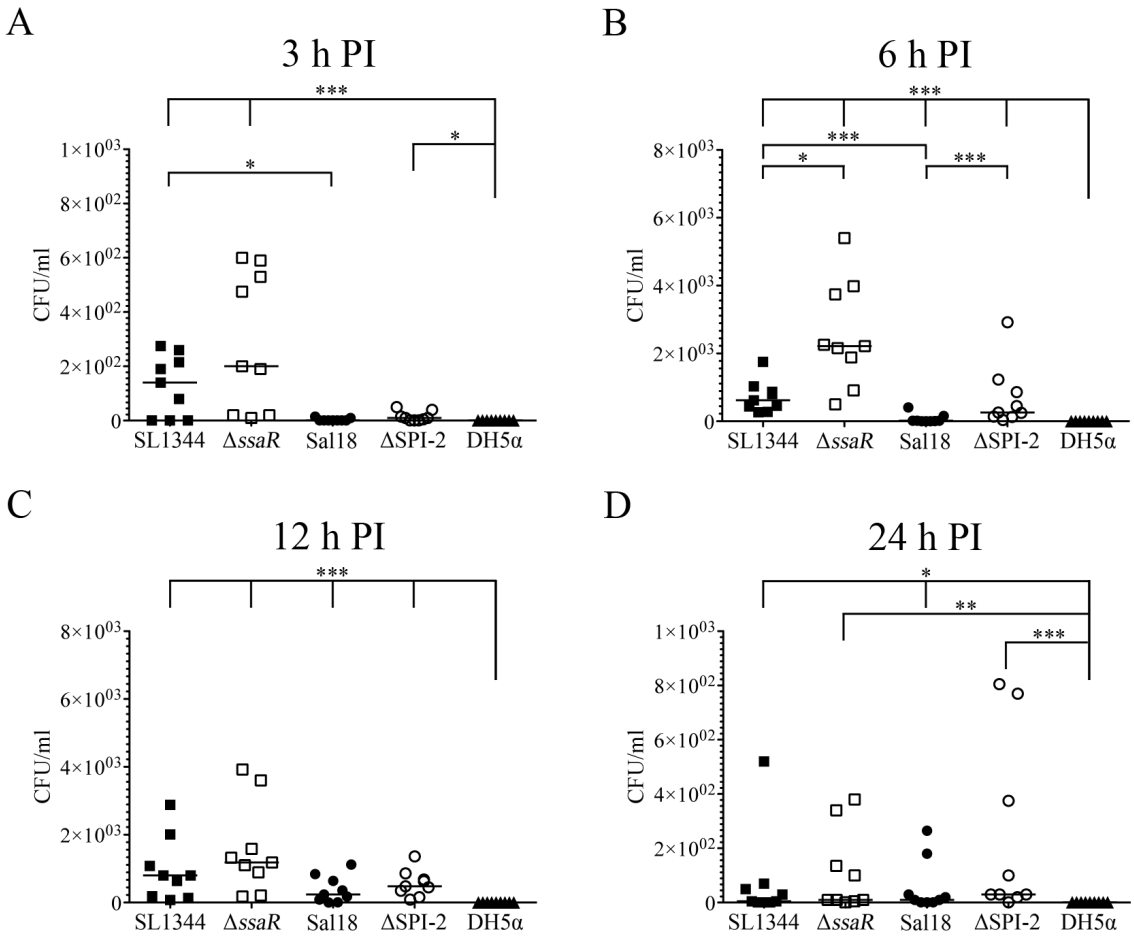

Figure 5.4 Recovery of *Salmonella* from the media fraction of HD11 cells over time

The ability of SPI-2 mutants (ST Δ*ssaR* and SE ΔSPI-2) to survive in the chicken macrophage HD11 cell line was compared to their parent WT strains (ST SL1344 and SE Sal18) as well as to the lab *E. coli* strain E. coli DH5α. Surprisingly, *Salmonella*, but not *E. coli*, was recovered from the media at all time points PI following the addition of gentamicin. **Panel A** shows the CFU/ml recovered from the media fraction at 3 h post-infection (PI) while **Panels B, C and D** show the CFU/ml recovered from the media fraction at 6, 12 and 24 h PI respectively. *, p-value < 0.05; **, p-value < 0.01; ***, p-value < 0.001. Note that the scale of the Y-axis is linear, and differs between time points.

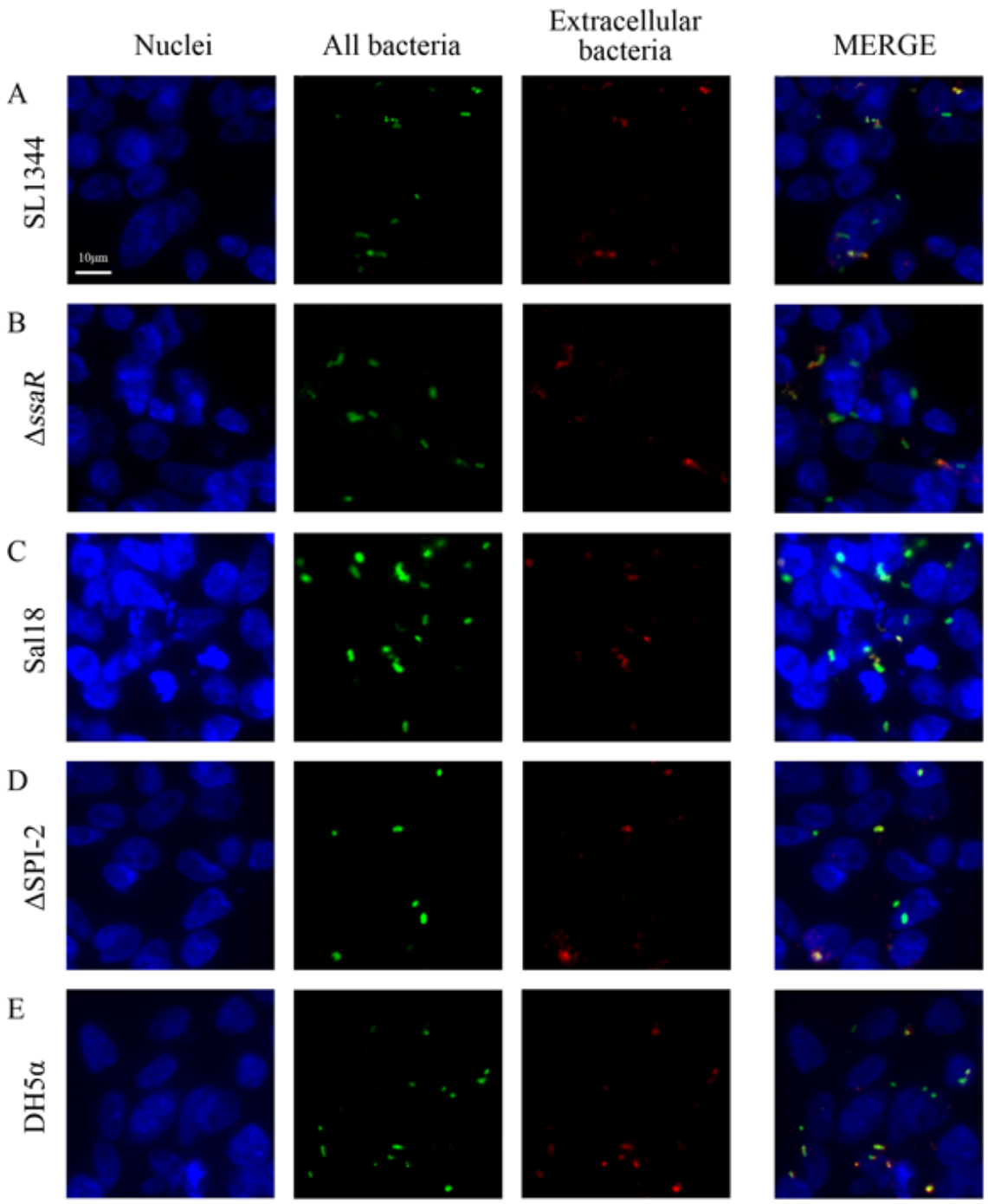

Figure 5.5 ***Salmonella*** **associated with the cell monolayer fraction at 0.5 h PI**

HD11 cell nuclei are stained blue, both intra- and extracellular bacteria are green, and extracellular bacteria appear red or yellow. **Panel A** shows HD11 cells infected with WT ST strain SL1344, **Panel B** shows HD11 cells infected with ST SPI-2 mutant Δ*ssaR*. **Panels C** and **D** show HD11 cells infected with WT SE strain Sal18 and SE SPI-2 mutant ΔSPI-2, respectively. **Panel E** shows HD11 cells infected with lab *E. coli* strain E. coli DH5α.

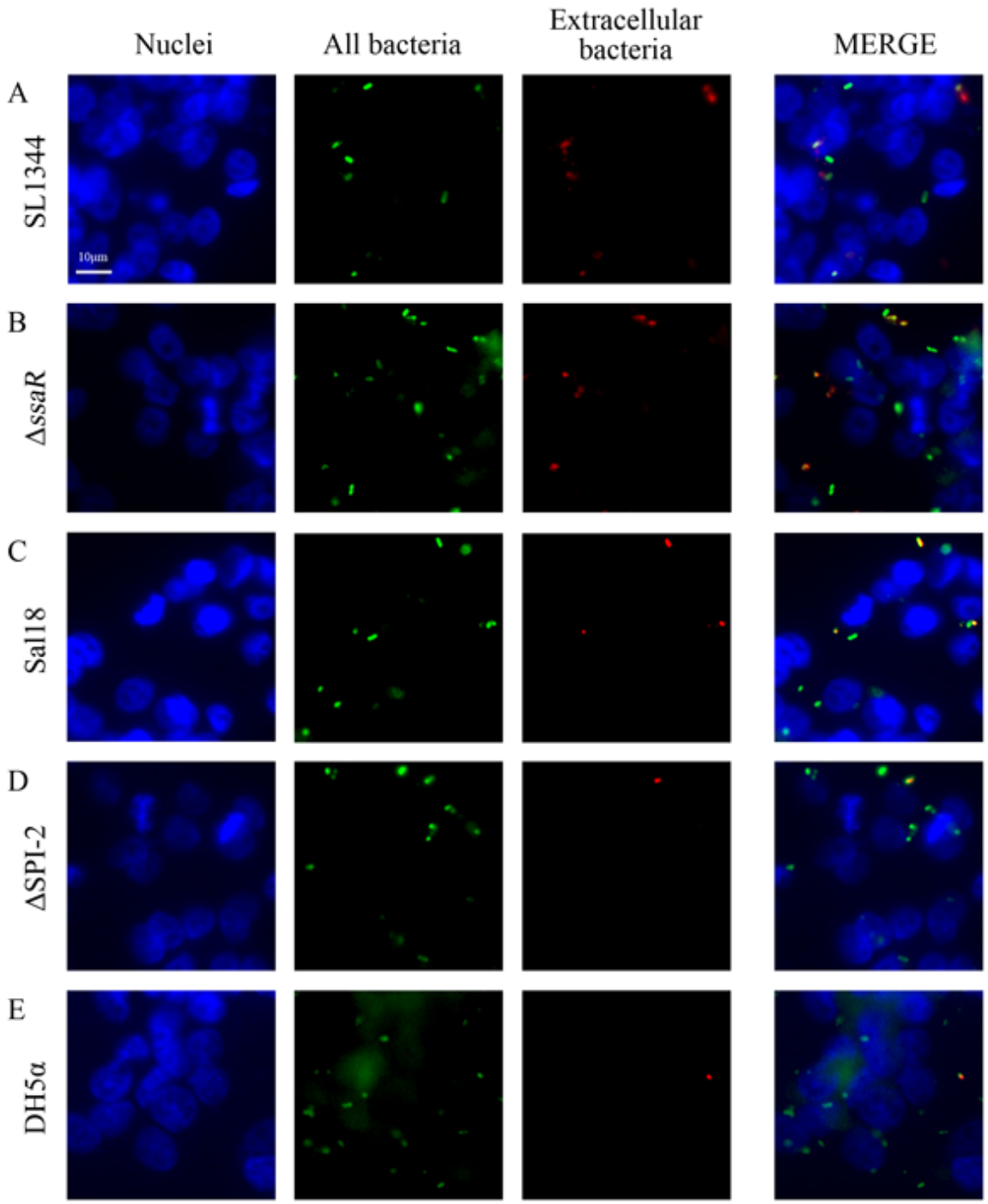

Figure 5.6 ***Salmonella*** **associated with the cell monolayer fraction at 3 h PI**

HD11 cell nuclei are stained blue, both intra- and extracellular bacteria are green, and extracellular bacteria appear red or yellow. **Panel A** shows HD11 cells infected with WT ST strain SL1344, **Panel B** shows HD11 cells infected with ST SPI-2 mutant Δ*ssaR*. **Panels C** and **D** show HD11 cells infected with WT SE strain Sal18 and SE SPI-2 mutant ΔSPI-2, respectively. **Panel E** shows HD11 cells infected with lab *E. coli* strain E. coli DH5α.

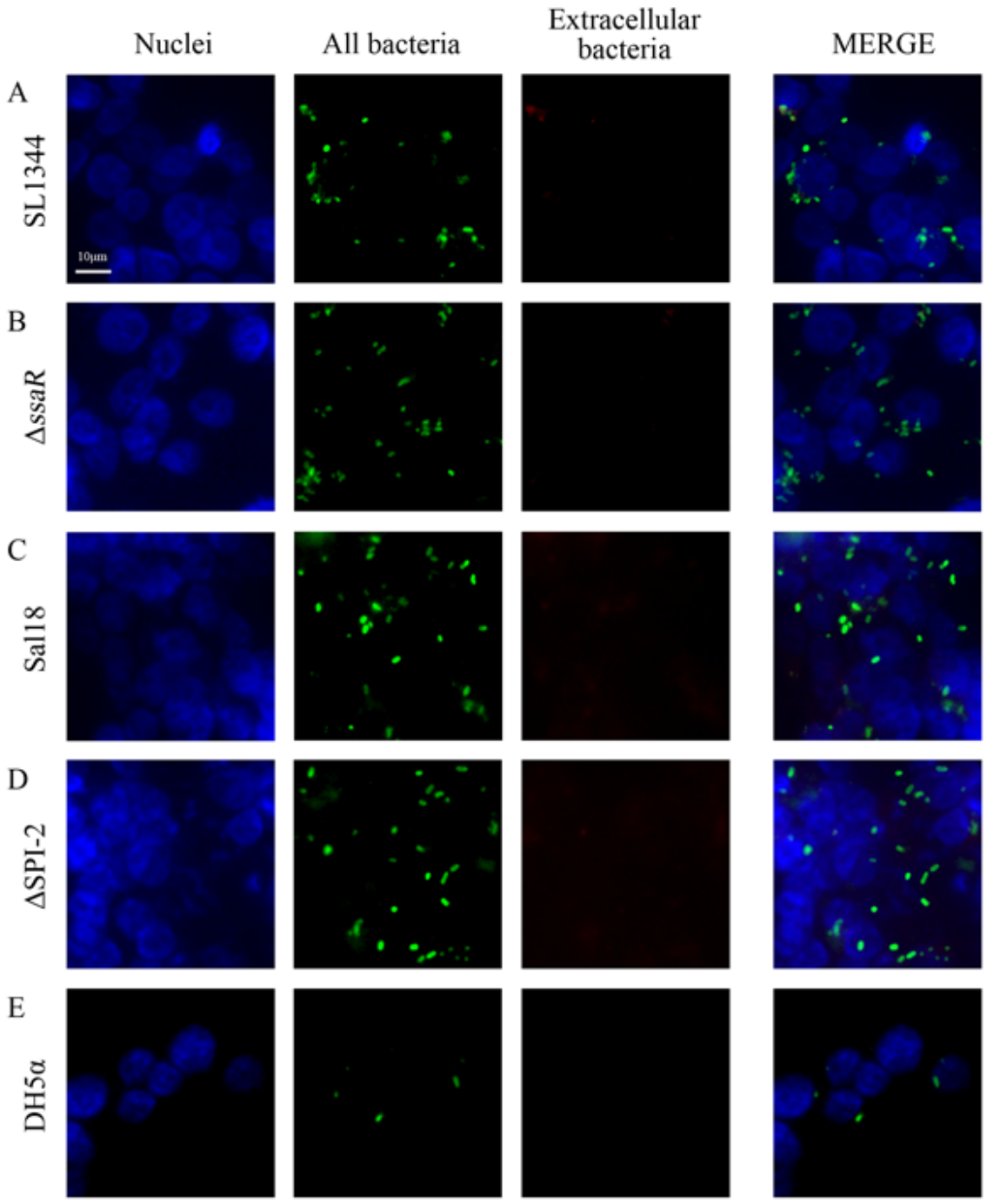

Figure 5.7 ***Salmonella*** **associated with the cell monolayer fraction at 6 h PI**

HD11 cell nuclei are stained blue, both intra- and extracellular bacteria are green, and extracellular bacteria appear red or yellow. **Panel A** shows HD11 cells infected with WT ST strain SL1344, **Panel B** shows HD11 cells infected with ST SPI-2 mutant Δ*ssaR*. **Panels C** and **D** show HD11 cells infected with WT SE strain Sal18 and SE SPI-2 mutant ΔSPI-2, respectively. **Panel E** shows HD11 cells infected with lab *E. coli* strain E. coli DH5α.

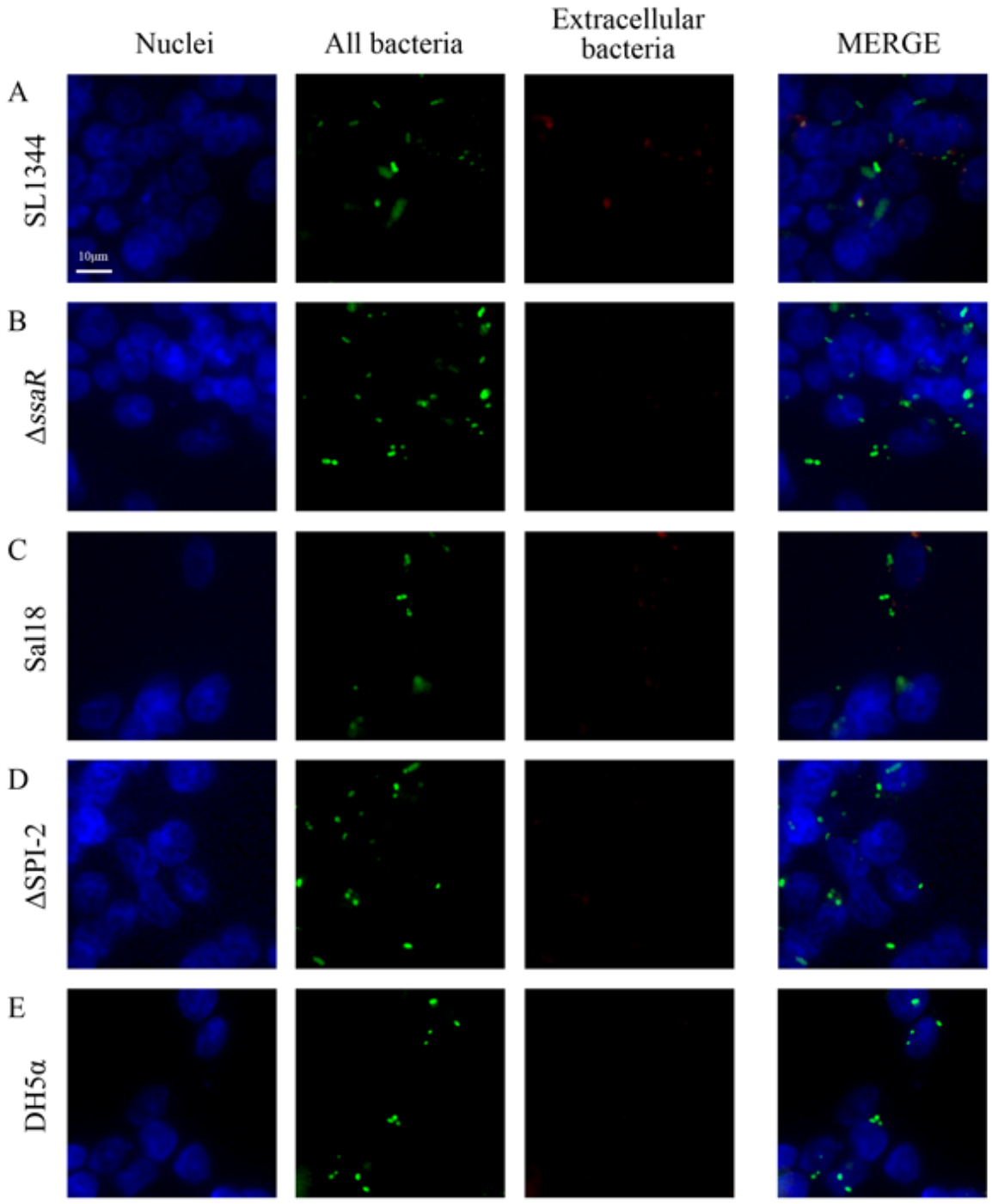

Figure 5.8 ***Salmonella*** **associated with the cell monolayer fraction at 12 h PI**

HD11 cell nuclei are stained blue, both intra- and extracellular bacteria are green, and extracellular bacteria appear red or yellow. **Panel A** shows HD11 cells infected with WT ST strain SL1344, **Panel B** shows HD11 cells infected with ST SPI-2 mutant Δ*ssaR*. **Panels C** and **D** show HD11 cells infected with WT SE strain Sal18 and SE SPI-2 mutant ΔSPI-2, respectively. **Panel E** shows HD11 cells infected with lab *E. coli* strain E. coli DH5α.

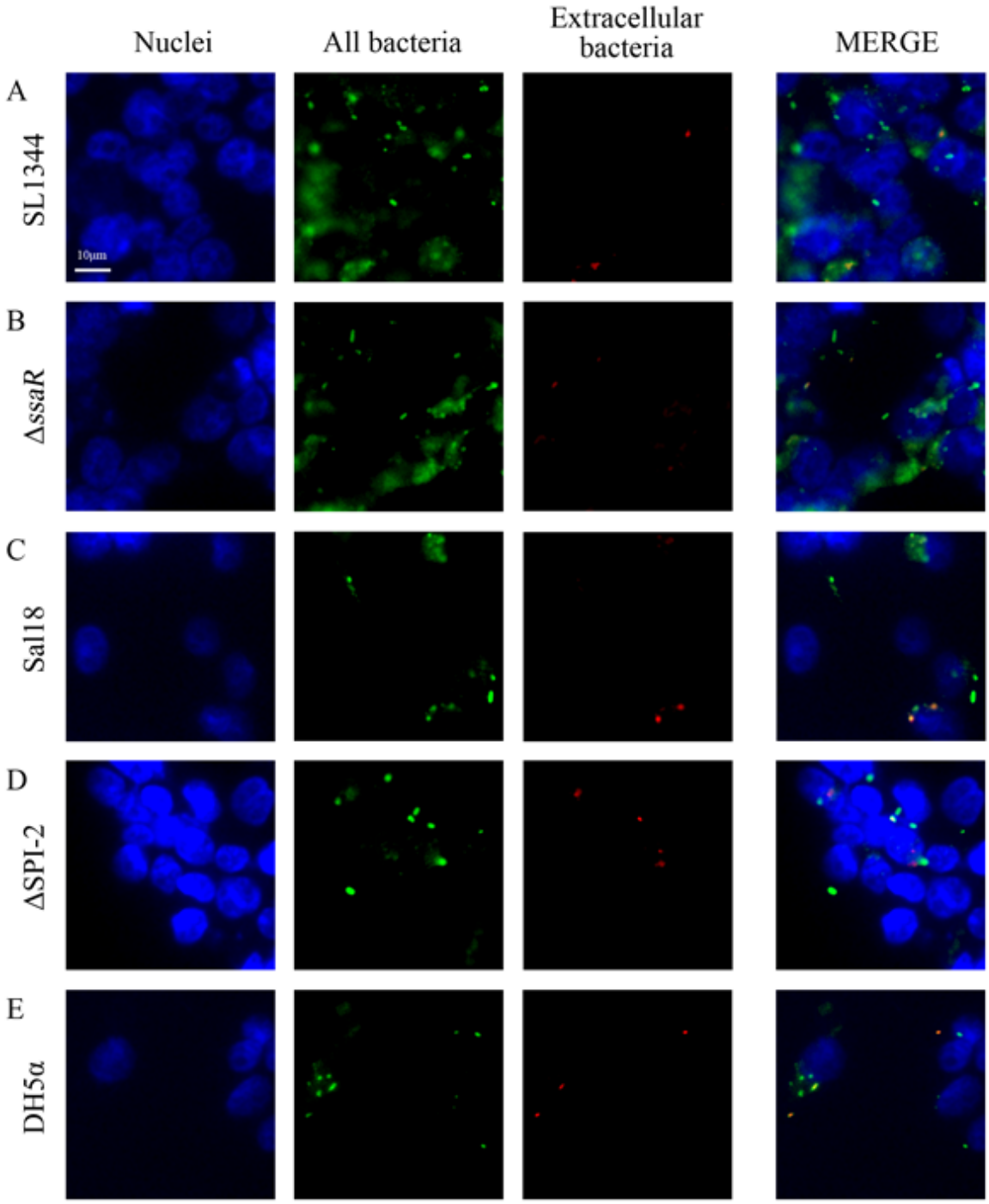

Figure 5.9 *Salmonella* associated with the cell monolayer fraction at 24 h PI

HD11 cell nuclei are stained blue, both intra- and extracellular bacteria are green, and extracellular bacteria appear red or yellow. **Panel A** shows HD11 cells infected with WT ST strain SL1344, **Panel B** shows HD11 cells infected with ST SPI-2 mutant Δ*ssaR*. **Panels C** and **D** show HD11 cells infected with WT SE strain Sal18 and SE SPI-2 mutant ΔSPI-2, respectively. **Panel E** shows HD11 cells infected with lab *E. coli* strain E. coli DH5α.

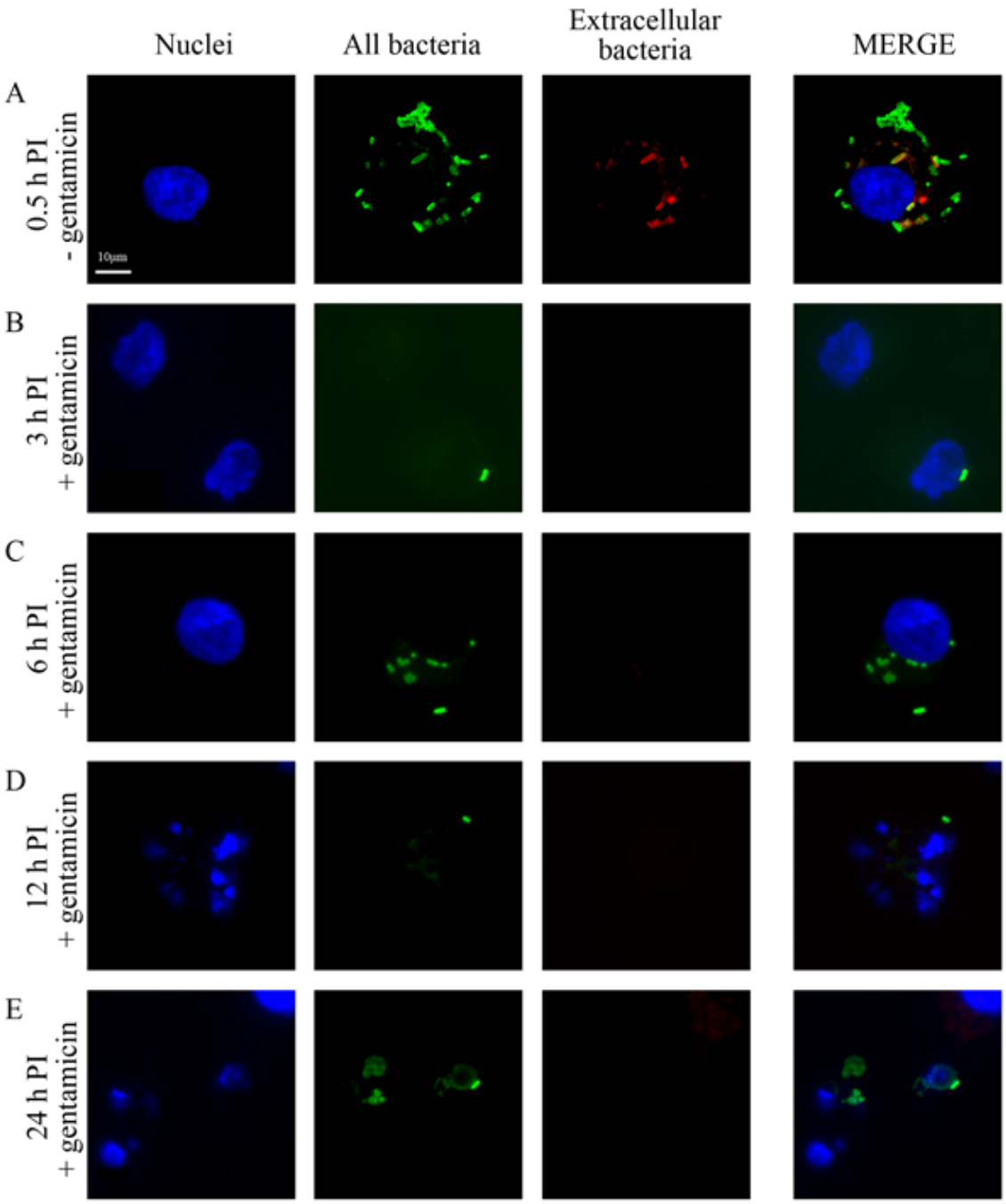

Figure 5.10 WT ST strain SL1344 in the media fraction over time

HD11 cell nuclei are stained blue, both intra- and extracellular bacteria are green, and extracellular bacteria appear red or yellow. **Panel A** shows an HD11 cell loaded with WT ST strain SL1344 at 0.5 h PI, just prior to addition of gentamicin. **Panels B** and **C** show whole HD11 cells containing SL1344 at 3 and 6 h PI, respectively, after addition of gentamicin. **Panels D** and **E** show fragmented HD11 cells containing SL1344 at 12 and 24 h PI, respectively, after the addition of gentamicin. Whole and fragmented cells containing SL1344 were visible at all time points in the media fraction.

HD11 cells than the *S.* Enteritidis strain. Importantly, at no time did the WT *S.* Typhimurium or *S.* Enteritidis strains survive better than their respective SPI-2 mutant strains, and, at 3, 6, and 12 h PI, the *S.* Enteritidis SPI-2 mutant (ΔSPI-2) out-performed the WT strain. All strains, including non-recoverable *E. coli* DH5α, were visible within macrophages at all time points PI by immunofluorescence, but it is likely that the immunofluorescence was detecting killed, as well as viable, bacteria (Figures 2.5 – 2.9). There were usually only one or two bacteria were seen within an individual cell, although a few instances of large bacterial load were also observed. After the addition of gentamicin, fewer macrophages containing *E. coli* were visible, when compared to those infected with *Salmonella* strains.

In a mouse model of infection, multiple groups have shown that various SPI-2 mutants are highly attenuated in virulence (measured by LD_{50}) [59, 61, 149, 338]. Initially, Hensel *et al.* showed that *S.* Typhimurium SPI-2 mutants replicated at similar levels to WT *S.* Typhimurium in the mouse macrophage-like RAW264.7 cell line [1]. However, later work by the same group (and others) indicated that SPI-2 mutants failed to replicate as well in mouse macrophages than WT strains if they were first grown to stationary phase and then opsonized before infection to enhance uptake of bacteria by macrophages [59, 61, 338]. In our experiments, bacteria were grown to mid-log phase before infection, and were not opsonized, because these conditions do not mimic the initial stages of infection. Many groups have recently published results in accordance with our findings. Forest *et al*. [339] determined that the absence of a functional SPI-2 T3SS in serovar Typhi (*S.* Typhi) did not affect survival in human macrophages. Aussel *et al.* [340] demonstrated that *S.* Typhimurium containing a non-functional SPI-2 or SPI-1 T3SS was able to survive similarly to WT *S.* Typhimurium in both mouse bone marrow-derived macrophages and RAW264.7 macrophages, but that the same mutants did not replicate to similar levels of the WT strain *in vivo*. A study by Helaine *et al.* [341] determined that while SPI-2 was important for replication of *S.* Typhimurium in macrophages, it does not affect the survival of phagocytosed bacteria within the SCV. Furthermore, it was shown in this study that most WT *S.* Typhimurium that are taken up by macrophages do not undergo replication at all, but rather enter a dormant state within the SCV. The number of bacteria in stasis did not differ between WT macrophages, *phox*$^{-/-}$ macrophages, or macrophages stimulated with IFNγ. Thus, *Salmonella* may be able to survive within macrophages without replication and disseminate to systemic sites, regardless of the presence of SPI-2. In fact, it has been shown that SPI-2 mutants are able to

reach the livers and spleens of mice, that similar numbers of spleen cells are infected, but mice infected with SPI-2 mutants have a reduced bacterial load in these organs [149, 341]. Previous work by our group showed that although *S.* Enteritidis SPI-1 and SPI-2 mutants were recovered in the livers and spleens of infected chickens in less abundance than the WT *S.* Enteritidis strain initially, the mutant strains reached comparable levels to the WT strain by day 4 PC [282, 312].

It is well known that the production of reactive oxygen species (ROS) by phagocytes is important for control of intracellular pathogens. Humans, or animals, with mutations in NADPH oxidase (Phox) are prone to severe recurrent infections by fungi and intracellular bacteria, including *Salmonella* [334, 342]. Phox assembles on phagosomes that contain intracellular pathogens, and is responsible for the production of superoxide (O_2^-). Superoxide is not readily able to cross the membranes of bacteria, but can spontaneously dismutate, or be dismutated by superoxide dismutases, into hydrogen peroxide (H_2O_2). Hydrogen peroxide can easily diffuse across bacterial membranes and can form highly reactive hydroxyl (HO) radicals in the presence of iron (Fe^{2+}) that damage bacterial DNA, proteins and lipids [334, 340]. *Salmonella* has developed multiple defenses to this process. *Salmonella* has two periplasmic superoxide dismutases (SodCI and SodCII) that combat exogenous superoxide. It also expresses three known cytosolic catalases (KatG, KatE, and KatN) and three cytosolic peroxidases (SodA, SodB, and Tpx) that degrade hydrogen peroxide within the bacterial cytoplasm. SodCI and SodCII, along with the cytoplasmic peroxidase Tpx, have been shown to be important for survival of *S.* Typhimurium in mouse macrophages [340, 342, 343]. It has also been previously shown that SPI-2 is important for vesicular trafficking and the association of Phox with the SCV in human and mouse macrophages; this observation led researchers to propose that the SPI-2 T3SS was essential in avoiding the oxidative burst [342, 344, 345]. However, recent work by Aussel *et al.* [340] indicates that SodCI, SodCII and Tpx are sufficient for *Salmonella* to overcome ROS, and that *S.* Typhimurium SPI-2 mutants perform similarly to WT *S.* Typhimurium *in vitro*. They found that, *in vivo*, the WT *S.* Typhimurium had increased replication in relation to the SPI-2 mutant in both WT and *phox*$^{-/-}$ mice (although both strains reached higher levels in the *phox*$^{-/-}$ mice), indicating that while SPI-2 is important for replication, it does not play a major role in evasion of ROS. While we showed that HD11 macrophages activated with PMA produced greater levels of hydrogen peroxide than non-activated macrophages (Figure 2.2), we did not see a major difference in survival or replication of SPI-2 mutants compared to WT in these activated

cells. This indicates that any avoidance of ROS in this case was independent of a functional SPI-2 T3SS. Slauch *et al.* [346] state that ROS in the SCV of infected cells may not be diminished by WT *Salmonella* as previously reported, so the role of SPI-2 in avoidance of ROS, as well as the importance of ROS in control of *Salmonella* infection, remains unclear.

SPI-2 has been shown to change cytokine and chemokine production by macrophages, including the HD11 cell line [333, 347-349]. In HD11 cells, PipB has been shown to be important in the down regulation of pro-inflammatory cytokines, indicating a role for SPI-2 in repression of the host's innate immune response [348]. SPI-2 has also been shown to be important in limiting the antigen presenting abilities of macrophages and dendritic cells to T cells [350, 351]. It may be that SPI-2 is less important for survival of *Salmonella* within macrophages, but more important in modulation of macrophage stimulation of other immune cells via cytokine/chemokine production and antigen presentation. This would better explain some of the differences seen between *in vitro* SPI-2 *Salmonella* mutant survival and *in vivo* SPI-2 *Salmonella* mutant vulnerability.

Surprisingly, in our study, *Salmonella* was recoverable in the media fraction at each time point after the addition of gentamicin. These bacteria were found to be associated with both whole detached cells and closed cell fragments. If the bacteria within the cell fragments are viable, this would be a novel way for *Salmonella* to avoid the host immune system between host cell death and uptake by other phagocytic cells.

Taken together, these results indicate that survival in activated chicken HD11 macrophage-like cells is likely SPI-2 independent. It would be interesting to see if similar results were found *in vivo*, looking to see if WT and SPI-2 mutant *Salmonella* strains could be recovered from macrophages of orally infected chickens. In addition, it would also be useful to include profiling of cytokines and chemokines produced by infected macrophages in order to determine the importance of ROS and RNS in bacterial clearance from chickens, as the vast majority of work has been carried out in mice. Further characterization of bacteria in the media fraction, in particular, whether those within macrophage fragments remain viable to infect naive macrophages, would be illuminating.

6.0 GENERAL DISCUSSION AND CONCLUSIONS

6.1 General discussion

Infections by *Salmonella enterica* subspecies *enterica* serovars Typhimurium (*S.* Typhimurium) and Enteritidis (*S.* Enteritidis) are one of the most common causes of bacterial food borne gastroenteritis (food poisoning) in humans worldwide [21]. *S.* Enteritidis and *S.* Typhimurium are passed to humans via consumption of contaminated poultry meat, contaminated water, and eggs [22]. Because chickens generally do not show symptoms of disease, *Salmonella* can spread throughout a poultry flock quite quickly and chickens will shed bacteria in their feces for extended periods of time [23-26]. Loss of consumer confidence in products because of *Salmonella* contamination can result in substantial economic loss to the poultry industry. Additionally, human cases of salmonellosis place a significant burden on the health care system [18]. In humans, *S.* Typhimurium and *S.* Enteritidis generally produce self-limiting gastroenteritis, but in rare occasions can cause typhoid-like systemic disease. In susceptible mice, *S.* Typhimurium and *S.* Enteritidis cause a lethal typhoid like disease, while resistant mice can develop chronic systemic infection. In gnotobiotic or streptomycin pre-treated mice, *S.* Typhimurium and *S.* Enteritidis cause symptoms resembling gastroenteritis. In most healthy adult chickens, infection with *S.* Typhimurium and *S.* Enteritidis result in an asymptomatic carrier state [13, 181, 204].

Salmonella uses two specialized type III secretion systems (T3SS) that facilitate invasion and survival within the host cell and are encoded within *Salmonella* pathogenicity islands 1 and 2 (SPI-1 and SPI-2). The SPI-1 and SPI-2 T3SS secrete effectors into the host cell, triggering a number of events in the infected cell. It is the current view that the SPI-1 T3SS is mainly involved in invasion of the host cell, while the SPI-2 T3SS plays a role in survival within the host cell and maintenance of the *Salmonella* containing vesicle (SCV) [59, 284, 285]. The SPI-2 T3SS has been reported to be necessary for systemic infection, intracellular proliferation and survival, and maintenance of the SCV. However, the majority of these studies have been performed in mice, where *S.* Typhimurium and *S.* Enteritidis produce a typhoid-like infection rather than gastroenteritis, and therefore may not be indicative of the course of infection in healthy adult humans and the asymptomatic carrier state in chickens [55, 286-288].

In order to determine the importance of the SPI-2 T3SS in cecal colonization and

systemic spread in chickens, the ability of *S.* Enteritidis SPI-2 mutants to colonize chickens was examined by us. When 35-day-old SPF chickens were challenged with both a WT strain and a strain impaired in both the SPI-1 and SPI-2 T3SSs, the WT strain began to out-compete the mutant strain in the liver and spleen by day 2 PC, but there was no detectable difference in the level of WT versus mutant strain in the cecal contents. Dieye *et al*. [315] used a similar experimental design in a recent study comparing colonization levels of a WT *S.* Typhimurium strain and either a ΔSPI-1 strain missing the entire SPI-1 region, a ΔSPI-2 strain missing a portion of the SPI-2 region encoding structural genes, or a combination ΔSPI-1ΔSPI-2 strain. Similar to our findings, this group recovered a greater amount of the WT *S.* Typhimurium strain from the spleen than the ΔSPI-2 and ΔSPI-1ΔSPI-2 strains. They also observed that colonization levels of the WT versus the mutant strains in the cecum were not different, again supporting our results. When one-week-old SPF chickens were challenged with a WT *S.* Enteritidis strain, a strain missing SPI-2, or a strain missing both SPI-1 and SPI-2, we observed that the mutant strains were impaired in their ability to infect the liver and spleen, but were recovered from the cecum at similar levels as the WT. On day 1 PC, both the WT and mutant strains had begun to spread to the liver and spleen, but while the WT strain reached peak colonization of the liver and spleen by day 2 PC, and was beginning to clear by day 3 PC, the mutant strains did not reach their peak until day 3 PC. By day 4 PC, both the WT and mutant strains were beginning to clear from the birds. Bohez *et al.* [319] observed that maximum presence of *Salmonella* in liver and spleen of SPF birds challenged with 10^8 CFU *S.* Enteritidis on the day of hatching occurred on day 2, supporting our observations. Others have also observed that SPI-2 mutant strains, while able to reach the liver and spleen of chickens, have a later peak colonization than the WT strain, and tended to be cleared faster from systemic sites [304, 314].

Live bacterial vaccines often produce long lasting immunity at both systemic and mucosal sites, however fear of reversion to virulence and release of genetically engineered organisms into the environment are a major concern. Inactivated bacterial vaccines (also known as bacterins) and subunit protein-based vaccines are often capable of inducing a strong humoral immune response, but tend not to induce production of mucosal IgA or a potent cell-mediated immune response. They are attractive over live vaccines as there is no chance of reactivated virulence or of live genetically modified bacteria entering the environment. Ideally, a vaccine for use in poultry to combat *Salmonella* should induce both strong humoral and cell-mediated immune

responses that confer protection against more than one serovar [15, 206, 220]. In our series of vaccine trials we found that vaccination of chickens with SPI-2 structural or effector components induced high levels of vaccine specific IgY that were long lasting and transferrable to progeny. Groups of chickens vaccinated with SPI-2 proteins, and progeny of vaccinates, exhibited lower overall numbers of *Salmonella* than unvaccinated controls.

In a mouse model of infection, multiple groups have shown that various SPI-2 mutants are highly attenuated in virulence, both *in vivo* and *in vitro*. Most of the *in vitro* work involved first growing *Salmonella* to stationary phase and then opsonizing it to facilitate uptake by macrophages [59, 61, 149, 338]. In our experiments, bacteria were grown to mid-log phase before infection, and were not opsonized, because these conditions do not mimic the initial stages of infection. Activated chicken macrophage-like HD11 cells were able to control infection with a non-pathogenic *E. coli* strain, eliminating recoverable bacteria by 3 h PI. However, both *S.* Typhimurium and *S.* Enteritidis SPI-2 mutants performed similarly to their parent strains, with significant numbers of *Salmonella* recovered up to 24 h PI. Similar results were observed by Hensel *et al.* [1] and Aussel *et al.* [340] using *S.* Typhimurium and *S.* Typhimurium SPI-2 mutants in mouse macrophage-like RAW264.7 cells primary mouse macrophage cells. Helaine *et al.* [341] observed that while SPI-2 of *S.* Typhimurium was important for replication of the bacterium within the SCV, it was not necessary for survival in mouse macrophages. SPI-2 has also been shown to modulate pro- and anti-inflammatory cytokine production in mammalian cells, as well as limit antigen presentation by dendritic cells and T cells [333, 347-349, 351].

Taking the observations of others into consideration, along with our observations, both *in vivo* and *in vitro*, it may be that *Salmonella* can survive within macrophages without replication and disseminate to systemic sites regardless of the presence of SPI-2. It may be that SPI-2 is less important for survival of *Salmonella* within macrophages, but more important in modulation of macrophage stimulation of other immune cells via cytokine/chemokine production and antigen presentation. This would better explain some of the differences seen between *in vitro* SPI-2 *Salmonella* mutant survival and *in vivo* SPI-2 *Salmonella* mutant vulnerability, as well as the differences in infection outcome in different hosts. Our work has increased knowledge about the role of the SPI-2 T3SS of *S.* Enteritidis in chickens and chicken macrophages. However, further research is needed to determine the exact mechanisms of systemic spread by *S.* Enteritidis and its role in immune modulation in chickens.

6.2 Future directions

Further research into the role of the SPI-2 T3SS in systemic spread in chickens is needed. Specific experiments involving challenge of chickens with labeled wild-type and *Salmonella* SPI-2 mutants would allow real-time observation of how the strains disseminate throughout the chicken. This would also allow for the surveillance of the specific cell types that the two strains are residing in within the chickens. Further, after isolation of infected phagocytic cells from challenged chickens, specific cytokine and chemokine profiles of these cells could be undertaken to see how SPI-2 effects immune cell function. Rather than using the available immortalized cell lines, isolation of a primary chicken macrophage cell line would be important for time course studies. Observations on how the cytokine and chemokine profiles of these cells change over time, when infected with wild-type *Salmonella* compared to the SPI-2 mutant, as well as whether the lack of SPI-2 affects replication of the bacteria within these cells would be helpful. Characterization of how *Salmonella* induces cell death in primary chicken macrophages, and the role SPI-2 plays, would be very useful and further study of the media fraction in the gentamicin protection assay would be interesting to see if *Salmonella* is truly contained in small membrane vesicles that are able to be taken up by naïve macrophages, and continue the infection process.

It would be interesting to see if there is any cross-reactivity in antibodies produced by hens vaccinated with either of the SPI-2 vaccines compared to the AviPro® vaccine. As the SPI-2 vaccines showed some promise, it would be worthwhile to try various vaccine formulations using some of the proteins, especially SseB. An oral vaccine would be the most desirable for use in poultry, and use in a situation more reminiscent to what they would see in the field, using the seeder-contact model, lower challenge doses, and a longer surveillance period, would be extremely useful.

6.3 General conclusions

- In a co-challenge situation the WT strain is more competitive than the SPI-1/SPI-2 mutant strains
- The SPI-2 T3SS is not important for cecal colonization in chickens
- The SPI-2 T3SS is important for systemic spread in chickens, but is obviously not the only factor involved

- SPI-2 T3SS structural and effector proteins are capable of eliciting a strong humoral immune response in chickens, as measured by protein specific serum IgY levels
- Antibodies generated through vaccination with either SPI-2 T3SS structural or effector proteins are transferrable from hens to progeny, as measured by egg yolk IgY
- SPI-2 T3SS proteins may be useful in a combination vaccine to protect chickens from colonization with *Salmonella*
- The SPI-2 T3SS is not important for survival of *S.* Typhimurium or *S.* Enteritidis in the activated chicken macrophage-like cell line HD11

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