Salmonella attenuation of cellular and systemic virulence

by

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Abstract

Salmonella enterica serovar Typhimurium causes a gastroenteritis in humans that is characterized by intestinal inflammation and diarrhea. In mice, serovar Typhimurium causes a systemic typhoid-like disease, providing a useful model for studying the systemic disease caused in humans by serovar Typhi. Host macrophages are absolutely essential for systemic infection, as *Salmonella* utilizes them to disseminate from the intestine to deeper tissues within the body. The ability to reside in macrophages also allows *Salmonella* to evade the host humoral immune response and provides a site for replication in the spleen and liver. *Salmonella* can also kill its own host macrophage, which may further aid bacterial dissemination. However, despite the fact that *Salmonella* is able to induce host cell death, bacteria can persist within macrophages for extended periods of time. To identify genes involved in these late stages of infection, a library of 50,000 serovar Typhimurium transposon mutants was selected for the ability to reside in host macrophages for 24 hours. This screen identified a previously uncharacterized gene, *sciS* that is homologous to a known virulence gene (*icmF*) in *Legionella pneumophila*. *SciS* is located in a 47-kb horizontally acquired locus in the serovar Typhimurium chromosome that contains several homologs to virulence genes in other gram-negative bacteria. This locus has been previously referred to as *Salmonella* Pathogenicity Island 6 (SPI6) in serovar Typhi.

A *sciS* deletion led to a significant increase in the number of intracellular bacteria at 24 hours post-infection, however no difference was observed at 6 hours post-infection. *SciS* transcription *in vitro* was relatively low. However, within infected macrophages, expression was substantially higher and peaked at 27 hours post-infection. The fact that *sciS* is maximally expressed and exhibits a phenotype after 24 hours post-infection correlates well with the timing of our selection process. *SciS* was found to be negatively regulated by SsrB, which is required for the survival and replication of *Salmonella* within macrophages. Furthermore, a *sciS* deletion exhibited a hypervirulent effect in a mouse model of infection. Based on our findings for *sciS*, we proceeded to assess the contribution of other genes in SPI6 to mouse virulence. Each SPI6 gene deletion that was tested demonstrated a hypervirulent phenotype. Together, these findings indicate a role for *sciS* in controlling intracellular bacterial numbers at late stages of infection and suggest that SPI6 is involved in attenuating systemic virulence.

Chapter 1: Introduction

I. *Salmonella* taxonomy and epidemiology

A. Taxonomy

Salmonella is a gram-negative rod shaped bacterium that causes a wide spectrum of disease in a variety of hosts. The taxonomy of the *Salmonella* genus has been repeatedly modified as new typing methods have become available. Previously, most strains of *Salmonella* were known by individual species names, i.e. *S. typhimurium* and *S. typhi*. However, despite vast differences in host range and disease symptoms caused, most of these strains are 96-99% identical in the DNA sequences of their housekeeping genes and 16S rRNA. As advancing technologies have provided more complete bacterial sequences, the nearly identical genomes can no longer justify separate species names for all the members of the *Salmonella* genus. Consequently, *Salmonella* was divided into two species, *S. bongori*, which contains subspecies V, and *S. enterica*, which has seven different subspecies, I, II, IIIa, IIIb, IV, VI and VII (70). Within each subspecies are serovars that are distinguished from one another by the Kauffman-White serotyping scheme based on antigenic polymorphisms of lipopolysaccharide (LPS) (O) and flagella (H)(101). Over 2,500 distinct *Salmonella* serovars have been identified to date. *Salmonella enterica*

subspecies I, which includes serovars Typhi and Typhimurium, is responsible for 99% of salmonellosis in warm-blooded animals. *S. bongori* and the remaining six subspecies of *S. enterica* are mainly restricted to cold-blooded hosts.

Despite their high degree of sequence similarity, the serovars of *S. enterica* subspecies I cause a wide array of disease, including but not limited to, gastroenteritis, enteric fever and bacteremia. The host range of these serovars varies from being highly host-adapted to having a broad host range. For example, serovars Typhi and Pullorum are limited to humans and chickens, respectively, while serovars Choleraesuis, Abortus-ovis and Dublin are associated with but not limited to, swine, sheep and cattle, respectively. The most studied serovar in *S. enterica* is Typhimurium, which also exhibits the broadest host range, infecting humans, cattle, horses, sheep, pigs, birds and rodents.

B. Epidemiology of non-typhoidal *Salmonella*

Infections with non-typhoidal salmonella are a common cause of food-borne illness worldwide. In humans, *Salmonella* species are believed to cause over one billion infections annually, with consequences ranging from acute gastroenteritis (food poisoning) to systemic, often fatal, typhoid fever. However, the number of cases each year is difficult to estimate, since in many instances the disease caused by these organisms is relatively mild and self-limiting. Yet, in susceptible,

individuals such as the very young, the elderly and those with suppressed immunity, the more common gastrointestinal disease can progress to a life-threatening septicemia. One study estimated that 600 deaths occur per year in the United States alone due to infections with nontyphoidal *Salmonella* serotypes (84). Further complicating treatment is the increased incidence of multi-drug resistant serovar Typhimurium in Europe and North America over the last decade (52). Also important to the spread of salmonellosis is the ubiquitous nature of the organism. It can infect a wide variety of animal species used for food, and can also thrive on various plant products, making human infections common.

II. Pathogenesis of Infection

Salmonella is a model organism for the study of host-pathogen interactions because of its broad host range and the variety of diseases it causes. *S. enterica* serovar Typhimurium is often the organism of choice for these studies because it causes only gastroenteritis in humans, making it relatively safe to work with in the laboratory. However, in a mouse model, serovar Typhimurium causes a systemic typhoid-like disease, providing a useful model to study typhoid fever. Furthermore, *Salmonella* is amenable to genetic manipulation and several genetic tools are available for its study. The following is a broad description of steps involved in a systemic infection and a description of the pathogenicity islands crucial for the disease process.

A. Course of Infection

The course of infection for *Salmonella* is classically divided into two phases, referred to as the intestinal phase and the systemic phase. *Salmonella* is ingested orally via contaminated food or water, passes through the stomach into the intestine where it can breach the intestinal epithelium to reach underlying tissues. This leads to an intense inflammation and an eventual destruction of the integrity of the mucosa. After reaching underlying tissues, *Salmonella* then spreads via the lymphatic system and bloodstream to the mesenteric lymph nodes, spleen and liver. In susceptible humans and animals, *Salmonella* replicates to high numbers within these tissues. The inability to control this replication leads to bacteremia and is ultimately lethal. (See Fig. 1-1 below).

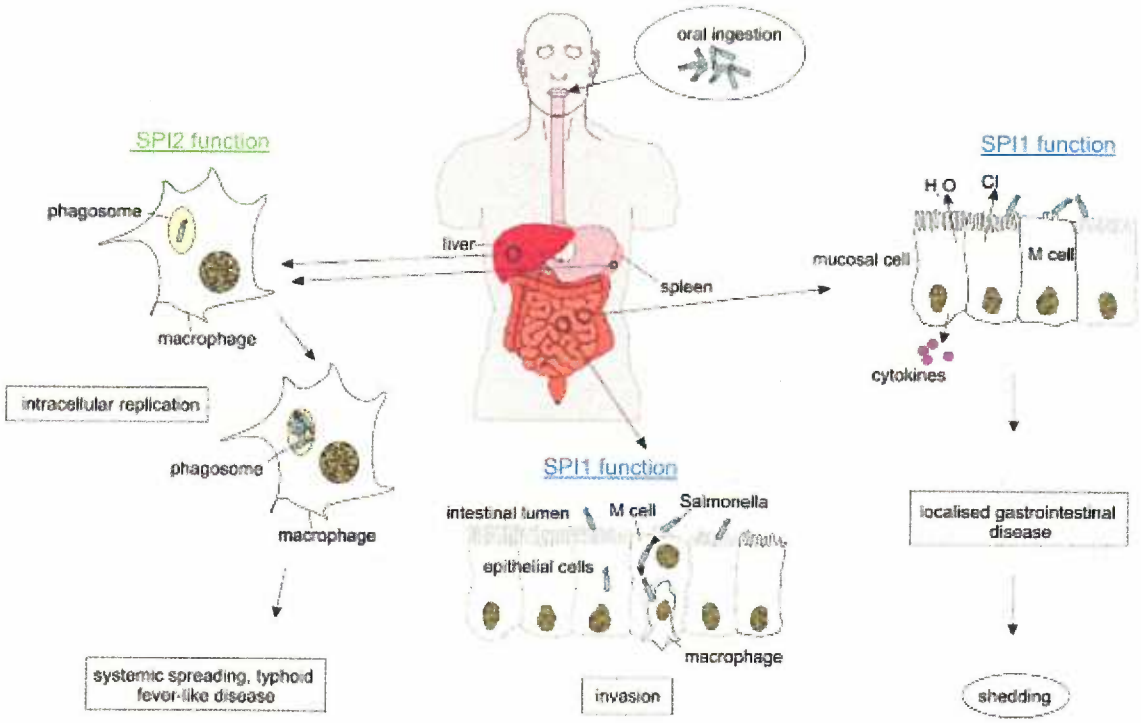

Figure 1-1. Schematic representation of host-pathogen interactions during *Salmonella* infections. Bacteria are ingested orally and then pass through the stomach to the intestine where they can breach the intestinal epithelium. Those bacteria that breach the intestinal epithelium are ingested by underlying macrophages and disseminate to the spleen and liver. [Reproduced from (46)]

B. Pathogenicity Islands

The ability of *Salmonella* to reside in many distinct tissues in several different hosts requires multiple virulence factors. It has been estimated that 4% of the serovar Typhimurium genome is required for a lethal infection of mice, which corresponds to roughly 200 virulence genes (7). The need for so many virulence genes illustrates the complex nature of *Salmonella* infection. *Salmonella* utilizes these virulence factors in a highly intricate and regulated

manner to cause disease in its hosts. Virulence genes in *Salmonella* are found on plasmids and the chromosome either as individual genes or clusters of genes. The largest clusters of genes that confer virulence are known as pathogenicity islands. These usually have a different GC content than the rest of the chromosome (53% for serovar Typhimurium), are inserted at tRNA loci, typically confer a specific virulent phenotype and are expressed at a specific time during the course of infection. Pathogenicity islands are thought to be horizontally acquired from other pathogenic bacteria or phages and are not present in closely related non-pathogenic bacteria.

A total of five *Salmonella* Pathogenicity Islands (SPIs) have been identified in serovar Typhimurium. SPI1 is principally involved in bacterial invasion of the intestinal epithelium while SPI5 is involved in inducing inflammation (intestinal phase). SPI2, 3, and 4 are primarily required for growth and survival of bacteria within the host (systemic phase). In addition to the five SPI's, serovar Typhimurium contains a virulence plasmid, the integrated prophages Gifsy 1&2, and several other horizontally acquired genes and gene clusters that contribute to virulence.

SPI1 and SPI2 are the most highly characterized pathogenicity islands. Both of these loci have been shown to encode type III secretion systems (TTSS) that deliver bacterial proteins directly into the host cell cytosol (18, 119). The TTSS is what is known as a "needle complex", a multi-protein structure that spans the inner and outer bacterial membranes (68). Both the SPI1 & 2 TTSS

secrete proteins that are essential for the viability of *Salmonella* pathogenesis of its host. However, the secretion systems are induced under different environmental conditions and phases of infection. This coordinated regulation of pathogenicity islands insures that the appropriate virulence factors will be expressed under the conditions requiring their function, leading to a successful infection.

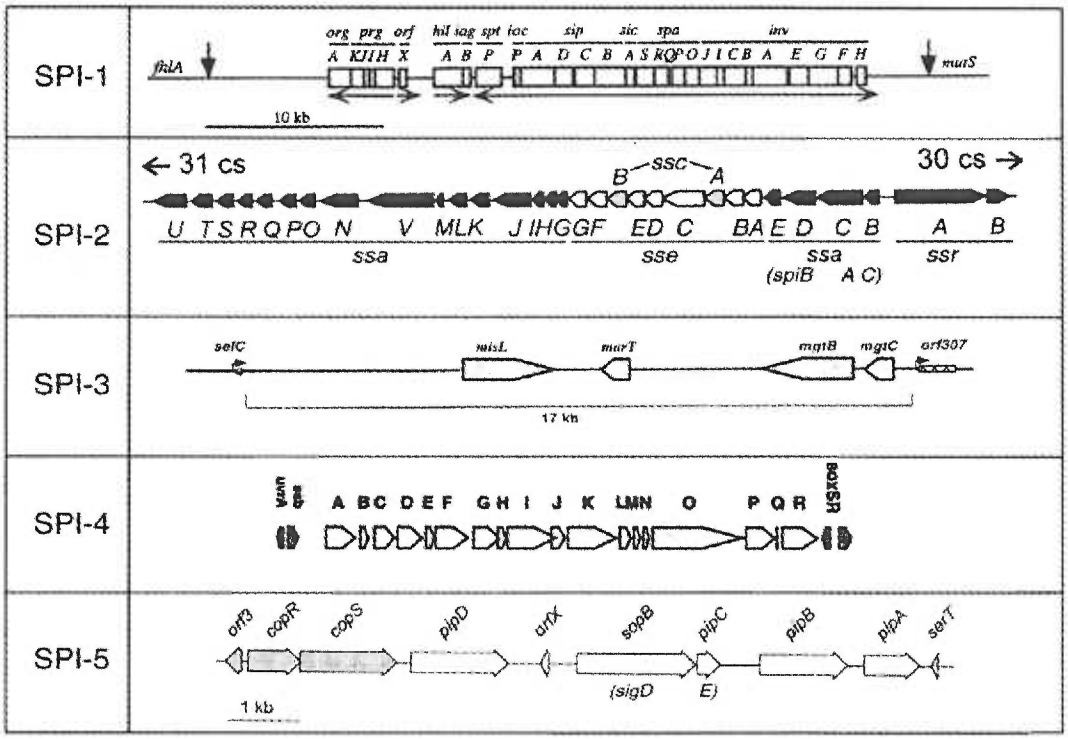

Figure 1-2. Schematic representation of *Salmonella* Pathogenicity Islands (SPI). [Reproduced from(78)].

1. Conditions of SPI1 expression

Several different factors affect the ability of *Salmonella* to colonize the intestinal epithelium including: oxygen levels, osmolarity, bacterial growth phase and pH. SPI1 expression is induced by a change from acidic to alkaline pH. This

response reflects the physiology of an *in vivo* infection as bacteria pass through the acidic environment of the stomach to the slightly alkaline environment of the small intestine (21). Under limiting oxygen conditions, *S. typhimurium* is highly invasive, while at aerobic conditions the bacteria are relatively non-invasive (72). Likewise, *Salmonella* grown in high osmolarity media are enhanced in their ability to invade eukaryotic cells (37). Consequently, the conditions present in the small intestine trigger the expression of SPI1 and thus the invasiveness of *Salmonella*, which is crucial for the intestinal phase of infection.

2. Conditions of SPI2 expression

SPI2 was first shown to be induced in bacteria contained within infected host cells (125). *Salmonella* resides in a phagosome in infected cells, which suggested that the conditions present in the intraphagosomal environment induce SPI2 expression. Further studies showed that growth of *Salmonella* in minimal media, which starves the bacteria for phosphate and Mg^{2+}, caused an increase in SPI2 TTSS expression (26). SPI2 is also maximally expressed during the bacterial stationary phase of growth, while SPI1 is maximally induced under early log phase conditions (126). This is an example of how both the SPI1 & 2 TTSS are inversely regulated; under conditions which fully induce SPI1 expression, SPI2 expression is at minimal levels and, and vice versa (46).

III. Intestinal Phase

A. Invasion

After reaching the intestine, *Salmonella* invade the cells in the underlying epithelium. *Salmonella* preferentially utilize M cells of the Peyer's patches to invade the intestinal epithelium (62). Regardless of whether the bacteria remain in the intestine or progress to cause a systemic infection, this step is crucial to *Salmonella* pathogenesis.

The invasion process is active on the part of the bacteria and several bacterial factors are produced to facilitate this process. The majority of genes that encode the proteins necessary for invasion are located on SPI1. All of the structural components of the TTSS are encoded within SPI-1, while the SPI-1 TTSS can secrete several different effector proteins that are encoded on SPI-1 and elsewhere in the chromosome. The secreted effectors allow *Salmonella* to invade eukaryotic cells by initiating several host cell-signaling cascades that lead to actin cytoskeletal rearrangements and uptake by the host cell. Pseudopods or "membrane ruffles" are induced and surround the bacterium, which is then engulfed, forming a phagosomal compartment. This process can be divided into 3 steps: (1) the activation of host cell Rho GTPases Cdc42 and Rac1, (2) the promotion of actin polymerization by bacterial effectors, and (3) the re-establishment of a normal host cell actin cytoskeleton.

The secreted effectors SopB, SopE and SopE2 are encoded outside of SPI-1 and are involved in activating the small G-proteins Cdc42 and Rac1 (47, 48, 122, 130). SopE and its homolog SopE2 act as nucleotide exchange factors for Cdc42 and Rac1, whose activation leads to the reorganization of the actin cytoskeleton. SopB is an inositol 3-phosphatase that increases cellular levels of 1,4,5,6-tetrakisphosphate, which leads to Cdc42 activation (134). SipA and SipC both are translocated effectors that bind host cell actin (51, 83). SipA inhibits depolymerization of actin filaments by binding to them and stabilizing them (74). SipC possesses dual functionality as it both nucleates actin and is required for translocation of all SPI-1 effectors (14). The exact mechanism of actin nucleation by SipC has not been delineated. It is likely that SipA and SipC act together to facilitate actin polymerization and bundling of F-actin at sites of invasion, which results in cytoskeletal rearrangements and the uptake of the bacterium (43). SptP is a SPI1 encoded tyrosine phosphatase that is secreted into the host cell cytosol. This protein helps to reverse the cytoskeletal rearrangements induced by the previously mentioned effectors during bacterial invasion (135). In addition to its tyrosine phosphatase domain, SptP has a GTPase-activating (GAP) domain that reverses the activation of Cdc42 and Rac1. This contributes further to the termination of the invasion process (36).

B. Inflammation

Two of the effector proteins that play a role in bacterial invasion of epithelial cells also induce an inflammatory response that is characterized by fluid secretion and polymorphonuclear leukocyte (PMN) influx into the intestine. The inositol phosphatase activity of SopB hydrolyzes phosphatidylinositol 3,4,5-triphosphate, an inhibitor of Ca^{2+}-dependent choloride secretion (94). The inability to control chloride secretion elicits the loss of electrolytes and fluid secretion from epithelial cells in the intestine, contributing to diarrhoeal symptoms (130). SipA is necessary and sufficient to induce epithelial cells to secrete a chemokine (PEEC), via an ARF6- and PLD-dependent lipid signaling cascade (19). The release of PEEC from epithelial cells induces the pertussis toxin-sensitive receptor on PMNs and elicits a Ca2+ signal that directs PMN migration across polarized epithelial cell layers (73, 82). Two other secreted effectors, SopA and SopD, also contribute to fluid secretion and PMN influx, but their biochemical activity is unknown (63, 131). The induction of inflammation aids in the dissemination of the bacterium through diarrhea and also compromises the integrity of the intestinal barrier, allowing growth and spread of bacteria throughout the intestinal mucosa. Once *Salmonella* gains access to the underlying tissues of the intestinal epithelium, it can be engulfed by resident phagocytes, then enter the lymphatic system, bloodstream and cause a systemic infection. (See Figure 1-3 below)

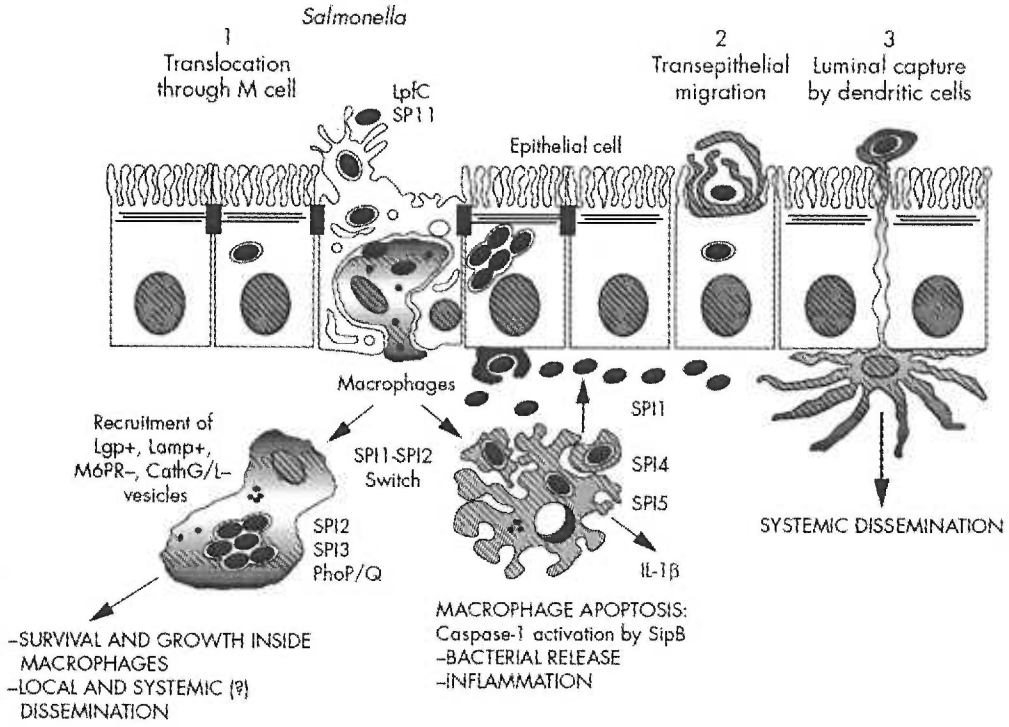

Figure 1-3. *Salmonella* routes for crossing the intestinal barrier and physiopathological scheme of infection. [Reproduced from (113)].

IV. Systemic Phase

A. Cell Types Encountered

During its course of infection, *Salmonella* has access to many host cell types including: PMNs, dendritic cells (DCs), fibroblasts and macrophages. PMNs are quite microbicidal for *Salmonella*, as the bacteria cannot avoid

phagocytosis or the intracellular oxidative burst (32). Mice that have been depleted of neutrophils (neutropenic) are more susceptible to *Salmonella* infection (128). DCs have been implicated in mediating bacterial transit across the epithelial layer as a method of antigen sampling (109). *Salmonella* was shown to be internalized by splenic DCs but remained a static, non-dividing population, hence intracellular bacterial numbers were quite low in mice infected orally (58). Fibroblasts, which are non-phagocytic, are permissive for *Salmonella* survival, but the bacteria do not proliferate within them (79).

While *Salmonella* can exist inside DCs and fibroblasts and even proliferate in epithelial cells, confocal microscopy analysis of sections of spleen and liver tissue from infected mice demonstrated that the majority of proliferating bacteria reside and multiply almost exclusively in CD-18 expressing phagocytes (27, 80, 110). While DCs may be important for transport from the intestinal lumen to the bloodstream, macrophages are critical for the systemic phase of *Salmonella* infection as they provide not only a means of dissemination for the bacteria, but also a replicative niche once the bacteria have reached the spleen and liver. *Salmonella* that cannot survive and replicate inside macrophages fail to establish a systemic infection (31). Furthermore, mice that have been depleted of macrophages are more resistant to *S. typhimurium* infection (44). However, the macrophage environment is very inhospitable for microorganisms. *Salmonella* must avoid being localized to the lysosomal compartment where free radicals and other toxic compounds would destroy it. It must also contend with the lack of

available nutrients inside of its host cell. *Salmonella* has evolved several defense mechanisms to survive in and ultilize macrophages to disseminate throughout its host.

B. Intracellular Virulence Systems

The intracellular phase of *Salmonella* infection is characterized by the formation of the *Salmonella* containing vacuole (SCV) and its manipulation by the bacteria to create a compartment permissive for survival and replication. Over 100 virulence genes have been identified to date that play a role in intra-macrophage survival and replication (78). The majority of these genes are members of two virulence systems: SPI2 and the two component regulatory system PhoPQ. Both SPI2 and PhoPQ are required for intracellular proliferation (88, 95). Despite the fact that numerous *S. typhimurium* genes have been identified that are expressed or required for bacterial replication in macrophages, the functions of only a few are known. The following sections will describe the SPI2 and PhoPQ systems and their roles in intracellular survival.

1. PhoPQ

The PhoP/Q locus encodes a two-component regulatory system controlling the expression of at least 40 genes (89), several of which were shown to be activated inside macrophages (1, 10). Two component regulatory systems are

found in a wide variety of bacteria and often regulate bacterial gene expression in response to environmental conditions. They are simple signal transductions systems composed of a membrane-spanning sensor/kinase protein that transfers a phosphate to the second, cytoplasmic component in response to environmental stimuli. The cytoplasmic component (in this case, PhoP) usually serves as a transcriptional regulator following phosphorylation. The primary signal activating PhoPQ is Mg^{2+} starvation, a condition that exists in the SCV. Growth in micromolar concentrations of Mg^{2+} promotes transcription of the regulon while growth in millimolar concentrations of Mg^{2+} represses transcription (39).

The PhoPQ system induces the expression of "PhoP activated genes" (pag) and represses the expression of others "PhoP-repressed genes" (prg) (88). Pags are expressed in the SCV and are required for intracellular survival while prgs are turned off in the SCV and include components of SPI1 (100). Both *PhoP* null and *PhoP* constitutive mutants are avirulent, suggesting that the appropriate timing of *pag* and *prg* gene expression is crucial for pathogenesis (88, 89). Expression of the PhoPQ regulon is necessary for resistance to acid pH and antimicrobial peptides, modification of antigen presentation, formation of the SCV and alteration of macrophage cell death (42). Some of the genes in the *Salmonella* PhoPQ regulon are ancestral (including PhoPQ itself), while others are the result of horizontal DNA transfer. This illustrates a common theme in bacterial pathogens: Relatively recently acquired virulence genes are controlled by more evolutionarily ancient regulators, thus exploiting the ability of these

regulators to sense simple physical and chemical cues to discriminate between host environments (97).

2. SPI2

The SPI2 TTSS is a horizontally acquired multifactorial virulence system that is activated upon entry of bacteria into eukaryotic cells and enables bacterial proliferation within them (17). *S. typhimurium* strains deficient for the SPI2 TTSS are highly attenuated in vivo and show a 10^4 higher LD_{50} (50% lethal dose) in a mouse model (119). The type III secretion system encoded by SPI2 has a central role in the interference with host cell functions by intracellular *Salmonella*. These effects are mediated by the translocation of several effector proteins by the SPI2 TTSS. Thirty-one genes encoding components of the type III secretion apparatus have been divided into four operons termed regulatory, structural I, structural II, and effector/chaperone (17, 53, 119). The designations of the genes of the SPI2 TTSS are based on their function; ssa (secretion system apparatus), sse (secretion system effector), ssc (secretion system chaperone) and ssr (secretion system regulator). However, some of the genes in SPI2 remain to be characterized. While the structural apparatus is entirely encoded within SPI2, most of the effectors secreted by SPI2 are encoded elsewhere in the chromosome (132). All of the genes in SPI2 as well as those outside are under the control of the two-component regulator SsrAB, encoded within SPI2 (119).

The precise environmental signals inducing SPI2 gene expression are not known in detail, however nutritional deprivation and reduced pH appear to play a role. There is also evidence for the role of global regulatory systems such as PhoPQ and OmpR/EnvZ in controlling SPI2 gene expression (26, 71). However, only OmpR/EnvZ has been definitively shown to activate SPI2 (64). Expression of SPI2 structural genes and effectors is activated soon after uptake by host cells and together they interfere with several host cell functions including: antimicrobial defense systems, intracellular transport, integrity and function of the cytoskeleton and cell death.

C. Intracellular Survival Strategies

The survival strategies of intracellular *Salmonella* can be broadly divided into two categories; (1) Establishment of a replicative niche and (2) Avoidance of and resistance to the phagocyte oxidative burst.

1. Establishment and maintenance of the SCV

The characterization of the SCV in macrophages has been a controversial subject and no consensus model has been determined. This is complicated by differences in macrophage activation states and differences in cell lines used. Most studies on the SCV have been performed in epithelial cell lines such as

HeLa, as it is a good model for studying cell biology. Invasion of epithelial cells depends on SPI1 but likely results in a temporary overlap of SPI1 and SPI2-mediated phenotypes thus complicating analysis of specific gene contributions. Furthermore, uptake of *Salmonella* into macrophages and subsequent changes in host cell biology vary considerably depending on the infection conditions used; invasion vs. receptor-mediated phagocytosis, *Salmonella* serovar, eukaryotic cell line, and bacterial pre-growth conditions. Thus, due to the differences in infection conditions and virulence gene expression, it is difficult to compare the models for biogenesis and modulation of the SCV between epithelial cells and macrophages. Furthermore, as macrophages are the dominant cell type during systemic infection by *Salmonella*, the study of the SCV in epithelial cells may be of limited relevance in understanding *in vivo* pathogenesis. As a result, this section will focus on what is known about the SCV in phagocytic cells.

a. SCV trafficking

Shortly after uptake, the SCV acquires the early-endosomal antigen 1 (EEA1), and the transferrin receptor (TfR), two early endosomal marker proteins (50, 106). Both of these proteins are quickly replaced by classical late endosomal or lysosomal marker proteins, such as lysosomal glycoproteins (lgps), LAMP-1, LAMP-2, LAMP-3 and the V-ATPase. The V-ATPase activity causes acidification of the SCV to a pH of 4.0-5.0 (107), leading to the secretion of SPI2 components

(2, 93). While some late endosomal and lysosomal markers are present on the SCV, *S. typhimurium* actively segregates its SCV from several other known late endosomal and lysosomal markers, forming its own unique intracellular compartment. SCVs containing live *Salmonella* have been shown to exclude the mannose-6-phosphate receptor (M6PR), normally present on phagosomes containing latex beads or dead *Salmonella* (50, 106). M6PR directs the delivery of cathepsins D and L (lysosomal hydrolases) from the TGN to late endosomes, however these proteins are absent from the SCV (40, 50, 106). The exclusion of cathepsin D and lysobisphosphatidic acid (LBPA) from the SCV in RAW264.7 macrophages was shown to be largely dependent on PhoPQ but not SPI2 (41). However, the majority of SCVs are positive for cathepsin L in murine bone marrow-derived macrophages, which are much less permissive for *Salmonella* survival and replication (96).

b. SifA and Membrane dynamics of SCV

Salmonella can alter the trafficking of the vacuole it resides in and prevent fusion with toxic lysosomal hydrolases. It must also ensure a net gain of membrane to accommodate the increase in vacuole size as bacterial replication proceeds. The formation of "*Salmonella* induced filaments" (sifs) occurs soon after uptake into host cells. These appear as long filamentous membrane structures and are dependent on the SPI2 secreted effector SifA (66). Sifs extend throughout the

cell and have the same protein markers as the SCV. SifA mutants have a replication defect in macrophages due to an inability to maintain the vacuolar membrane (3). SifA is also necessary to maintain the vacuole in epithelial cells but the mutant bacteria grow at a faster rate than wild type (4, 9). This paradox occurs because the macrophage cytosol is quite toxic for bacteria, whereas the epithelial cell cytosol is permissive for bacterial growth, giving the bacteria more space to replicate. It has been proposed that the function of SifA is the recruitment of LAMP-1 containing vesicles to the bacterial microcolony and fusion of these with the SCV membrane (3, 111). However, the exact mechanism of SifA has not been elucidated.

c. Manipulation of actin cytoskeleton - formation of VAPs

Salmonella has been shown to initiate a second round of actin polymerization shortly after the initiation of bacterial replication (3-6 hours post-infection) (85) (Meresse, 2001). This process is SPI2-dependent and occurs by *de novo* polymerization of actin monomers on the SCV membrane itself, leading to a cluster of F-actin around the bacterial microcolony (85). These structures are referred to as vacuolar-associated actin polymerizations (VAP). Two SPI2 secreted effectors SspH2 and SseI (SrfH) were shown to localize to the VAP but were not required for its formation (86). The exact role of VAP in intracellular survival of *Salmonella* has not been established, but it is possible that it serves to

stabilize the SCV membrane, and or provide a framework to allow the recruitment of membrane vesicles for their fusion with the SCV (57).

(See Fig. 1-4 Below)

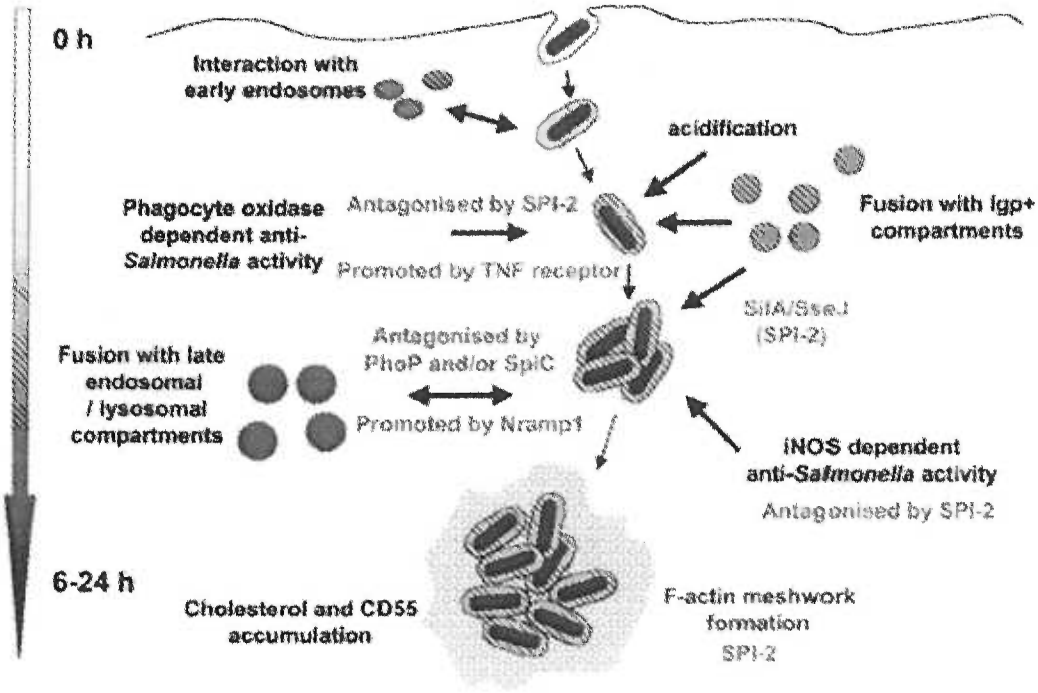

Figure 1-4. *Salmonella* growth and survival in murine macrophages. A time-course of the maturation of the SCV is represented schematically, in which *Salmonella* virulence proteins that promoted defined steps are indicated, and those that antagonize macrophage-driven processes. 'Lgp' refers to the lysosomal membrane glycoproteins, LAMP-1 and LAMP-2. [Reproduced from (76)].

d. Nramp1 – host factor hindering *Salmonella* intracellular survival

Natural resistance-associated macrophage protein (Nramp1) has been determined to be vital for resistance to intracellular pathogens such as *Salmonella, Leishmania* and *Mycobacterium* [reviewed in (5)]. Most of the studies on intracellular behavior of *Salmonella* in macrophages have utilized cells or animals lacking a functional *Nramp1* gene. The gene encoding this protein is the determinant for the *bcg/ity/lsh* host resistance phenotype in mice. Nramp1 is an integral membrane protein with 12 membrane-spanning domains and is expressed in the spleen, liver, lungs and peripheral blood leukocytes. Within these tissues, Nramp1 is found highly expressed almost exclusively in a few populations of phagocytic cells. It is targeted to the phagosome after phagocytosis of a pathogen and colocalizes with LAMP1 in late endosomes and lysosomes. It is upregulated upon exposure to IFN gamma or bacterial LPS and is a pH-dependent transporter of divalent cations. In *Nramp1*$^{+}$ cells, SCVs exhibit a much higher association with M6PR, but acidification of the SCV and acquisition of LAMP-1 were unaffected by Nramp1 (20). This study implies that Nramp1 interferes with the ability of *Salmonella* to modify its vacuole, increasing fusion with late endosomal and lysosomal degradative compartments. The exact mechanism by which Nramp1 antagonizes SCV trafficking is unknown but it is important to take into account that animals and cells with a functional *Nramp1* gene are much less susceptible to *Salmonella* infection.

2. Avoidance of intracellular antimicrobial compounds

In addition to being able to alter its intracellular compartment to avoid lysosomal hydrolases, *Salmonella* must also contend with antimicrobial compounds in the macrophage. Several reactive oxygen species (ROS) and reactive nitrogen intermediates (RNI) are produced in response to *Salmonella* infection of macrophages (28, 129). The enzymes responsible for the creation of these reactive compounds are NADPH oxidase and inducible nitric oxide synthase (iNOS). SPI2 was shown to be required for preventing the co-localization of the NADPH oxidase with the SCV (38). *S. typhimurium* lacking SPI-2 will not cause a lethal infection in mice, however in mice without the $gp91^{phox}$ subunit of the NADPH oxidase, the mice are susceptible (129). SPI2 also is important in limiting the bacteriostatic effects mediated by iNOS, as SPI2 mutants show association of iNOS with the SCV at 8 and 16 hours post-uptake (13). Although SPI2 is required for inhibiting delivery of both iNOS and NADPH to the SCV, no specific SPI2 TTSS effector has been implicated in this process.

The PhoPQ regulon also assists in protecting intracellular *Salmonella* from antimicrobial compounds, but these compounds consist of peptides rather than ROS or RNI. The activation of the PhoPQ regulon leads to widespread modifications in the protein and LPS components of the bacterial inner and outer membranes. These surface modifications promote *Salmonella* survival in the stressful environment of the phagosome by providing resistance to antimicrobial

peptides located in the macrophage (45). These peptides are small, amphipathic molecules that kill bacteria by disrupting the bacterial membrane (103). PhoP activated genes catalyze covalent modifications of the lipid A component of LPS, possibly discouraging antimicrobial insertion into the outer membrane by altering membrane fluidity and surface charge density (45, 97).

D. Macrophage killing by *S. typhimurium*

S. typhimurium can induce three distinct forms of cell death in infected macrophages. A rapid SPI1-dependent form of cell death takes place within 40 minutes of infection and is mediated by the interaction of the SPI1 effector SipB with the host cysteine protease caspase-1 [reviewed in (65)]. However, caspase-1 was not absolutely required for *Salmonella* induced cell death as cas pase-1 deficient macrophages still undergo a delayed cell death (61). This type of cell death occurred at 4-6 hours after infection, involved caspases 2,3,4,6 and 8, was associated with cytochrome c release from mitochondria and was dependent on SPI1 and SipB. A third type of delayed cell death similar to apoptosis was SPI1-independent and began around 6 hours post-infection and was visually evident by 12-14 hours post-infection (126). SPI2 and the regulator *ompR* were required for this delayed cytotoxicity (69, 90, 126). Caspase-1 was involved in the delayed cell death and led to the release of the pro-inflammatory cytokines interleukin IL1-ß and IL-18 (90). It is likely that all three of these forms of *Salmonella* induced cell death or combinations thereof are used during host

infection. During the intestinal phase of infection, it could be advantageous for bacteria to induce rapid cell death and inflammation to recruit macrophages to allow for systemic spread. During the systemic phase, the delayed *Salmonella*-induced cell death could allow intracellular spread within apoptotic bodies. However it is not known whether macrophage cell death is triggered by *Salmonella* to counteract host defense mechanisms or whether it is a host response to halt bacterial spread.

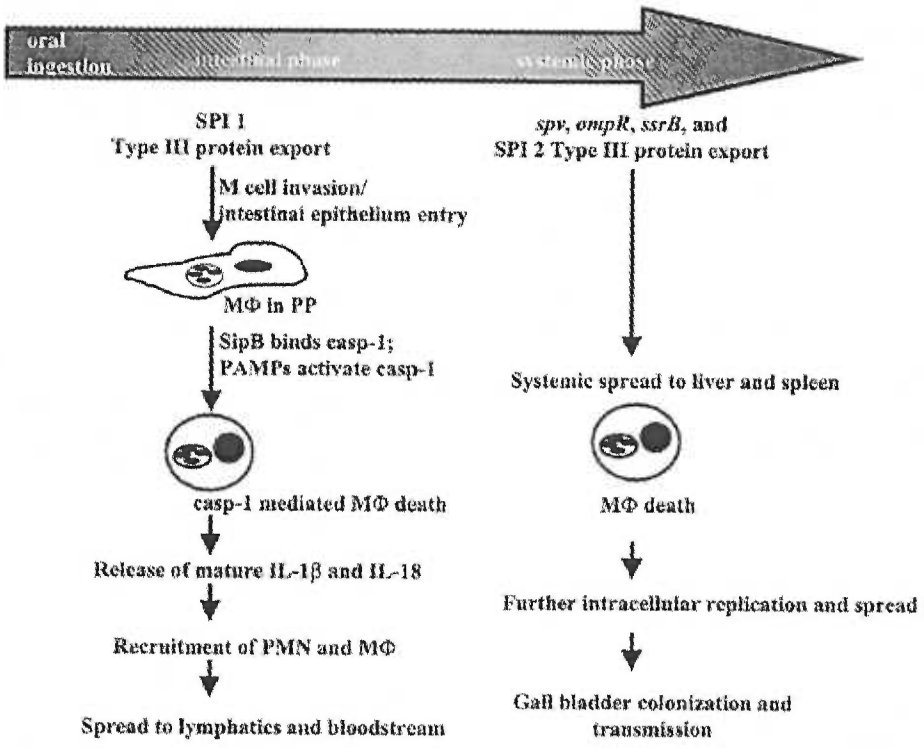

Figure 1-5. Model of serovar Typhimurium-induced macrophage death *in vivo*. The rapid and delayed macrophage deaths occur under discrete *in vivo* conditions at distinct times and locations. SPI1-dependent induction of caspase-1 activation in macrophages of the GALT results in increased inflammation and recruitment of phagocytes that may be required for systemic spread. SPI2-dependent macrophage death occurs at distant sites of infection and may aid in intracellular spread. [Reproduced from (90)].

V. Research Goals

Despite the fact that *Salmonella* can kill its host macrophage, it can persist for long periods of time in its host macrophage. My research began by identifying genes involved in long-term *Salmonella enterica* serovar Typhimurium survival within macrophages. One of the genes identified was chosen for further study to determine its role in pathogenesis. This gene is located in an unexplored pathogenicity island (SPI6) and has an atypical effect on systemic virulence in mice. The possibility that other genes in SPI6 have a similar phenotype was then tested.

Chapter 2.

SciS, an *icmF* homolog in *Salmonella typhimurium*, limits intracellular replication and decreases virulence

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Abstract

Salmonella typhimurium utilizes macrophages to disseminate from the intestine to deeper tissues within the body. While *S. typhimurium* has been shown to kill its host macrophage, it can persist intracellularly beyond 18 hours post infection. To identify factors involved in late stages of infection, we screened a transposon library made in *S. typhimurium* for the ability to persist in J774 macrophages at 24 hours post infection. Through this screen, we identified a gene, *sciS*, found to be homologous to *icmF* in *Legionella pneumophila*. *IcmF*, which is required for intracellular multiplication, is conserved in several gram-negative pathogens and its homolog appears to have been acquired horizontally in *S. typhimurium*. We found that a *sciS* mutant displayed increased intracellular numbers in J774 macrophages when compared to the wild type strain at 24 hours post-infection. *SciS* was maximally transcribed at 27 hours post-infection and is repressed by SsrB, an activator of genes required for promoting intracellular survival. Finally, we demonstrate that a *sciS* mutant is hypervirulent in mice when administered intragastrically. Taken together, these data indicate a role for SciS in controlling intracellular bacterial levels at later stages of infection and attenuating virulence in a murine host.

Introduction

Salmonella typhimurium is an enteric pathogen that causes a self-limiting gastroenteritis in humans and a systemic typhoid-like disease in mice. Macrophages are an essential vehicle for the pathogenesis of *S. typhimurium*, which utilizes them to disseminate from the intestine to the spleen and liver. *S. typhimurium* survives inside and eventually kills its host macrophage. Much has been published on how *S. typhimurium* manipulates the macrophage phagosome to facilitate survival (41, 57, 85, 121, 129), as well as how it kills macrophages (8, 16, 54, 59, 91, 126), but little is known about the factors that allow it to persist intracellularly beyond 24 hours. This study provides evidence that a horizontally acquired gene, *sciS*, limits intracellular replication of *S. typhimurium* in macrophages at late stages of infection.

Pathogenicity islands, which are horizontally acquired pieces of DNA that confer virulence traits, are especially crucial for the interaction of *S. typhimurium* with eukaryotic host cells. *S. typhimurium* contains two highly studied pathogenicity islands on its chromosome--Salmonella Pathogenicity Islands 1 and 2 (SPI 1&2)—which encode separate type III secretion systems (TTSS) that facilitate invasion and survival, respectively. In the absence of SPI1, infected macrophages are not killed at early timepoints (1-6 hours post-infection) (75, 126). A second killing pathway, mediated by SPI2, results in cell death at 18-24 hours post-infection (126). Despite this second killing pathway, bacteria can still

persist inside intact macrophages beyond 24 hours. To identify factors involved in the long-term persistence of *S. typhimurium* in macrophages, we isolated mutants with enhanced survival in macrophages at 24 hours post-infection. One of the mutants identified was *sciS*, a homolog of *icmF* that is contained in several gram-negative pathogens.

IcmF was first studied in *L. pneumophila*, where it is part of the Dot/Icm cluster of genes that form a type IV secretion system (T4SS) required for host cell killing and intracellular multiplication (104, 116). Three recent studies have shown *icmF* to be critical for allowing *L. pneumophila* to replicate in its own modified vacuole by maintaining an intact T4SS. *IcmF* was shown to be "partially required" for replication in human macrophages and essential for intracellular growth in amoeba (136). Another study showed that DotU and IcmF are required for the formation of replicative vacuoles and the translocation of the T4SS substrate, SidC (127). Additionally, DotU and IcmF serve to prevent degradation of type IV secretion components, indicating a role in stabilizing the T4SS (117).

A conserved cluster of 15 genes surrounding *icmF* in *Vibrio cholerae* has been designated IcmF-associated-homologous proteins (IAHP) (24). Gram-negative pathogens containing the *icmF* homolog have from six to fourteen of the fifteen genes in this cluster, but there is some variability in the composition and arrangement between the species. *IcmF* in *V. cholerae* is induced under *in vivo* conditions as measured in a rabbit ileal loop model (23). An *icmF* insertion mutant showed a nearly 2 fold increase in IL-8 mRNA levels in *V. cholerae*

infected intestinal epithelial cells when compared with wild type (114). The same insertion mutant showed reduced motility, increased adherence to epithelial cells and a higher conjugation frequency leading to speculation that it is involved in bacterial cell surface reorganization (22). The importance of *icmF* homologs is highlighted by their conservation in nine different gram-negative pathogenic species (24); however, their exact function remains unclear. Most bacteria that contain an *icmF* homolog are pathogenic and maintain close contact with eukaryotic cells. Therefore, it is likely that *icmF* homologs and associated proteins play an important role in bacterial pathogenesis.

The *icmF* homolog in *S. typhimurium*, *sciS*, named for *Salmonella* centisome 7 island, is located within a 44kb genomic island, which contains 9 of the 15 IAHP genes. A deletion of the entire island causes a defect in the ability of *S. typhimurium* to enter Hep-2 cells (34). The only individual genes studied in this island constitute the *Salmonella* atypical fimbrial (*saf*) operon, that was not required for mouse virulence (33). The remaining open reading frames in this island have not been characterized but several encode putative proteins with homology to known virulence proteins.

In this study, we identified *sciS* in a transposon mutant screen and investigated its role in the long-term persistence of *S. typhimurium* inside macrophages. We determined that SciS limits intracellular growth in macrophages only at late stages of infection and attenuates the lethality of *S. typhimurium* in a murine host. Together, these data constitute a unique example

of a horizontally acquired, temporally regulated gene that controls *S. typhimurium* virulence in mice.

Materials and Methods

Bacterial strains, plasmids, and DNA manipulations. All strains were grown in Luria-Bertani (LB) broth overnight at 37^{0}C. Antibiotics, when added, were used at the following concentrations: choramphenicol (Cm), 30μg/ml; kanamycin (Kan), 60μg/ml; carbenicillin (Carb), 100μg/ml; and tetracycline (Tet), 20μg/ml. All strains and plasmids used or constructed in this study are listed in Table 2-1. The construction of the *sciS* non-polar deletion (DP103) was performed using the pMAK705 temperature-sensitive plasmid based system (6). A fragment containing the mutation in sciS, plus at least 2kb of flanking DNA on either side was cloned into suicide plasmid pMAK705. The two fragments were amplified from the chromosome using primers [TCTAGAGCATCCAGCTTCATGAATGTCGATGAG and CTGCAGGATAC-TCAACTGGTCGGCCAC] for fragment 1 and primers [CTGCAGCAGATCGCTTCGC-TGCTGACC and AAGCTTGCACATAGTCGCGCTGGTCCG] for fragment 2. Fragment 1 contains an N-terminal XbaI restriction site and a C-terminal PstI restriction site and ends 1413bp into the SciS open reading frame. Fragment 2 contains an N-terminal Pst1 restriction site and a C-terminal HindIII site and begins 2959bp into the 3871bp SciS open reading frame. These two fragments were cloned separately into pCRBlunt (Invitrogen, Carlsbad, CA), excised and ligated together into pMAK705 creating a 1545 bp deletion in sciS. The resulting plasmid, pMAKf12

was transformed into 14028 at 30^{0}C on LB-Cam plates. Resulting colonies were then selected for growth on Cam plates at the non-permissive temperature 42^{0}C, which forces the integration of the plasmid into the chromosome via homologous recombination. To select the rare double crossover event, ampicillin enrichment was used to remove those colonies that still contained the plasmid. Any chloramphenicol-sensitive bacteria remaining were then screened via PCR for the correct mutation. The resulting strain, DP103, contains a 1546bp non-polar deletion spanning bp 1413-2959 in the 3870bp *sciS* open reading frame.

The sciS chromosomal lacZ fusion (DP137) was also made using pMAK705 but the resulting co-integrate was not resolved, leaving a sciS upstream fragment transcriptionally fused to lacZ and an intact sciS gene. A DNA fragment covering the promoter region of sciS was amplified from the chromosome via PCR using the primers [GGTACCCCCTGTCAGAGGACAG-ACTCC and GGATCCCAGCGGCCCGACAAACCAGAC]. The sciS promoter fragment spanning positions –1000 through +95 was ligated into the suicide plasmid pSC433 (pMAK705::lacZ), generating plasmid pDP136 (sciS::lacZ). This plasmid was eletroporated into 14028 and the resulting colonies were grown at 42^{0}C to select for single crossovers into the chromosome. The resulting chromosomal construct was transferred into a new 14028 background by P22 transduction. The resulting strain was confirmed via PCR before use in intracellular ß-galactosidase assays.

TABLE 2-1. *S. enterica* serovar Typhimurium strains used in this study

Strain	Description	Reference
ATCC 14028	Wild type	ATCC
DP101	14028 *invA*::*cat*	This study
DP103	14028 Δ*sciS*	This study
DP114	DP103 + pEGFP	This study
DP137	14028 *sciS*-lacZ transcriptional fusion	This study
DP148	14028 + pEGFP	This study
MJW129	14028 *ssrB*::*cat*	(38)

Eukaryotic Cells and Infection Procedure. The murine macrophage cell line, J774A.1 (American Type Culture Collection [ATCC], Manassass, Va.) was maintained in Dulbecco modified Eagle medium (DMEM; Gibco-BRL, Rockville, Md.) supplemented with 10% fetal bovine serum (FBS; Gibco-BRL), 0.2 mM MEM sodium pyruvate (Gibco-BRL) and 0.1 mM non-essential amino acids (Gibco-BRL). J774 macrophages in tissue culture plates were infected with overnight cultures grown rolling at 37°C. Prior to infection, cultures were adjusted to an OD_{600} of 1.0 and resuspended in PBS. After the addition of bacteria, the plates were centrifuged at 25°C for 5min and placed in a 37°C-incubator with CO_2 for 25min. Cells were then washed 3x with PBS to remove extracellular bacteria and fresh DMEM containing 100μg/ml gentamicin was added for 1 hour at 37°C to kill extracellular bacteria. The plates were washed once with PBS and then incubated with 20μg/ml gentamicin at 37°C.

Selection and screen for persistent mutants in J774 macrophages. A mutant bank was generated in DP101 (*invA*::cam) using MudJ transposon mutagenesis with P22 (60). The bank was plated on large M9 minimal media agar plates to reduce the number of auxotrophs. Approximately 50,000 mutants were generated on 12 separate plates. Each plate was scraped, the bacteria resuspended in 2mls of LB with 10% glycerol and frozen at -80^{0}C. An overnight culture was grown from each pool and J774 macrophages in 6-well plates were infected (as described above) at an MOI of 1 bacterium to 10 macrophages. After 24 hours of infection, the twelve infected wells were washed with PBS and the remaining macrophages were lysed with 1% Triton-X (Sigma Chemical, St. Louis, MO). These lysates were used to start overnight cultures and 12 new wells were infected. This process was repeated 3 times to enrich for mutants that remained inside the macrophage. Four of the twelve pools showed a sharp increase in bacterial numbers after 3 passages. Individual mutants from these pools were scored for cytotoxicity on J774 macrophages in 96-well plates. Three candidate mutants from each of the four pools were selected and their MudJ insertions sequenced by inverse PCR and the results are listed in Table 2.

Cytotoxicity Assays. These assays were performed as previously described by van der Velden *et. al.* (126). Briefly, J774 macrophages seeded in 96-well plates, were infected with overnight stationary phase cultures of *S. typhimurium* at an MOI of approximately 50. At the indicated timepoints, the supernatant was

removed and used to determine the release of cytoplasmic lactate dehydrogenase (LDH) using the CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega, Madison, WI). Cytoxicity was determined for each strain by calculating the LDH released as a percentage of the maximal release from lysed macrophages.

Microscopy. J774 macrophages were grown on glass coverslips placed in 6-well plates prior to infection with *Salmonella* strains containing the plasmid pEGFP. Cells were infected at an MOI of 80 or 10. After 24 hours of infection, cells were washed with PBS and fixed with 3.6% paraformaldehyde in PBS for 15 minutes at 25^{0}C. The coverslips were then stained with either 4',6-Diamidino-2-phenylindole (DAPI, Sigma Chemical) alone or with FM4-64 (Molecular Probes, Eugene, OR) and DAPI at 25^{0}C for 1 hour. Ten fields were randomly chosen for each coverslip and images were taken using an Applied Precision Image Restoration System (Advanced Precision Instruments, Issaquah, WA). All images were taken at 60x. Image stacks of 5 steps spaced 0.2 micron apart were taken and deconvolved. Selected images were saved as TIFF format and imported into Adobe Photoshop to be formatted for publication. The number of bacteria per infected macrophage was counted double blind for each field.

Intracellular ß-Galactosidase Assays. Activity of ß-galactosidase was assayed as described (87). To measure the ß-galactosidase activity from

intracellular bacteria, macrophages were grown in 6-well plates and infected with DP137 at an MOI of 50, as described above. Cells were collected from 3 wells and lysed with 1% Triton-X at the indicated time points. Lysates were serially diluted and plated to determine the number of intracellular bacteria. ß-galactosidase activity was expressed in Miller units per 10^9 bacteria.

Screen for Regulators of SciS. A modified tetracycline-inducible transposon (T-POP) was constructed by replacing the original Tn10 ends with Tn5 ends (105). The T-POP transposon using the primers 5'-CAGCTGTCTCTTATACACATCTCCATTAAGGTTACCATCACGGA-3' and 5'-CAGCTGTCTCTTATACACATCTGTGATCTCGGGAAAAGCGTTGGTGA-3'. The resulting fragment was cloned into the pCR2.1-TOPO (Invitrogen) to yield a vector containing the Tn5 T-POP. The transposon was excised from the vector with PvuII and purified. This Tn5 T-POP transposon was used to mutagenize the *sciS::lacZ* strain (DP137). A complex containing the Tn5 T-POP transposon and purified transposase was electroporated into electrocompetent DP137. Transposition of the transposon then occurred randomly within the bacterial chromosome. Bacteria containing transposon insertions were then selected by plating on tetracycline and X-gal plates. Those mutations that caused a color change from DP137 (light blue) were transduced into DP137 and retested for color change. Chromosomal DNA was purified from those colonies that retained a color change and the transposon insertion site was sequenced.

Real-time PCR Analysis. Total RNA from *S. typhimurium* infected J774 macrophages (MOI of 50) was isolated using hot phenol extraction and cleaned with a RNeasy Mini column (Qiagen, Valencia, CA) and used as a template to make cDNA using the iScript cDNA synthesis kit (BioRad, Hercules, CA). Quantitative real time PCR was performed on the cDNA using primers to *sciS* and *gyrB* as a control. *rpoD* was initially used as a control in our real-time PCR assays, as this gene has been established as a steadily transcribed housekeeping gene for use as an internal control (115). However, the primers to this gene seem to cross-react with eukaryotic cDNA, so *rpoD* could not be used as an internal control for RT-PCR performed on cDNA from infected macrophages. *gyrB* was suggested as a housekeeping gene to use as an internal control (personal communication with Ferric Fang). Primers to gyrB were tested in RT-PCR experiments on cDNA from bacterial cultures in parallel with rpoD primers and both genes were transcribed equally in all conditions tested. Each real-time PCR reaction was run in quadruplicate and values were expressed as fold induction to gyrB. The fold induction of wild type infected macrophages at 5 hours was set to 1. Values are the average of three separate experiments.

Mouse Studies. Mice were inoculated intragastrically with 200μl of an overnight culture of either 14028 or ΔsciS that was resuspended and diluted in PBS. The

following doses were given to groups of 4 mice: $3.1 \times 10^{4,5,6}$ for 14028 and $3.3 \times 10^{4,5,6}$ for ΔsciS. This experiment was repeated to groups of 8 mice with the following doses: $3.64 \times 10^{4,5,6}$ for 14028 and $3.70 \times 10^{4,5,6}$ for ΔsciS. Mice were monitored for 28 days for survival and LD_{50} values were calculated according to the method of Reed and Muench (108).

Results

A selection and screen for mutants that persist in macrophages identified *sciS*.

To investigate the contribution of *S. typhimurium* genes at late stages of infection, MudJ transposon mutants that survived in J774 macrophages for 24 hours without causing host cell lysis were selected. The bacteria used to infect the macrophages were from a bank of 50,000 MudJ transposon mutants divided into 12 separate pools. To prevent early killing of the macrophages, mutants were generated in a strain deleted for *invA*. The SPI-1 TTSS has been shown to induce rapid apoptosis in macrophages and InvA is required for its formation (77, 123). J774 macrophages were infected at an MOI of 0.1 and at 25 minutes post-infection, gentamicin was added to the extracellular media to eliminate those bacteria that didn't invade or escaped their host cell via lysis. By infecting at an MOI of 0.1, the majority of infected macrophages would contain a homogeneous population of replicating bacteria derived from a single progenitor. Following 24 hours of infection, the remaining macrophages were lysed with 1% Triton-X and the intracellular bacteria were recovered, used to start an overnight culture and infect new macrophages. This process of serial passaging was repeated three times, enriching the pools for persistent mutants. Four of the original twelve pools showed an increase in bacteria recovered from infected macrophages after three passages. Individual mutants from each of these four "enriched" pools

were then isolated and screened in J774 macrophages for persistence. Three MudJ insertions from each pool were sequenced giving a total of 12 individual sequences (Table 2-2). These sequences included *sciS* as well as genes involved in DNA repair and propanediol metabolism. Based on its homology to a known virulence gene in *L. pneumophila* and its location in a genomic island, *sciS* was chosen for further characterization.

Table 2-2. MudJ Transposon insertions from selection for persistent mutants

Pool#	Gene Description	STM#
4	ND[a]	ND
4	*mutH*	3005
4	*mutH* (sibling)	3005
6	*pduO*	2050
6	*pduO* (sibling)	2050
6	*pduO* (sibling)	2050
9	*ycfO*- putative glycosal hydrolase	1209
9	*sciS*	0285
9	*umuD* homolog	1998
11	*mutH*	3005
11	*mutH* (sibling)	3005
11	ND[a]	ND

[a] ND, no data as the insertion was not retransduced or sequenced

A *sciS* mutant is more numerous in J774 macrophages at 24 hours post-infection.

Surprisingly, none of the mutants isolated had measurable defects in cytotoxicity on J774 macrophages (data not shown) as measured by LDH release and crystal violet staining (126). Because the mutants were cytolytically normal, their persistence suggested that they had differences in intracellular numbers that led to their selection. Mutations that caused reduced intracellular replication would lead to an inability to break out of the phagosome, thus leading to a persistent state that would allow their selection. Conversely, mutations that cause overgrowth would be preferentially selected due to a higher representation of this population. To determine the effect of a *sciS* mutation on intracellular bacterial numbers we constructed a non-polar deletion of *sciS* (DP103) and infected J774 macrophages to determine its effect on intracellular replication. We used two methods to enumerate intracellular bacteria: 1) quantitation of intracellular bacteria and 2) visual inspection of the bacteria per infected macrophage via microscopy. The first method is standard for measuring intracellular replication and involves lysing macrophages at 24 hours post-infection and plating intracellular bacteria. The wild-type strain and DP103 had similar intracellular numbers of viable bacteria at 6 hours post-infection. However, *Salmonella* 14028 infected macrophages contained 2 to 3 fold less cfu's than DP103 infected macrophages past 24 hours post-infection (Fig. 2-1:

A). DP103 was also re-tested for cytoxicity on J774 macrophages and showed no significant difference from wild type at 6 or 24 hours post-infection (Fig. 2-1: B). To more directly visualize what was occurring in individual host cells, wide-field deconvolution fluorescence microscopy was used to enumerate intracellular bacteria. For these studies, 14028 and DP103 were transformed with the GFP containing plasmid pEGFP, resulting in strains DP148 and DP114 respectively. J774 macrophages were infected with either DP148 or DP114 for 24 hours, fixed and then stained with DAPI (DNA; blue) and FM4-64 (membrane; red). Microscopy experiments were then performed using MOI's of 10 and 80 (Fig. 2-2: A-D). In each experiment, the number of bacteria per infected cell was determined double blind for each strain in 10 separate fields (Fig. 2-2: E&F). The results show that macrophages infected with a *sciS* mutation at an MOI of 10 had 63% more intracellular bacteria than wild type at 24 hours post-infection while an MOI of 80 showed a 94% increase. All experimental MOI's in this study fall within the range of 10 to 80 established with the microscopy results. Differences in intracellular bacterial numbers were not evident between the strains at 6 hours post-infection, as macrophages infected with a *sciS* mutation showed 7.9 bacteria per infected cell versus 8.4 for wild type at an MOI of 80 (data not shown). However, the *sciS* mutant had significantly increased intracellular bacterial numbers when measured at 24 hours post-infection, correlating well with the selection procedure.

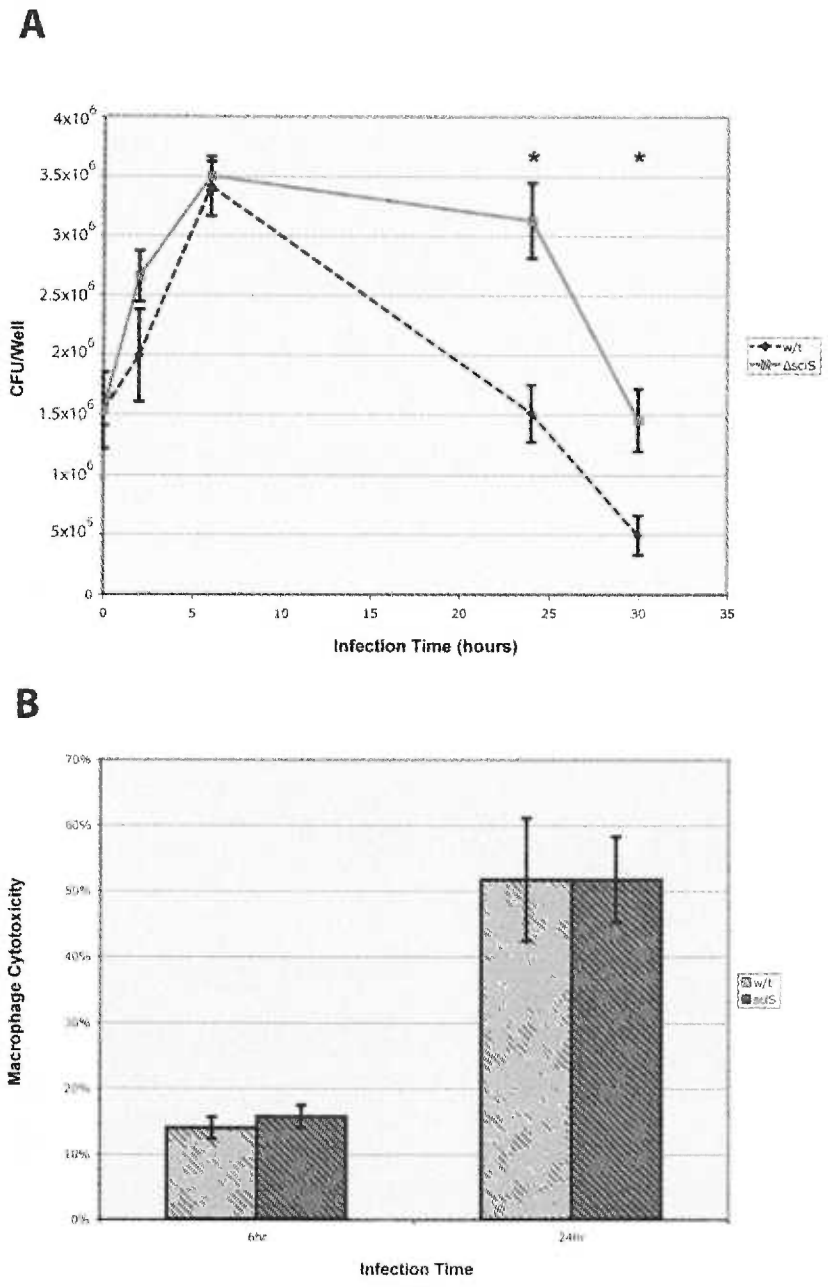

FIG. 2-1 – A *sciS* mutant displays increased intracellular numbers at late stages of infection in J774 macrophages but shows no difference in cytotoxicity. (**A**) Cells were infected with 14028 (diamonds) and DP103 (squares) at an MOI of 35. At the indicated timepoints, the macrophages were lysed with 1% Triton-X and intracellular bacterial numbers were determined. Each data point is the average of three individual wells. The asterisks indicate a P-value of < 0.10 as measured by a student's *t* test. (**B**) Cytotoxicity to J774 macrophages by 14028 or *sciS* was quantified by measuring LDH release at 6 and 24 hours post-infection. The differences between the strains in panel B were not statistically significant. Data from panels A & B are the average of three independent experiments with each time-point measured in triplicate. Error bars indicate the standard deviations of the mean.

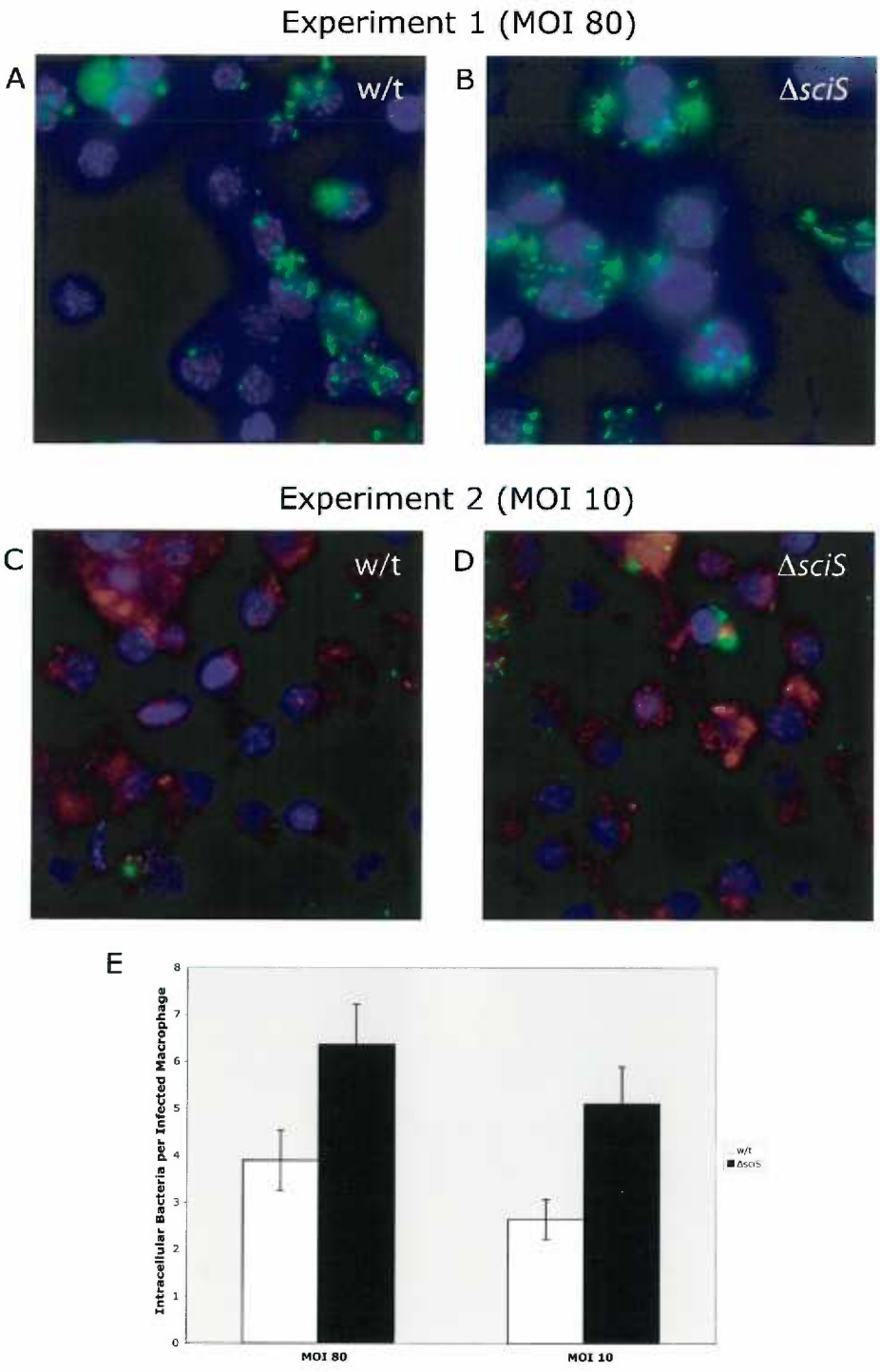

FIG. 2-2 - A *sciS* mutant is more numerous in J774 macrophages at 24 hours post-infection. These images are representative fields from experiments performed at two separate MOI's. (**A&B**) Macrophages were infected on glass coverslips in 6-well plates for 24 hours at an MOI of 80 and stained with DAPI (DNA stain; blue). (**C&D**) Macrophages were infected on glass coverslips in 6-well plates for 24 hours at an MOI of 10 and stained with DAPI and FM4-64 (membrane stain; red). Macrophages were infected with 14028 (A&C) and *ΔsciS* (B&D). (**E**) The average number of bacteria per infected cell for 10 individual fields ($P < .01$, student's *t* test). Data from the graph in Panel E represent results from three separate experiments.

SciS is maximally expressed in host macrophages at 27 hours post-infection.

The difference in intracellular numbers at late but not early timepoints suggested that *sciS* might not be expressed until late during the infection process. To investigate the temporal expression of *sciS* inside cells, we constructed a *sciS* promoter fusion to *lacZ* (DP137). Macrophages were infected with DP137 and ß-galactosidase activity was measured at seven timepoints between 10 and 34 hours post-infection (Fig. 2-3). *In vitro*, *sciS* was transcribed at undetectable levels above background; it was also minimally transcribed inside macrophages before 10 hours post-infection (data not shown). *SciS* induction is markedly different than that of SPI-2 genes as expression levels for 7 SPI-2 genes encoding effectors or structural proteins have either peaked or leveled off at 8 hours post-infection (17). We found that *sciS* was maximally transcribed inside macrophages at 27 hours post-infection. The timing of *sciS* transcription correlates well with the finding that a *sciS* mutant selected at 24 hours post-infection shows increased intracellular numbers at the same time but not at 6 hours post-infection. To verify this finding, we isolated total RNA from 14028 infected macrophages at 5 and 24 hours post-infection. We measured *sciS* mRNA levels using quantitative real-time PCR. We found that *sciS* mRNA levels were 7-fold higher at 24 hours than at 5 hours post-infection, in agreement with our previous observations (Fig. 2-4).

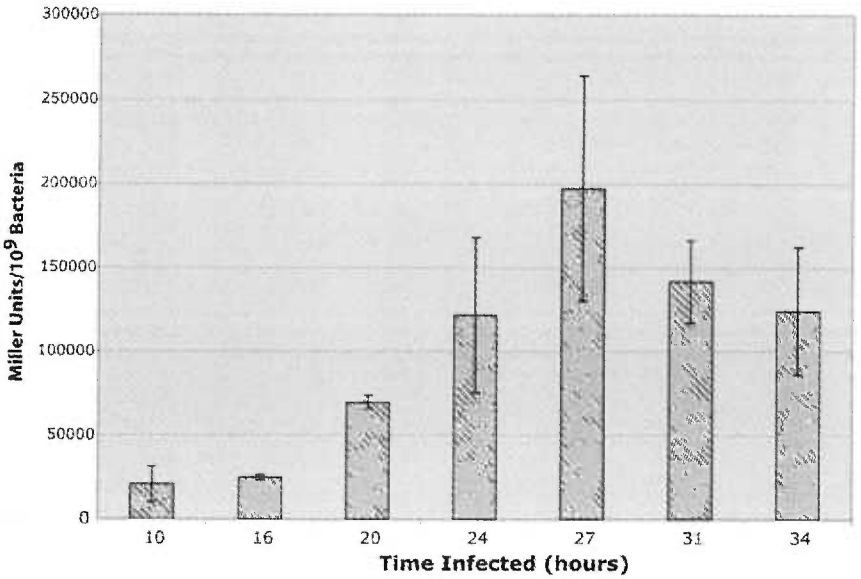

FIG. 2-3 – *SciS* is maximally expressed in host macrophages at 27 hours post-infection. J774 macrophages were infected with DP137 at an MOI of 50. Intracellular ß-galactosidase activity was determined at the indicated timepoints. Miller Units are expressed per 10^9 intracellular bacteria. *In vitro* cultures of DP137 showed ß-galactosidase activity below background. Data shown represent arithmetic means of three independent experiments. Error bars indicate the standard deviations of the mean.

SsrB negatively regulates SciS.

The late transcription of *sciS* in host cells raised questions as to how it was regulated. To identify genes that affected expression of SciS, DP137 (*sciS*::*lacZ* promoter fusion) was mutagenized with a modified T-POP transposon. The six thousand colonies from the mutagenesis procedure were screened for differences in transcription of *sciS* from the parental strain. The parental strain (DP137) appears light blue on an LB plates containing X-gal, so those colonies that were white or dark blue were selected for further analysis. A dark blue colony was isolated containing a transposon insertion was within *ssrB*. It was predicted to be a negative regulator of SciS because the transposon

insertion within *ssrB* leads to increased expression of *sciS*. To verify this, real-time PCR was performed on total RNA was isolated from macrophages infected with either an *ssrB* mutant or wild type *S. typhimurium* at 5 hours post-infection. There wasn't a sufficient number of intracellular bacteria at 24 hours post infection to provide the RNA necessary to do real-time PCR, because *ssrAB* mutations impair intracellular replication in macrophages (17). Real-time PCR showed a 5-fold increase in *sciS* transcription in an *ssrB* mutant when compared to wild type at 5 hours post-infection, confirming that SsrB negatively regulates *sciS* (Fig. 2-4). However, is not known if SsrB directly or indirectly regulates *sciS*.

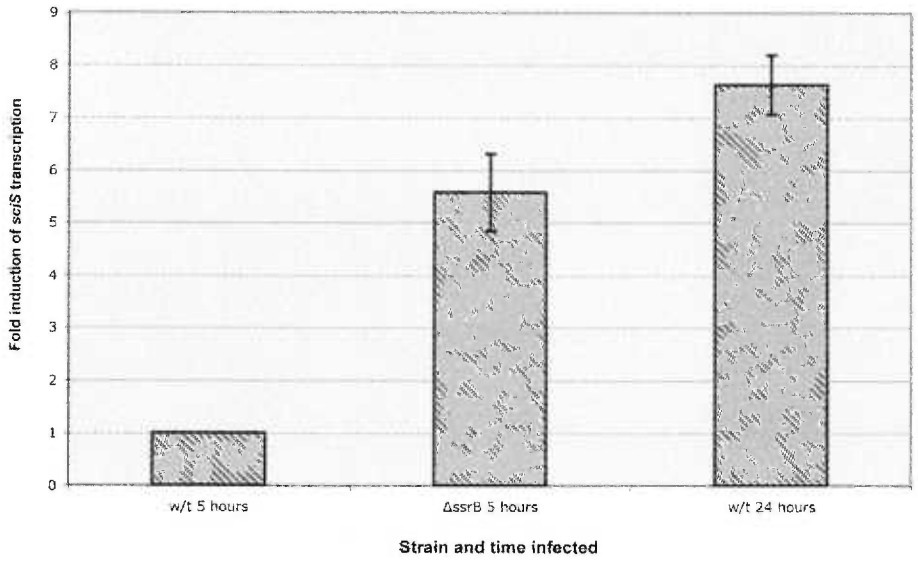

FIG. 2-4 – *SciS* is more highly transcribed at 24 hours post infection and is negatively regulated by SsrB. *SciS* RNA levels were quantified via real time PCR with total RNA isolated from w/t and ΔssrB infected J774 macrophages (MOI 50). Values were normalized to the levels of *gyrB* RNA and subsequently to the 5-hour timepoint from the w/t infected J774's. SciS is 7.5 fold more highly transcribed at 24 hours post-infection than at 5 hours. An *ssrB* mutant shows a 5.5 fold increase in *sciS* message at 5-hours post infection over the wild type strain. Data shown represent arithmetic means of three independent experiments. Error bars indicate the standard deviations of the mean.

A *sciS* mutant is hypervirulent in mice.

Having shown that *sciS* is important in *S. typhimurium* pathogenesis of tissue culture macrophages, we tested its contribution to *S. typhimurium* virulence in an *in vivo* mouse model. Groups of eight Balb/c mice were injected intra-gastrically with either 14028 or DP103 (Δ*sciS*) and monitored for survival over a 28-day period. Mice began dying at approximately the same day, regardless of the inoculating strain, however only half of the wild-type inoculated mice had died by day nine while all of the DP103 inoculated mice died by day eleven (Fig. 2-5). The LD_{50} calculated for DP103 was eight fold lower than that of 14028, indicating that the mutant was hypervirulent. This experiment was also done with groups of 4 mice, showing a nine-fold reduction in LD_{50} for DP103 (data not shown). This was an unexpected result, given that only two other mutations in *S. typhimurium* (*grvA*, *pcgL*) have shown an increase in virulence (56, 92).

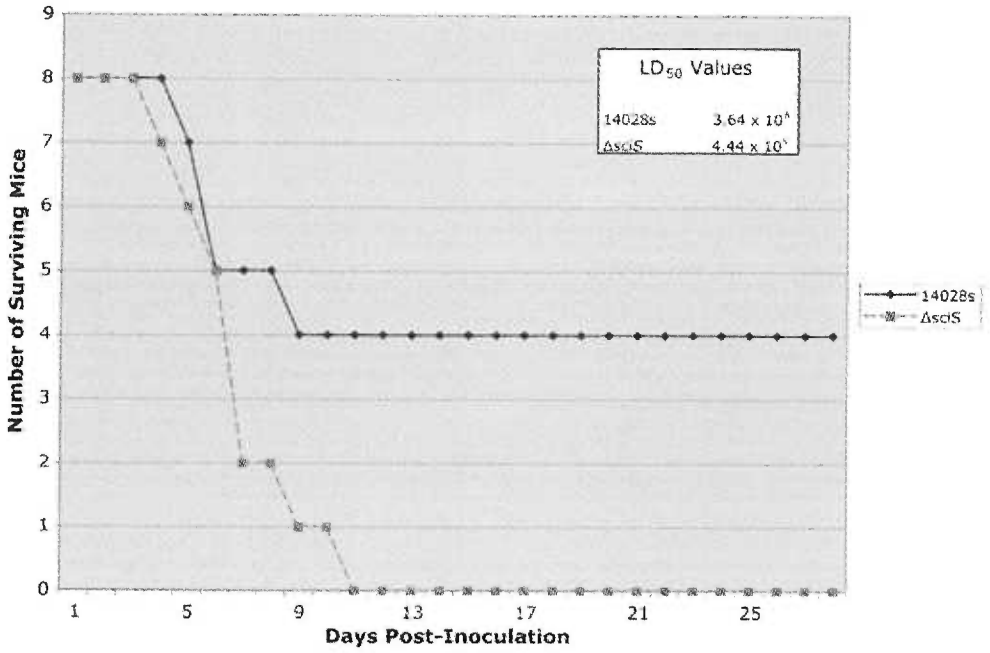

FIG. 2-5 – A *sciS* mutant is hypervirulent in Balb/c mice when administered intragastrically. Groups of 8 mice were inoculated intragastrically with either 14028 or DP103 and monitored for 28 days. The graphed results are from the groups inoculated with the 10^6 doses.

Discussion

To identify genes associated with the later stages of *S. typhimurium* infection of macrophages, mutants were selected that persisted inside macrophages at 24 hours post-infection. This selection isolated a gene, *sciS*, which limits replication in host macrophages and decreases virulence in mice. The increase in bacterial numbers seen with a *sciS* mutant at 24 hours post-infection was not observed in macrophages at 6 hours post-infection or in epithelial cells at any time. In addition to *sciS*, several other genes were identified in this study that were primarily involved in DNA repair and propanediol metabolism. These genes were most likely selected because the mutations attenuated bacterial growth, preventing them from lysing their host cells.

There are homologs of *sciS* in several other gram-negative pathogens, but the first identified and best characterized is *icmF* in *L. pneumophila*. Recently, *icmF* was isolated in a screen for mutants that have a growth defect because of an inability to lyse out of their host macrophage at the end of an infection cycle (127). It is interesting that *icmF* was isolated in much the same way that *sciS* was identified, by enriching pools of mutagenized bacteria in macrophages with gentamicin in the extracellular media. Previous studies have implicated *icmF* as being partially required for survival in human macrophages, which would seem to contradict the phenotype for *sciS* reported in this study. However, differences in the way the multiplication assays are performed can explain this discrepancy.

Multiplication assays performed in *L. pneumophila* typically measure numbers of bacteria in the supernatant, while replication assays in *S. typhimurium* typically measure only the bacteria residing intracellularly. As *icmF* is involved in escape from the vacuole, a delay or inability to escape its host phagosome would result in a decreased bacterial load in the supernatant. It is tempting to speculate that *sciS* could play a similar role in *S. typhimurium*, and would help explain the increased bacterial loads in infected macrophages. While there is an abundance of studies on *S. typhimurium* induced host cell killing, egress of the bacteria from the phagosome, and events during the latter stages of infection of macrophages have not been studied extensively.

SciS was induced only in host cells and its expression peaked at 27 hours post-infection, correlating well with the selection procedure and the increased bacterial loads seen only at 24 hours of infection. Interestingly, *sciS* transcription is negatively regulated by SsrB, a two-component regulator that activates Salmonella Pathogenicity Island 2 (SPI2) genes and additional genes outside of SPI2 that are primarily involved in promoting systemic infection of the host (13, 17, 35, 129, 133). Soon after uptake of *S. typhimurium* in macrophages, SsrB is expressed and induces several proteins that facilitate intracellular replication (26, 118, 132). SsrB RNA levels are reduced later during infection of macrophages (unpublished data, J. Rue), likely leading to the de-repression of *sciS* at 24 hours post-infection. The early expression of SsrB and its subsequent downregulation would help explain the delayed expression of SciS. Previously reported SsrB

regulated genes are activated, making this the first evidence for SsrB mediated repression. However, it isn't clear if repression of SciS by SsrB is direct, or if there are intermediate factors involved. SsrB facilitates *S. typhimurium* survival and replication inside macrophages after uptake, and perhaps its subsequent downregulation allows *sciS* to be expressed, limiting the bacteria from overgrowing their host cell or allowing for escape from the vacuole.

To our knowledge, *sciS* is the first example of a gene involved in limiting intracellular replication of *S. typhimurium* in macrophages. Previously, several genes were identified that prevent overgrowth of *S. typhimurium* in fibroblasts (11). The master regulator PhoP-PhoQ had the most profound effect on controlling replication, which was unexpected because in other cell types, PhoP-PhoQ is associated with promoting survival and replication. These differences in replication were measured at 24 hours post-infection, paralleling what was seen with *sciS* in macrophages. While *S. typhimurium* replicates more efficiently in macrophages than in fibroblasts, our results provide evidence that *sciS* is involved in attenuating bacterial growth in host macrophages at later stages of infection.

It is important for pathogens to limit their effects upon the cells they infect in order to achieve a balance with their host. An example of this phenomenon on a cellular level of *S. typhimurium* pathogenesis is demonstrated with its invasion process. *S. typhimurium* injects SopE and SopE2 into epithelial cells to activate Cdc42 and Rac1 which induces ruffling and promotes uptake of the bacteria into

host cells (47). Another secreted protein, SptP reverses these effects to restore a normal actin cytoskeleton once the bacterium is inside of the cell (36). These proteins are delivered simultaneously in equal amounts, however the SptP protein is degraded much more slowly than SopE, allowing it to reverse the effects of SopE (67). It is interesting that this process is temporally regulated, as a process set in motion by the bacterium can be purposely modulated at a later time. This is a recurring theme in pathogenesis and one that is clearly illustrated with *sciS*.

The attenuation of deleterious effects upon a host cell can be further extended to an animal model, as a *sciS* deletion showed an 8-fold increase in lethality with a mouse model. This hypervirulent phenotype in mice was initially a surprise, given that almost all known *S. typhimurium* mutations reduce virulence. Only two other *S. typhimurium* mutations have been shown to increase virulence in mice (56, 92). A null mutation in *grvA* increased virulence, as measured by competitive index experiments in mouse spleens and small intestine. Interestingly, *gvrA* is carried on the lamdoid phage Gifsy-2 near *srfH*, which was shown to be activated by the SsrAB regulon (132). However, it is not known whether SsrB regulates GrvA. Another study determined that inactivation of the dipeptidase *pcgL*, led to an accumulation of D-Ala-D-Ala, which caused an increase in bacterial numbers in mouse liver and spleen 24 hours post inoculation. The occurrence of the phenotype within 24 hours of inoculation suggested that the accumulation of D-Ala-D-Ala somehow compromised the

innate immune system leading to faster bacterial growth in host tissues. Both of these studies demonstrate that it is possible for an inactivated gene to lead to an increase in bacterial numbers host tissues. Increased bacterial loads in a murine host would likely lead to more rapid sepsis and toxic shock thus increasing lethality. The increased intracellular numbers observed in a *sciS* mutant appear to be consistent with this idea, as a *sciS* mutant is hypervirulent in orally infected mice. Most studies in bacterial pathogenesis are directed toward finding genes that promote virulence in the host. *SciS* is a unique example of a horizontally acquired gene that is temporally regulated, and limits *S. typhimurium* virulence.

Acknowledgements

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Chapter 3.

Contribution of *Salmonella enterica* serovar Typhimurium centisome 7 to mouse virulence

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Abstract

The centisome 7 genomic island of *Salmonella enterica* serovar typhimurium is a horizontally acquired region that contains several genes that are broadly conserved in pathogenic bacteria. In this study, we deleted nine genes in this locus and tested their individual contributions to mouse virulence. All genes tested led to an increase in virulence from wild type, implicating this region in restricting serovar Typhimurium virulence in mice.

Results and Discussion

The ability to survive and replicate in macrophages is critical for the systemic phase of *Salmonella* infection. By manipulating host cell functions to create a unique intracellular vacuole, *Salmonella* can thrive within macrophages and utilize them to disseminate to the spleen and liver (76, 97). Many virulence factors contribute to this process and are expressed in a highly coordinated and complex pattern to promote disease. However, it is important for pathogens to also express proteins that limit the harmful effects of the disease process in order to maintain an equilibrium with their host. Recently, a gene was identified (*sciS*) that plays a role in controlling growth of *Salmonella enterica* serovar Typhimurium in host macrophages at late stages of infection (99). A deletion in *sciS* also led to an increase in mouse virulence, suggesting a role in controlling systemic virulence. *SciS* resides in a 47-kb, horizontally acquired section of centisome 7 in serovar Typhimurium. This region is also present in *S. typhi* and has been named Salmonella Pathogenicity Island 6 (SPI6) (98).

Genes in the centisome 7 island of serovar Typhimurium were acquired separately from several different gram-negative pathogens, creating a mosaic of insertions. The open reading frames encoding putative proteins in this locus have been named *sci A* to *Z*, for *Salmonella enterica* centisome 7 genomic island (34). With the exception of *sciS*, no phenotype has been attributed to any of the individual genes in SPI6. The *saf* fimbrial operon in SPI6 was shown not to be

required for mouse virulence (33). A deletion of the entire 47-kb genomic island exhibited a defect in the ability to invade HEp2 cells but showed no defect in mouse virulence (34). However, neither of these studies tested doses below the 50% lethal dose (LD_{50}), so a mutation that increased virulence would have been overlooked.

SPI6 is present only within *S. enterica* subspecies I, which comprises 99% of the *Salmonella* serovars that cause disease (102). Genes that encode homologs of the putative *sci* proteins are always in close proximity to one another, indicating that they perform their function in concert. A cluster of 15 genes surrounding the *sciS* homolog *icmF* in *Vibrio cholera* has been designated IcmF Associated Homologous Proteins (IAHP) (24). A core set of proteins in serovar Typhimurium Centisome 7 that are present in most gram-negative bacterial pathogens has been defined and includes homologs of SciB, C, G, H, I, O and S (34). Given the broad conservation of these genes in pathogenic bacteria and the virulence phenotype reported for *sciS*, we investigated the role of SPI6 genes in mouse virulence.

Construction of Deletions in SPI6

The genes deleted in this study encompass the nine IAHP genes encoded in SPI6. These include the previously mentioned core proteins SciB, C, G, H, I, O and S as well as Sci N and P. Each of these genes was deleted according to

the method of Datsenko and Wanner (25), creating a non-polar deletion of the predicted open reading frame. The primers used to make these deletions are listed in Table 3-1. The resulting strains are listed in Table 3-2 and were numbered according to the STM number assigned to the individual deleted ORF in the serovar Typhimurium sequencing project (81). None of the mutations caused *in vitro* growth defects (data not shown).

Table 3-1. Primer Sets for Deletion of SPI6 genes

Primer Name	Primer Sequence	Deletion of STM#
SciB- F	GCTTACCGGTTTAGTCAGGGACATGTCCATATCATTACTCGTGTAGGCTGGAGCTGCTTC	267
SciB- R	GACCTGGCCGGTCAGAACCGGGCAACGGGGGAGCGTATGACATATGAATATCCTCCTTAG	267
SciC- F	GCCAGGGGGTTTTCTGCGCCTGGTTGAGAGCGCCGGTCATGTGTAGGCTGGAGCTGCTTC	268
SciC- R	GCTGAGCCGCATTACGTTCTTTTAATACAGGGCATTATTCCATATGAATATCCTCCTTAG	268
SciG- F	GCATTATTTTTATGAATTTTTATGTCACAAGGCATAACACGTGTAGGCTGGAGCTGCTTC	272
SciG- R	GTTTTTAAAATCGCACTACGGACACTTCGAACGGCCGGTTCATATGAATATCCTCCTTAG	272
SciH- F	CCTTGCGGCAACCAGTTGACTAAAAAGGAAATGAAGGATTGTGTAGGCTGGAGCTGCTTC	273
SciH- R	ATATTACTGTTTGCCATGAAAATTCCTTAAAAATTCGACGCATATGAATATCCTCCTTAG	273
SciI - F	ATGAATCAGCGGAATAACGTCGAATTTTTAAGGAATTTTCGTGTAGGCTGGAGCTGCTTC	274
SciI - R	TTCTCTGACTTCACTCGCTTAAACATTCACAAAATATATACATATGAATATCCTCCTTAG	274
SciN- F	TCTGGCCGGATTTATTATTTTGATTTTAAAGGAATTTACAGTGTAGGCTGGAGCTGCTTC	280
SciN- R	GTCCTTCACTCCAGACTACGCGGTCATTCCAGCTCATAATCATATGAATATCCTCCTTAG	280
SciO- F	ACAAAGCGCCATTGTAATTACGGAACGGGATAAATGAATTGTGTAGGCTGGAGCTGCTTC	281
SciO- R	TCATATCCGCCGCTGGCGGCGTCAGGGTACTGTCTGTCATCATATGAATATCCTCCTTAG	281
SciP- F	GGGGCTGGAGACAGAACTGTGGGGAGTGCGCGATAAATGAGTGTAGGCTGGAGCTGCTTC	282
SciP- R	ATGATGAGACACATCATAGGTTGATTTTTTCATCATTCATCATATGAATATCCTCCTTAG	282
SciS- F	ATTTTAAAATATTGATTATTGGGTGGCGAGTCGAAATATAGTGTAGGCTGGAGCTGCTTC	285
SciS- R	CATCGCCGGGGCTGATACCACTGCTGGCCGGCGCATTACACATATGAATATCCTCCTTAG	285

TABLE 3-2. *S. enterica* serovar Typhimurium strains used in this study

Strain	Description	Reference
ATCC 14028	Wild type	ATCC
MA6054	14028 *ara-907 araD 901*::MudJ	(55)
DP267	14028 Δ*sciB*	This study
DP268	14028 Δ*sciC*	This study
DP272	14028 Δ*sciG*	This study
DP273	14028 Δ*sciH*	This study
DP274	14028 Δ*sciI*	This study
DP280	14028 Δ*sciN*	This study
DP281	14028 Δ*sciO*	This study
DP282	14028 Δ*sciP*	This study
DP285	14028 Δ*sciS*	This study

Contributions of Deletions to Mouse Virulence

To measure the contribution to virulence of the 9 IAHP genes in SPI6, the LD_{50} was determined in six to eight week old female Balb/c mice. Bacterial cultures were grown overnight in Luria-Bertani broth at 37°C and diluted in phosphate buffered saline. Doses of approximately 2×10^5, 2×10^6, and 2×10^7 were administered intragastrically to groups of 5 mice. The mice were monitored for 28 days and the LD_{50} was calculated for each strain (108). All strains tested exhibited a LD_{50} below that of wild type and most strains led to death in all mice tested (Table 3-3). For these strains, the LD_{50} was estimated below the lowest dose given.

TABLE 3-3. LD_{50} Values for SPI6 Genes

Strain	Description	LD_{50} Value
ATCC 14028	Wild type	1.36×10^6
DP267	14028 Δ*sciB*	[a] $<3.27 \times 10^5$
DP268	14028 Δ*sciC*	[a] $<3.22 \times 10^5$
DP272	14028 Δ*sciG*	[a] $<3.42 \times 10^5$
DP273	14028 Δ*sciH*	2.11×10^5
DP274	14028 Δ*sciI*	3.00×10^5
DP280	14028 Δ*sciN*	[a] $<3.30 \times 10^5$
DP281	14028 Δ*sciO*	[a] $<2.08 \times 10^5$
DP282	14028 Δ*sciP*	2.14×10^5
DP285	14028 Δ*sciS*	[a] $<2.62 \times 10^5$

[a] These strains were lethal in all mice tested; the LD_{50} was estimated below the lowest dose administered

The same deletion strains were used to study the effects of SPI6 on *in vivo* replication in infected mice. Balb/c mice were injected intra-peritoneally with 10^4 CFU of a test strain mixed with a wild-type reference strain MA6054. The animals were sacrificed at 3 days post-innoculation, the spleens were recovered, homogenized and plated. The bacterial numbers in each spleen were expressed as a ratio between the bacterial strain being tested to the wild-type reference strain. The value for the wild-type test strain was much higher than the expected value of 1, indicating that the reference strain was compromised *in vivo*. The competitive index ratios of the deletion strains were not significantly different from that of the wild-type strain.

TABLE 3-4. Competitive Index Ratios for SPI6 Genes

Strain	Description	CI Value[a]
ATCC 14028	Wild type	3.99
DP267	14028 Δ*sciB*	1.51
DP268	14028 Δ*sciC*	2.62
DP272	14028 Δ*sciG*	2.01
DP273	14028 Δ*sciH*	5.14
DP274	14028 Δ*sciI*	3.53
DP280	14028 Δ*sciN*	3.19
DP281	14028 Δ*sciO*	4.85
DP282	14028 Δ*sciP*	1.77
DP285	14028 Δ*sciS*	2.98

[a]- Competitive Index Values (CI) are represented as the strain tested divided by the wild-type reference strain.

It is puzzling that there weren't large replication differences *in vivo* given previous data from cell culture that showed that a *sciS* deletion showed an increase in intracellular numbers(99). However, differences observed *in vitro* aren't always conserved *in vivo*. The fact that every deletion displays an increase in virulence supports the idea that these genes perform their function together and are interdependent. It has been proposed that the core proteins in SPI6 may form a type of structure such as a novel secretion or organelle biosynthesis system (34). Further evidence for the collective nature of the genes this area may be found by investigating the timing of expression and regulation of these genes. In a previous study, *sciS* was shown to be transcribed only in host

cells at 24 hours post-infection and is regulated by *ssrB* (99). As these genes seem to exhibit a similar phenotype, they may also be coordinately expressed and regulated. It is significant that several genes in SPI6 are involved in attenuating virulence. It seems that *Salmonella* has not only acquired pathogenicity islands to promote disease, but also to limit the harmful effects of the disease process. It will be of great interest to determine the exact function and mechanism of action of the genes in SPI6.

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Chapter 4. Summary and Conclusions

A crucial element of systemic salmonellosis is the ability of bacteria to survive and replicate within macrophages. *Salmonella* utilizes these cells to avoid the humoral immune response, to disseminate to deeper tissues within the body and as preferred sites of replication in the spleen and liver. *Salmonella* can have several effects on host cells; it can kill them rapidly via apoptosis, induce a delayed cell death or persist within them for long periods of time. While much is known about the early steps in *Salmonella* host cell infection, the process of long-term macrophage infection remains largely unstudied. To identify factors involved in this process, we infected macrophages with a serovar Typhimurium transposon mutant library for 24 hours and selected mutants that remained within their host cell.

Selection of persistant mutants in macrophages

We identified several mutations that enabled bacteria to persist within host macrophages. These included DNA repair genes (*umuD*, *mutH*), a propanediol utilization gene (*pduO*) and a previously uncharacterized gene (*sciS*). Interestingly, *sciS* and *pduO* reside in horizontally acquired regions of the chromosome. Horizontally acquired genes often have specific purposes during pathogenesis. The mutations selected highlight the importance for *Salmonella* to repair DNA damage and utilize alternate carbon sources in the harsh

environment of the macrophage. We speculate that the loss of DNA repair genes or the inability to process propanediol leave the bacteria in a compromised state, unable to kill the host macrophage or replicate well, leading to persistence. However, we focused on characterizing *sciS* because of its homology to a known virulence gene (*icmF*) in *L. pneumophila* and its location in an unexplored pathogenicity island.

Controlling intracellular growth in the host and bacterial egress

A deletion in sciS was found to increase the number of bacteria in host macrophages at 24 hours post-infection. This effect was not observed at earlier timepoints during infection or in epithelial cells. This result was unexpected as mutations in *Salmonella* that affect intracellular numbers are usually found to decrease survival. To our knowledge, this is the first report of a mutation that causes an increase in intracellular bacteria in macrophages. However, mutations that increase intracellular numbers in fibroblasts have been reported (12). *Salmonella* appears to be in a natural state of persistence in fibroblasts that is at least partially mediated by bacterial factors. The infected fibroblasts retain integrity and viability for long periods of time, up to 3 weeks after bacterial entry (12). However, the relevance of fibroblasts in systemic infection remains to be determined as *Salmonella* predominantly colonizes macrophages in the liver and spleen (110, 112).

In contrast to some reports of Salmonella reaching high numbers in cultured macrophages (49), *Salmonella* does appear to limit its intracellular growth in macrophages *in vivo*. A recent study showed that more than 90% of infected cells in the mouse liver contain less than 3 bacteria (120). Interestingly, the bacterial load in the liver increases by 3-logs during this time. While this may seem paradoxical, *Salmonella* increases its overall numbers by increasing the number of infected foci rather than the number of bacteria per macrophage or the size of the foci. In fact, the infected foci seem to reach a critical size, after which the bacteria must redistribute to uninfected cells, forming new foci (120). Thus, it is crucial for *Salmonella* to be able to escape its host cell to spread systemically.

It is not known whether the increase in intracellular numbers observed for a *sciS* mutation is due to an increase in replication, an inability to exit the host cell or another unidentified mechanism. A replication increase seems unlikely because no difference from wild type intracellular numbers is observed before 24 hours. As replication is a constant process, any change in replication would likely be seen early during infection. Host cell killing defects were not observed for *sciS*, so any defect in host cell egress would likely be a non-lytic event. This is an attractive possibility as *L. pneumophila* was shown to escape host macrophages non-lytically (15). In fact the *sciS* homolog, *icmF*, was isolated in a screen for *L. pneumophila* mutants that were delayed in host cell escape (127). It is interesting that *icmF* was isolated in much the same way that *sciS* was identified, by enriching pools of mutagenized bacteria in macrophages with

gentamicin in the extracellular media. Previous studies have implicated *icmF* as being partially required for surival in human macrophages, which would seem to contradict the phenotype for *sciS*. However, differences in the way the multiplication assays are performed can explain this discrepancy. Multiplication assays performed in *L. pneumophila* typically measure numbers of bacteria in the supernatant, while replication assays with *Salmonella* measure only the bacteria residing intracellularly. A delay or inability of bacteria to escape their host phagosome would result in a decreased bacterial load in the supernatant. It is tempting to speculate that *sciS* could be involved in non-lytic escape in serovar Typhimurium. This would help explain the increased bacterial loads observed in macrophages infected with *sciS* bacteria (99). While there is an abundance of studies on serovar Typhimurium-induced host cell killing, egress of the bacteria from the phagosome and events during the latter stages of macrophage infection of have not been studied extensively. Demonstration of non-lytic cell to cell spread by *Salmonella* would be a significant finding. During phases of infection where inducing an inflammatory response would be detrimental to a pathogen, non-lytic escape from host cells could be a good means of dissemination.

SciS: timing and regulation of expression

The timing and manner of expression of *sciS* are somewhat unusual. This gene was expressed at very low levels outside cells, leading us to believe that *sciS* is specifically adapted for the host cell environment. In host cells, expression levels did not exceed background levels until 10 hours post-infection. This is in contrast

to the expression profiles shown by other intracellularly induced genes. Seven SPI2 genes encoding effectors or structural proteins showed maximal expression by 8 hours post-infection in macrophages (17). *SciS* expression peaked at 27 hours post-infection, which correlates well with the timing of our selection procedure and manifestation of the increased bacterial numbers at 24 hours post-infection.

Interestingly, *sciS* transcription is negatively regulated by SsrAB, a two-component regulator that activates Salmonella Pathogenicity Island 2 (SPI2) genes and additional genes outside of SPI2 that are primarily involved in promoting systemic infection of the host (13, 17, 35, 129, 133). Soon after uptake of *S. typhimurium* by macrophages, SsrB induces the expression of several proteins that facilitate intracellular replication (118, 132). The negative regulation of *sciS* by SsrB seems appropriate when the timing of *sciS* expression is considered. SsrB helps to initiate the formation of the SCV and survival of *Salmonella* during early stages of infection. During this time SsrB represses *sciS* transcription. Recent data from our lab indicates that *ssrB* expression is reduced after 8 hours post-infection (unpublished data, JoAnn Rue). The early expression of SsrB and its subsequent downregulation could explain the delayed expression of *sciS*. All previously reported SsrB-regulated genes are activated, making this the first evidence for SsrB-mediated repression. However it isn't known if *sciS* repression by SsrB is direct or indirect. SsrB facilitates *S. typhimurium* survival and replication inside macrophages after uptake, and we hypothesize that its

subsequent downregulation allows *sciS* to be expressed, thereby limiting intracellular bacterial numbers or permitting non-lytic cell-to-cell spread.

Hypervirulence resulting from a sciS deletion and occurrence of other Salmonella antivirulence genes

The importance of limiting replication can be seen on a systemic level as a *sciS* deletion caused a 8-fold increase in mouse virulence. This was an unforeseen result, as most previously reported *Salmonella* mutations decrease virulence in mice. However, previous studies have demonstrated that mutations in *grvA* and *pcgL* increase virulence as measured by competitive index assays in mouse spleens but these mutations did not cause increased lethality (56, 92). While the mechanism of action of *grvA* is not known, a mutation in *pcgL* causes a buildup of the dipeptide D-Ala-D-Ala in the periplasmic space. When a transporter was expressed that removed D-Ala-D-Ala to the cytoplasm, the virulence phenotype disappeared. D-Ala-D-Ala serves as a 'pathogen-associated molecular pattern' (PAMP) that stimulates the immune system to control bacterial growth.

Both of these studies demonstrate that it is possible for inactivation of a gene to lead to an increase in bacterial numbers in host tissues. It is possible that increased bacterial load in macrophages could lead to more rapid sepsis and toxic shock, thus increasing lethality. Increased intracellular numbers observed in *sciS* mutants are consistent with this idea, as a *sciS* mutant causes increased intracellular bacteria in vitro and is hypervirulent in orally infected mice. The

timing of virulence gene expression in *Salmonella* is highly regulated. If *sciS* is involved in host cell escape, its absence could cause the bacteria to be released at inappropriate times or locations during host infection, triggering deleterious effects in the host. Perhaps, *sciS* plays a role in assuring the proper delivery of bacteria to preferred sites of replication.

Salmonella Pathogenicity Island-6 role in restricting virulence

In addition to its role in pathogenesis, the location of *sciS* in a horizontally acquired locus was of great interest. The *sci* genes were named for their location in the *Salmonella enterica* serovar Typhimurium Centisome 7 Island (34). This region was also named SPI6 in serovar Typhi (98) and has all the hallmarks of a pathogenicity island: a large region containing homologs to virulence proteins in other organisms, a GC content differing from that of the core genome, and insertion at a tRNA locus. While the composition of SPI6 is a mosaic, some of these genes are conserved collectively in pathogenic bacterial species. I deleted the conserved core genes in SPI6 and observed a hypervirulent phenotype for all genes tested. The same result for each gene suggests the possibility that these genes act in concert. To lend further support to this idea, it would be interesting to test whether the timing of expression and regulation of these core genes is similar to that observed for *sciS*. The core proteins in this locus were previously predicted to form a novel secretion system or biosynthetic organelle (34). It is not known if other genes in SPI6 play the same role in pathogenesis as the core

subset. Due to the mosaic structure of SPI6, it is likely that the surrounding genes play a variety of roles during *Salmonella* infection.

Conclusions

SPI6 mediated attenuation of virulence correlates with the notion that it is beneficial for pathogens to reduce virulence in their hosts. There is a general view that pathogens will naturally evolve toward a less-virulent, symbiotic relationship that does not kill the host and thereby allows their continued propagation (29, 30, 124). Although this argument is affected by the transmissibility of the pathogen, the lower virulence of nontyphoid *Salmonella* than of typhoid *Salmonella* is cited as a specific example of evolution towards decreased virulence (29).

While specific *Salmonella* genes have been identified that attenuate virulence, their exact mechanism of action remains a mystery. It will be of interest to determine if SsrB regulates SPI6 genes in the same manner as *sciS.* If any of the genes in SPI6 act together, they could be a part of the same regulatory circuit. It also remains to be demonstrated if *Salmonella* can utilize non-lytic cell-to-cell spread to decrease its lethality. Further analysis of the SPI6 locus is warranted to determine its exact function and role in pathogenesis.

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