Investigating *Salmonella*'s type III secretion: identification of effectors and their targets

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Abstract

Salmonella is an important human pathogen that causes thousands of infections every year due to ingestion of contaminated food. The need to develop new strategies to control *Salmonella* spread and combat disease are of paramount interest because of the rise in the frequency of antibiotic resistant *Salmonella* strains. A key to developing new therapeutic strategies is a better understanding of the pathogenesis of Salmonellosis. Although animal and cell culture models have provided much insight into *Salmonella* infection, many aspects of Salmonellosis warrant further analysis. This dissertation describes the investigation of one of *Salmonella*'s critical virulence determinants, its type III secretion systems (T3S).

T3S are syringe like structures that allow pathogenic bacteria to directly inject virulence factors, called effector proteins, into the cytosol of host cells. *Salmonella* has two T3Ss, encoded in *Salmonella* pathogenicity island 1 (SPI-1) and *Salmonella* pathogenicity island 2 (SPI-2). The SPI-1 encoded T3S (T3S-1) mediates cell invasion and inflammation during the intestinal phase of *Salmonella* infection, while the SPI-2 encoded T3S (T3S-2) is essential for intracellular survival within phagocytes during the systemic phase of disease. Although, recent research has provided much insight into the molecular mechanisms by which T3S secreted proteins promote *Salmonella* infection, the T3S effectors mediating several T3S dependent phenotypes have yet to be identified. Therefore a strategy was devised to identify secreted effectors, using a transposon that creates random fusions to the calmodulin dependent adenylate cyclase (CyaA') from *Bordetella pertussis*. This strategy was used to identify three new T3S effectors, SteA, SteB and SteC. Analysis of these proteins revealed that only SteA is required for

virulence in mice. Further characterization of SteA demonstrated that it is targeted to the Golgi network in infected cells.

Analysis of the effector proteins that were isolated in the screen for new effectors revealed that they have different secretion patterns. Some effectors are secreted only via T3S-1, some are secreted only by T3S-2, and some are secreted by both T3S-1 and T3S-2. While the current paradigm is that T3S-1 is only involved in the intestinal phase of disease and T3S-2 is only involved in the systemic phase of the disease, some evidence suggests that this may not be the case. Therefore, the analysis of the *in vivo* secretion patterns of effectors during acute mouse infection was undertaken. Using *Salmonella* strains that express β-lactamase fusions to severeral different effectors, spleen cells targeted by T3S could be identified by cleavage of a fluorescent substrate. Only effectors secreted via T3S-2 were secreted into spleen cells, supporting the existing views that T3S-1 is not involved during the systemic phase of infection. FACS analysis of the targeted cells determined secretion was detected in T-cells, B-cells, monocytes and neutrophils, but not in mature macrophages. Most of the secretion and viable bacteria were actually found in neutrophils, indicating that neutrophils are an important niche for bacterial growth. This result challenges the current view that macrophages are the primary site of intracellular growth.

Chapter 1: Introduction

I. Background and significance

Salmonellosis is one of the most common forms of food-borne illness (1). In developing countries, with limited access to antibiotics, *Salmonella* infections are estimated to result in hundreds of millions of infections and thousands of deaths every year. In industrialized nations, Salmonellosis remains a critical health concern, resulting in millions of infections every year and imposing a significant burden on their health systems and economies. In the United States, an estimated 1.4 million non-typhoidal *Salmonella* infections occur and result in approximately 580 deaths annually. These infections account for 168,000 patient visits to physicians and an estimated cost of 3 billion dollars. The prevalence of Salmonellosis can be attributed largely to the association of the 2,501 known serotypes of *Salmonella* with livestock (1, 2). In addition, the rise of multi-antibiotic resistant strains of *Salmonella*, due to overuse of antibiotics in livestock, has limited the effectiveness of antibiotic therapy in humans.

In addition to its importance as a human pathogen, *Salmonella* has potential to be developed for other medical uses. Due to the relative ease with which *Salmonella* can be cultured and genetically modified, it has become one of the most highly developed systems for the analysis of bacteria-host interactions. The availability of diverse research tools has encouraged scientists to attempt many novel and creative applications. For example, several researchers are attempting to develop strains and methods to use *Salmonella* as a means to deliver antigens for vaccination against various diseases (3-5) or as a means to help kill cancer cells (6).

Research in the *Salmonella* field promises to help improve treatment and prevention of human disease. Spread of *Salmonella* can be prevented by developing improved methods for treating infected livestock, and by development of vaccines and new antibiotic strategies. In addition, *Salmonella* has proven amenable to development of new biotechnologies that help further research in all fields of biological sciences. Continued investigation of animal models, bacterial pathogenic mechanisms, and basic bacterial processes are essential in order to fully meet the potential that *Salmonella* research has to offer.

II. Salmonellosis in humans

Salmonella infections in humans are acquired by ingestion of contaminated food or contact with infected animals or humans. (See Figure 1-1) Two forms of disease are associated with *Salmonella* infections in humans: Typhoid fever and enteritis. The outcome of the disease is dependent on the serotype of *Salmonella* that an individual contracts. For example, *Salmonella enterica serovars typhi, paratyphi,* and *sendai* are exclusively associated with Typhoid fever, whereas, most other serotypes of *S. enterica*, such as *typhimurium* and *enteritidis*, are associated with enteritis in humans (1, 2, 7). The microbial factors that determine whether systemic or enteric disease results are not fully understood. However, it is clear that host specific factors must play an important role, because certain *Salmonella* strains, such as *S. typhimurium*, can cause either systemic or enteric disease depending on the host species (8).

A. Typhoid fever

Typhoid fever is initiated by ingestion of 10^3-10^6 organisms (see Figure 1-1) (9). The bacteria adhere to and invade the intestinal mucosa, most likely targeting the M cells

of the Peyer's patches (10). *Salmonella* are then engulfed by phagocytic cells and replicate within them in the lymphoid follicles, liver, and spleen (8). The invasion of the intestinal mucosa does not result in acute inflammation or diarrhea and patients remain relatively asymptomatic during the 7-14 days of this initial phase of infection (8, 10). Sometime after 7-14 days of incubation, bacteria are released from the intracellular environment and enter the systemic circulation, establishing itself at secondary systemic sites of infection such as the spleen, liver, bone marrow, gall bladder and the Peyer's patches (10). This bacteremia is associated with fever and general malaise, involving a wide range of symptoms and manifestations. Abdominal tenderness, pain, and constipation are common symptoms, however, in children and patients with HIV, diarrhea can occur (11, 12). In the most severe cases, gastrointestinal bleeding can occur as a result of perforation of the ileal intestine (13, 14).

Due to the variety and non-specificity of symptoms, blood or bone marrow cultures remain the preferred method for diagnosis (15). In most cases, antibiotics, usually fluoroquinolones, are prescribed and the infection resolves, although relapses can occur in 5-10 percent of the cases. In severe cases involving intestinal perforation, surgery is required to remove the affected tissue (13, 14). The mortality associated with typhoid fever has been greatly reduced largely due to antibiotic treatment and surgical procedures.

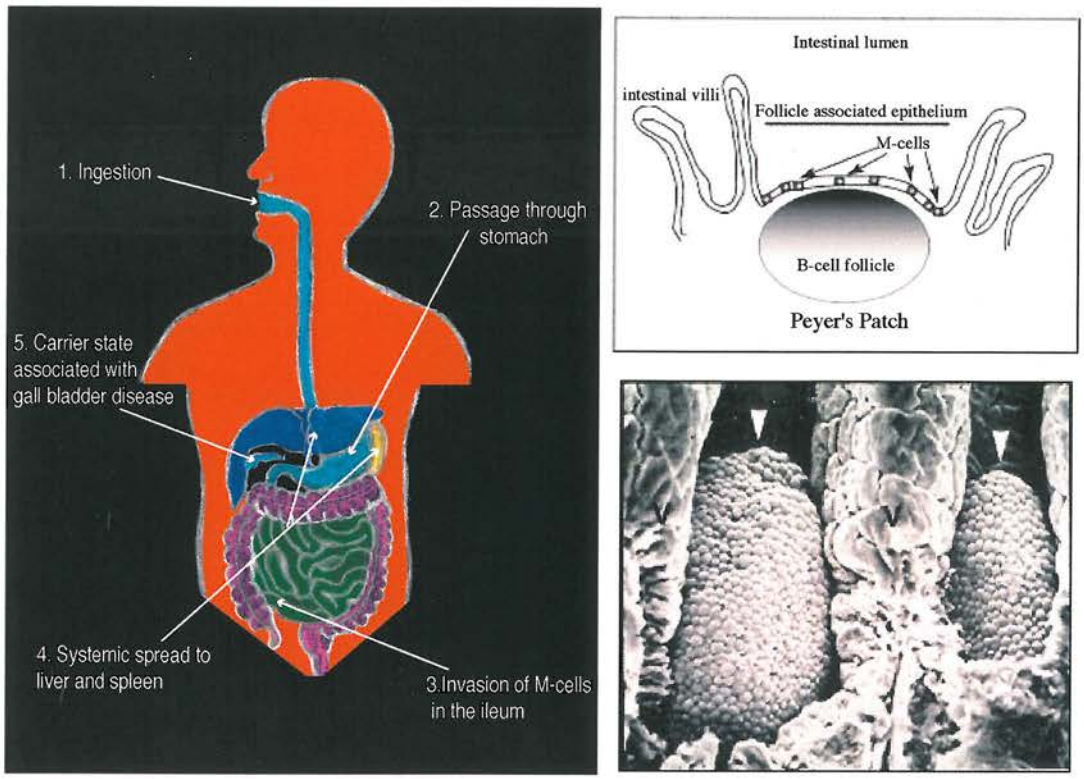

Figure 1-1. Anatomical sites of a *Salmonella* infection. 1. Infection is initiated by ingestion of contaminated food. 2. *Salmonella* passes through and survives the low acid environment of the stomach. 3. *Salmonella* invades M-cells of Peyer's patches in the ileum. In gastroenteritis, invasion results in massive inflammation and fluid secretion resulting in a self-limiting diarrhea. In Typhoid fever, *Salmonella* invades M-cells causing little if any inflammation. The diagram in the upper right corner shows the location of M-cells within Peyer's patches, the preferred site of *Salmonella* infection. M-cells are specialized cells involved in sampling intestinal antigens. 4. During typhoid, *Salmonella* spreads to systemic sites of infection such as the spleen, liver and lymphoid follicles where it survives and replicates within phagocytic cells. Gut associated lymphoid follicles (or Peyer's patches) from a cow are shown in the scanning electron micrograph in the lower right. After 7-14 days of incubation, bacteria are released from the intracellular environment and enter the systemic circulation, establishing itself at secondary systemic sites of infection such as the spleen, liver, bone marrow, gall bladder and the Peyer's patches. 5. Occasionally, *Salmonella* infection results in a carrier-state that is associated with gall bladder disease. [The image in the upper right was obtained from the following website: http://www.neurome.com/technology/images/mcells.jpg. The image in the lower right was downloaded from http://www.johnes.org/gif/photos-dairy/Peyers_patch_SEM.jpg]

Occasionally, systemic *Salmonella* infections result in a quiescent carrier state. An asymptomatic individual with no history of typhoid fever can shed bacteria and remain infectious for months to years, as in the infamous case of 'Typhoid' Mary Mallon (10). A higher prevalence of the carrier state occurs when gall disease is present (16). This may be due to *Salmonella*'s ability to form biofilms on gallstones or in the presence of bile (17). In some cases where antibiotic treatment is not effective, removal of gallstones is required to resolve carrier state associated *Salmonella* shedding (18, 19). Interestingly, women are more likely to become asymptomatic carriers than men, however the reason for this gender bias is not known (20). It is possible that this bias is due to a higher frequency of gall bladder disease in women versus men.

B. Enteritis

Symptoms associated with *Salmonella* induced enteritis begin 8-48 hours after ingesting contaminated food (see Figure 1-1) (7). These symptoms are largely due to a robust inflammatory response, characterized by a massive neutrophil influx into the intestinal lumen, resulting from colonization of the intestinal epithelium by invasive *Salmonella* (21-23). Symptoms include: nausea, vomiting, abdominal pain and diarrhea. These symptoms can last 3-5 days for gastroenteritis or can last 2-3 weeks for enterocolitis. Positive clinical diagnosis is usually achieved by culturing stool samples. In most cases the enteritis is self-limiting and antibiotics are generally not prescribed unless patients are at an increased risk of sepsis. Fluid and electrolyte replacement therapy may be applied to individuals exhibiting signs of dehydration. In addition, a constipating agent and dietary changes may be recommended to alleviate symptoms. Infants, the elderly and immuno-compromised individuals are at the greatest risk of developing bacteremia (7,

24) and in the absence of antibiotics, especially in developing nations, the outcome is often fatal (1).

III. Model systems for studying *Salmonella* infection

A. Animal models of *Salmonella* infection

Salmonella is known to infect and cause disease in a wide range of host species: pigs, cows, sheep, chickens, reptiles, amoeba, and many other animals are known to harbor *Salmonella*. Some *Salmonella* serovars are host restricted and are generally only associated with specific species. For example: *S. choleraesuis*, *S. abortusovis*, *and S. dublin* are associated with pigs, sheep and cattle, respectively (2). In contrast, certain strains of *Salmonella*, such as *S. typhimurium* appear to be capable of infecting practically anything that moves. A wide variety of experimental models have been established for studying *Salmonella* infections. However, for the sake of brevity, only two of the most popular models will be discussed: the cow and mouse models.

1. The bovine model of *Salmonellosis*

The bovine model of non-typhoidal *Salmonellosis* is considered the model that most closely recapitulates the pathology seen in humans (25, 26). In calves orally infected with *S. typhimurium* an acute diarrhea develops associated with a neutrophil influx seen in the intestinal mucosa (22). This inflammation results primarily from invasion of the M cells of Peyer's patches and to a lesser extent from invasion of the enterocytes overlying absorptive villi (25, 27). Within 1-4 hours of infection, *S. typhimurium* penetrates the intestinal epithelium and gains access to the lamina propria, where it resides within mononuclear cells and neutrophils (27, 28).

In addition to oral infection, a ligated ileal loop infection model has been developed in order to reduce variations seen between animals (25). This model allows for testing of multiple strains of *S. typhimurium* simultaneously in the same animal and has the added advantage of reducing the number of animals required for studies. *S. typhimurium* tested in both the calf oral challenge and ligated ileal loop models have shown that mutants manifest similar phenotypes in both systems.

The calf infection models allow for quantitative analysis of intestinal invasion, inflammation, fluid secretion, mucosal damage, histopathology, cytokine secretion and much more (22, 25). The cow model has provided invaluable insight into pathological processes involved in Salmonellosis both from the perspective of the host and the pathogen. Much of our understanding of the molecular mechanisms behind enteritis caused by non-typhoidal *Salmonella* is due to studies performed in calves. However, this model has not gained wide popularity among researchers due to constraints imposed by the size of the animal, the costs of housing and maintenance, and the lack of inbred or genetically modified strains of cows.

2. The murine model of *Salmonellosis*

The most popular animal model for studying *Salmonella* infection is the murine model. The relative ease of maintenance, the accessibility of well-established genetically inbred strains, and the availability of mutant strains make mice an extremely attractive model. One major caveat to the murine model of *S. typhimurium* infection however, is that the course of infection is very different from that seen in humans.

In mice, *S. typhimurium* causes a typhoid-like systemic disease with very little inflammation of the gastro-intestinal tract (25, 29). Although a streptomycin pre-treated

mouse model has been shown to exhibit symptoms more closely resembling human enteritis, the inflammation in these mice is restricted to the colon, and systemic disease remains a prominent feature in these mice (30-32). Despite the disparity between the human and mouse manifestations of *S. typhimurium* infections, the murine model is considered important for study because of its similarities to typhoid fever, and also because of its relevance for livestock infections since mice are considered a natural reservoir for *Salmonella* (33-36).

The LD_{50}, duration of the infection, titers in infected organs, and the general outcome of the disease all depend on the route of inoculation, the strain of bacteria used and genetic background of the mouse that was infected. Two popular mouse strainsm are C57BL/6 and Balb/c, both of which are susceptible to *S. typhimurium* infection and die following both intra-gastric (i.g.) and intra-peritoneal (i.p.) infection with strain 14028s (LD50 ~10^6 i.g., LD100 < 10^1 i.p.). The presence of natural resistance associated macrophage protein-1 (Nramp1) plays a crucial role in determining the susceptibility of mice to *Salmonella* infection (37). C57BL/6 and Balb/c mice do not have functional Nramp1. In contrast, 129sv mice, that have functional Nramp1, usually do not die following high-dose oral inoculation and instead develop a chronic infection that lasts up to 1 year, and possibly longer (36).

Although the natural route of infection is oral (or i.g.), i.p., intravenous, subcutaneous and other means of inoculating mice with *S. typhimurium* are frequently used. However only i.g. and i.p. inoculation will be discussed because those are the most common procedures. Following ingestion or i.g. inoculation, *Salmonella* passes through the stomach where it is capable of surviving the low pH environment. After passing

through the stomach, *S. typhimurium* enters the distal ileum and caecum and invades intestinal epithelial cells and M cells of Peyer's patches (25, 38, 39). *S. typhimurium* mainly gains access to deeper tissues through the M-cells where *Salmonella* is capable of surviving within and killing macrophages associated with Peyer's patches (39, 40). *S. typhimurium* then survives within phagocytic cells and disseminates to systemic sites of infection such as lymph nodes, the liver, and spleen in a process that takes approximately 24 hours. A second pathway for accessing systemic sites has also been described. In this pathway, within 30 minutes of oral inoculation, a relatively small number of *S. typhimurium* is transported to the systemic circulation within CD18 expressing monocytic cells (41). Regardless of the mechanism by which it reaches systemic sites of infection, *Salmonella* replicates within cells at these sites until a certain titer is achieved and then, in susceptible hosts, bacteremia ensues and the mouse becomes moribund and dies in a process that usually takes 6-10 days from the initial time of inoculation.

Following i.p. injection, the pathology of the disease is thought to follow a similar course as the systemic disease seen following i.g. inoculation, the main difference being that the intestinal phase involving interactions with Peyer's patches is circumvented. Due to the lack of the intestinal phase the course of infection is much shorter. Higher titers are achieved at systemic sites more rapidly, and as a result susceptible animals die within 3-5 days. Although i.p. inoculation does not represent the natural course of infection, i.p. inoculations are often preferable because there is greater overall consistency of the course of infection and also because it allows for isolated analysis of the systemic phase of the disease.

Murine models of Salmonellosis have allowed the study of the interaction of *S. typhimurium* with specific cell types during the course of infection. During the intestinal phase, *Salmonella* interacts primarily with the intestinal epithelial cells and M cells of Peyer's patches (25, 38, 39). During the systemic phase of infection *Salmonella* is found within monocytic cells, dendritic cells (DC), macrophages and neutrophils (36, 42-45). At earlier times of infection (1-2 days), most of the intracellular *Salmonella* at systemic sites is found within neutrophils (43), whereas at later times of infection macrophages are the major reservoir of intracellular bacteria (36, 44). Despite the association with neutrophils at early stages of infection, the prevailing dogma is that the preferred site of systemic replication is within macrophages (46). In addition, researchers interested in immune responses and in vaccine development have identified *in vivo Salmonella* interactions with DCs, T-cells, and to a lesser extent B-cells (45, 47, 48). *Salmonella* has been shown to interact with and invade practically every cell type that has been investigated.

In both susceptible and resistant hosts, lesions can be seen containing *Salmonella* in the infected organs, such as the spleen and liver (36, 42-45). Although the bacterial titers indicate evidence of extensive bacterial replication and the majority of the replication is thought to occur in an intracellular compartment, most infected cells only contain a single bacterium (49). This apparent lack of evidence for intracellular replication *in vivo* may be partially explained if cells containing multiple bacteria die as a result of infection. In support of this hypothesis, dying macrophages and neutrophils have been observed in infected mouse tissues, and these are the cell types associated with bacterial replication (40, 43, 44).

Studies involving the murine model of Salmonellosis often involve identification of bacterial virulence factors through different screening techniques. Various methods have been developed to identify specific *Salmonella* mutants that are attenuated for virulence. Although initial studies involved testing mutants one at a time, new technologies have allowed for the development of strategies to identify attenuated mutants when large numbers of mutants are simultaneously injected into mice (50-53). These mutant screens rely on the concept of competitive infection using mixed infections. In a competitive infection of a mouse, attenuated mutants will be absent or present at significantly lower titers at systemic sites of replication when competing against non-attenuated strains. This is because they are not able to compete for niches for replication. Competitive infection can be used to test a collection of mutants or to individually test a single mutant verses a reference strain allowing for very sensitive detection of *in vivo* virulence defects.

B. Cell culture models of *Salmonella* infection

Studying *Salmonella* infection in cell culture infection models has provided much insight into the molecular mechanisms underlying different aspects of disease. The study of *Salmonella* infection in cell culture generally is divided into two categories: the study of interactions with epithelial cells and the study of intracellular survival in phagocytic cells.

In vitro infection of epithelial cells, such as the HeLa cells, represents a specific aspect of the intestinal phase of *Salmonella* infection: the invasion of the intestinal epithelium. This model has allowed for in-depth analysis of how *Salmonella* manipulates host cell processes during the invasion of epithelial cells. Much of what is known about

how bacterial and host factors interact at a molecular level during the invasion of epithelial cells is due to infection studies performed in cultured epithelial cells (54).

Macrophage infection models represent a specific aspect of the systemic phase of infection: survival within phagocytic cells. Infection of macrophages, such as the J774 macrophage-like cell line, has helped identify many *Salmonella* virulence factors required for replication and survival within vacuolar compartments of these cells. These virulence factors contribute to changes in infected macrophages that promote bacterial survival within these cells. Furthermore, *Salmonella* mutants that are attenuated for growth in cultured macrophages are also attenuated in mouse infections, therefore this model has allowed efficient identification of bacterial factors required for virulence in the mouse model (55).

Some groups have also developed cell culture models that involve multiple cell types in order to study how *Salmonella* affects interactions between different types of host cells. For example, one model allows the study of how *Salmonella* infection induces neutrophil trafficking across polarized epithelial cells (56). This model uses polarized T-84 cells that are seeded on trans-wells. Following infection of the polarized epithelial layer, neutrophils are added to the trans-wells and then the rate of transepithelial migration is measured by determining the number of neutrophils that migrate across the epithelial cell layer. Using this model, factors involved in eliciting neutrophil migration across *Salmonella* infected polarized epithelial cell layers have been identified and investigated.

Cell culture models are an indispensable tool for isolating and analyzing specific aspects of *Salmonella* infection. However, it is important to recognize that cell cultures

are performed under artificial conditions and the host cells are usually immortalized cells with many abnormal properties. Despite these important caveats, cell culture models will continue to provide invaluable insight into the host cell and bacterial factors involved in the infectious process.

IV. The Role of Type III Secretion in *Salmonella* pathogenesis

A. An overview of type III secretion in *Salmonella*

Salmonella possess many virulence factors, however, the type III secretion system (T3S) is arguably the most fascinating. T3S are virulence factors found in many plant and animal Gram-negative bacterial pathogens. *Salmonella typhimurium* has two T3S encoded in *Salmonella* pathogenicity island-1 (SPI-1) and *Salmonella* pathogenicity island-2 (SPI-2). The T3S encoded in SPI-1 (T3S-1), plays a pivotal role during the intestinal phase of infection while the T3S encoded in SPI-2 (T3S-2) is essential for the systemic phase of the disease (54, 57, 58).

T3S is a *sec* independent secretion system meaning that the *sec* genes, involved in the general secretory pathway, are not required for type III protein export. Although some components of the secretion apparatus require *sec* for assembly in the inner and outer membranes of the bacteria, export of proteins by the secretion system itself is *sec* independent and relies on a T3S specific ATPase associated with the bacterial inner membrane for its energy source. The type III secretion system is composed of approximately 20 proteins (Figure 1-2). A subset of these proteins form pores in the inner and outer bacterial membranes and form a needle like structure that spans the periplasm and projects from the bacterial surface. This needle apparatus is the minimum structure required for export of proteins into the culture medium. The tip of the needle apparatus

makes contact with target host cells and another group of proteins, called the translocon or translocases, form a pore in the host cell membrane (58, 59).

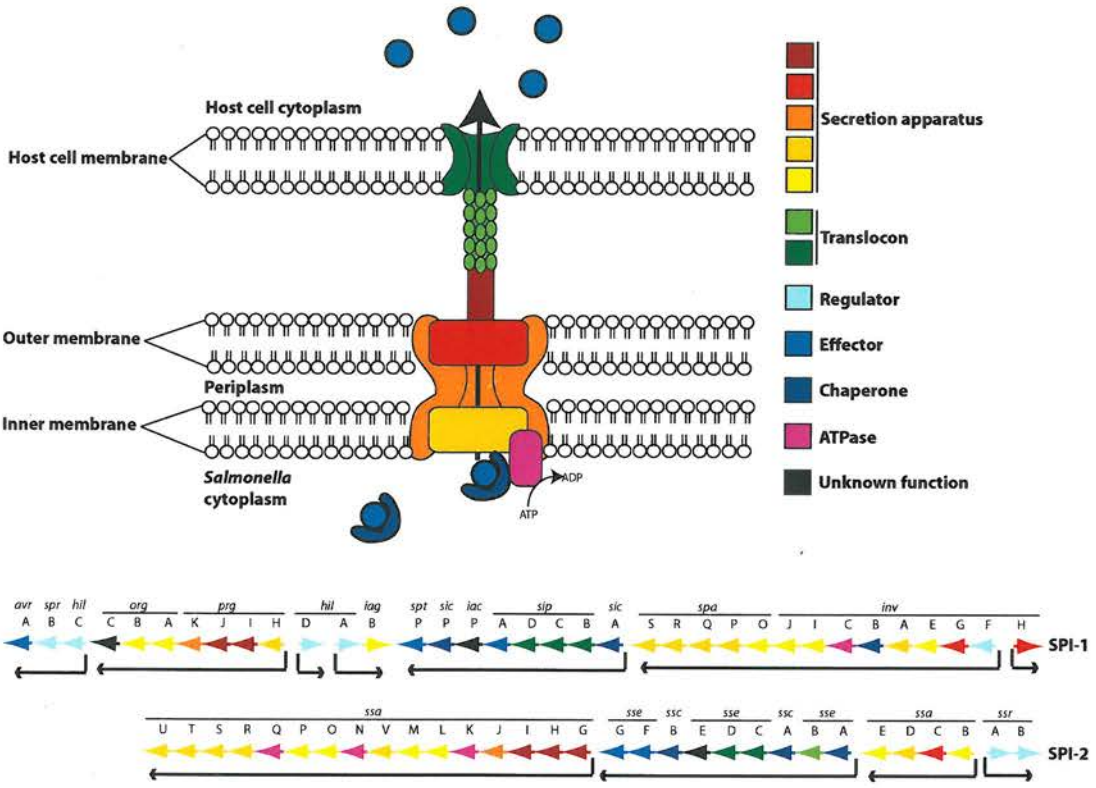

Figure 1-2. Schematic representation of the *Salmonella* pathogenicity island-1 and *Salmonella* pathogenicity island-2 type III secretion systems. Genetic maps of the SPI-1 and SPI-2 T3Ss are shown at the bottom with colors of the genes, represented by arrowheads, corresponding to the colors of the proteins in the diagram of the secretion apparatus above. The thin black lines with arrowheads indicate genes within operons that share the same promoter. [The illustration of the apparatus was based on a diagram from Kuhle and Hensel (58) and additional references were used to gather information for this figure (54, 59-63)]

The needle complex and translocon enable the bacteria to directly inject virulence factors into the cytoplasm of host cells. The injected virulence proteins, the so-called effectors, interact with targets in the host cell cytosol and disrupt normal host cell processes. These secreted effectors subvert host cell function in order to promote bacterial invasion and overall survival within the host. *Salmonella* has many known secreted effector proteins as well as several chaperone proteins that are required for the export of effectors. The regulation of *Salmonella*'s T3Ss in response to different environmental signals ensures that T3S-1 and T3S-2 will be active during appropriate stages of infection and will be described in the following sections. In addition, the ways in which T3S-1 and T3S-2 effectors contribute to virulence will be discussed in further detail.

B. The role of the SPI-1 encoded type III secretion system

T3S-1 is thought primarily to play a role in the gastric phase of Salmonellosis. In cultured epithelial cells, the presence of T3S-1 and some of its effectors is required for a dramatic membrane ruffling phenotype that facilitates bacterial entry into these cells (54). Consequently, T3S-1 is required for efficient invasion of M-cells *in vivo* (64) and T3S-1 mutants are avirulent in calf infection studies (65-67). In bovine models of *Salmonella* infection T3S-1 also plays an essential role in eliciting cytokine release by the intestinal epithelium and inducing neutrophil influx into the intestinal ileum (30). In cell culture models, infection of T84 epithelial cells with *Salmonella* can direct neutrophil trafficking across a polarized epithelial cell layer in a T3S dependent manner (56). Additionally, infected phagocytes can undergo apoptosis in a T3S-1 dependent manner, promoting dissemination of the bacteria from initial sites of infection (68-70). Therefore, it appears

that T3S-1 plays a critical role in mediating the invasion of the intestinal epithelium, in enhancing the subsequent development of an inflammatory response, and in dissemination to secondary sites of infection or to other hosts (see Figure 1-3).

1. Regulation of T3S-1

a. Regulators encoded in SPI-1

The regulation of T3S-1 involves a complex network of regulators encoded both within and outside SPI-1. For this review, I am only providing a brief summary of the major regulators and how these regulatory systems relate to the infectious process. A recent review by Altier provides a complete overview of the known SPI-1 regulators (60). Four transcriptional regulators are encoded within SPI-1: HilA, HilD, HilC and InvF (Figure 1-2). HilA is the central regulator involved in activating T3S-1 expression, and activates expression of the *prg* and *inv* operons (71-75). HilA activation also results in the activation of a second regulator, InvF. InvF in turn controls the expression of secreted effectors and translocases encoded in the *sip* operon within SPI-1, as well as SopB an effector encoded elsewhere in the chromosome (73, 74, 76). The two remaining regulators encoded in SPI-1, HilD and HilC/SprB are members of the AraC/XylS and LuxR/UhaP family of transcriptional regulators (2, 77-79). HilC and HilD are both capable of regulating *hilA* gene expression, however only HilD is indispensable for cell invasion (77, 78).

b. Regulators encoded outside of SPI-1

In addition to the transcriptional regulators encoded within SPI-1, regulators encoded elsewhere in the chromosome can activate or repress T3S-1 expression. One two-component regulator not encoded in SPI-1, BarA/SirA, activates *hilA* expression and

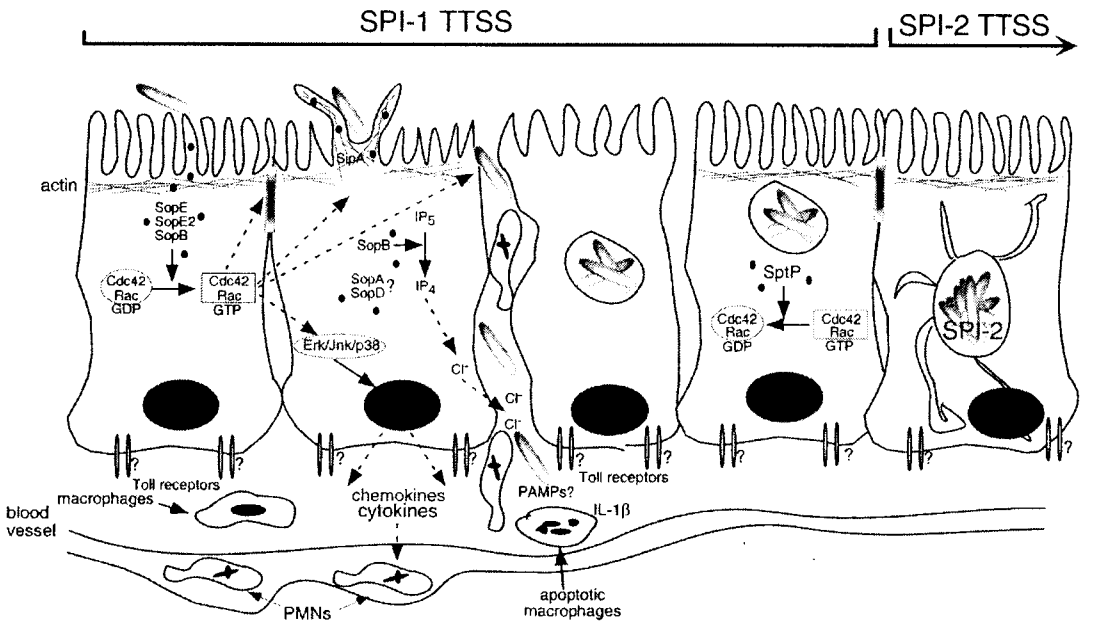

Figure 1-3. Model for *Salmonella*-induced enteropathology. Upon interaction with the brush border of the intact intestinal epithelium, *Salmonella* delivers a battery of effector proteins through its SPI-1 type III secretion system (T3S). Among these effectors are the guanine nucleotide exchange factors SopE and SopE2 and the inositol phosphatase SopB, which activate the Rho-family GTPases Cdc42 and Rac. Activation of these GTPases leads to a series of downstream events that result in actin cytoskeleton rearrangements, MAP kinase (Erk, Jnk, and p38) activation, and the destabilization of tight junctions. The actin cytoskeleton rearrangements, further modulated by the actin-binding protein SipA, result in bacterial uptake. The stimulation of Erk, Jnk, and p38 results in nuclear responses leading to the production of a variety of chemokines, which attract neutrophils to the site. The destabilization of tight junctions opens a paracellular pathway for bacterial penetration and allows neutrophil transmigration. The depolarization of the intestinal epithelium may expose Toll receptors, presumably located in the basolateral side. Consequently, these receptors can be stimulated by a variety of bacterial products such LPS, lipoprotein, and flagellin (generally known as pathogen associated molecular patterns (PAMPs)), which will further amplify the inflammatory response. The ability of *Salmonella* to kill macrophages releasing IL-1β may also contribute to this process. SopB, through its inositol phosphatase activity, causes the accumulation of Ins(1,4,5,6)P4, which stimulates Cl- secretion. Two T3S-1 effectors, SopA and SopD, contribute to intestinal pathology via unknown mechanisms. In most cases, infection with *Salmonella* is self-limiting. In these cases, the activity of the GTPase-activating protein SptP, restoring of the integrity of the intestinal cell by reversing the activation of Cdc42 and Rac, may be crucial. Once inside the cell, expression of SPI-2 T3S is stimulated. Through its effector proteins, T3S-2 mediates the building of an intracellular niche permissive for *Salmonella* intracellular growth. [Reproduced from (54)]

is required for cell invasion (79, 80). BarA/SirA regulation of SPI-1 gene expression appears to act both directly through binding of *hilA* and *hilC* DNA as well indirectly through controlling CsrA/CsrB/CsrC expression. CsrA is a post-transcriptional regulator whose presence is required for normal SPI-1 expression but has the ability to destabilize RNA molecules required for SPI-1 expression when it is over expressed (81, 82). The presence of the untranslated CsrB and CsrC RNA molecules appear to suppress CsrA activity allowing for proper SPI-1 gene expression. It is not clear whether CsrA/CsrB/CsrC control of T3S-1 expression is direct or indirect, but this mechanism of post-transcriptional control most likely provides a mechanism to quickly respond to environmental changes and appropriately express or repress SPI-1 genes. Finally, another two-component regulator encoded outside SPI-1, PhoP/PhoQ, represses expression of SPI-1 genes through repression of *hilA* expression (83, 84).

c. Regulation of T3S-1 by environmental signals

Several environmental signals activate or repress SPI-1 gene expression. Low oxygen, high osmolarity, and neutral pH activate SPI-1 gene expression through HilA and acetate activates SPI-1 genes through SirA (84-88). Whereas low Mg^{2+} levels and cationic peptides repress SPI-1 genes through PhoP/PhoQ activation, bile represses SPI-1 gene expression through BarA/SirA (89-91). The incorporation of all these signals enables *Salmonella* to express T3S-1 at the appropriate physiological locale following oral ingestion. The preferred site of initial *Salmonella* colonization, the distal ileum, is a low oxygen, neutral pH environment, with relatively high levels of acetate and a low bile concentration. Activation of T3S-1 in this environment promotes invasion of M-cells and enterocytes. Repressing conditions such as limiting magnesium concentrations and

cationic peptides are associated with the intracellular environment of macrophages and other phagocytes, where SPI-1 expression does not normally occur. It is important to note that although T3S-1 is not likely to be active within acidified phagocytic vacuoles, that T3S-1 can still inject proteins into phagocytes from the extracellular environment or during early stages of cell invasion before the vacuole matures into an environment that would repress SPI-1 gene expression. In summary, this complex regulatory mechanism activates T3S-1 expression during invasion of the intestinal epithelium and allows T3S-1 to play a role in the initial interaction with phagocytes that are found in Peyer's patches. However during the later stages of infection where *Salmonella* is replicating within phagocytes, T3S-1 is not likely to be active.

Simply growing *Salmonella* in LB broth to the logarithmic phase of growth can activate T3S-1. The activation of SPI-1 genes in LB broth is dependent on the growth phase, as *Salmonella* grown to the stationary phase no longer display T3S-1 dependent phenotypes. Cell cultures infected with *Salmonella* grown to the logarithmic phase of growth display phenotypes associated with T3S-1 including invasion of non-phagocytic epithelial cells and rapid induction of apoptosis in macrophages (92, 93).

2. T3S-1 mediated invasion of epithelial cells

T3S-1's role in *Salmonella* invasion of epithelial cells is one of the most thoroughly investigated aspects of pathogenesis of any bacterial pathogen (Figure 1-3). Several reviews provide intimate details of the molecular actions of T3S-1 secreted effectors proteins and how these proteins mediate actin cytoskeleton rearrangements to facilitate *Salmonella* invasion of non-phagocytic cells (54, 94-102). The 'membrane ruffling' associated with *Salmonella* invasion of epithelial cells was first described in

1967 by Takeuchi (21). Although several genes from SPI-1 had been shown to be required for invasion of epithelial cells by 1989 (103), it was not until 1995 that Kaniga et al. formally recognized that SPI-1 genes encoding components of a T3S were the cause of this dramatic phenotype (104).

a. T3S-1 mediates actin rearrangements through Rho Family GTPases

T3S-1 secreted effectors proteins, SopE, SopE2, and SopB, target host cell signaling pathways that regulate actin polymerization (105). Injection of these proteins by T3S-1 results in localized membrane protrusions that engulf *Salmonella* and ultimately lead to internalization of the bacteria in a vacuolar compartment. SopE and SopE2 both directly target Rho family GTPases, which are key regulators of eukaryotic actin cytoskeleton dynamics (106). SopE and SopE2 act as Rho guanine nucleotide exchange factors (GEFs) and phosphorylate two Rho family GTPases, Cdc42 and Rac1 (107-111). Although SopE and SopE2 are closely related, they display different substrate preferences with SopE2 primarily acting through Cdc42, and SopE acting through both Cdc42 and Rac1 (107, 109).

SopB is an inositol phosphate polyphosphatase that indirectly activates the Rho family GTPases Cdc42 and RhoG by generation of second messengers through its processing of phosphoinositide phosphate (PIP) and inositol phosphate (IP) (105, 112-116). The generation of second messengers leads to activation of an SH3 containing GEF (SGEF), which in turn leads to activation of Cdc42 and RhoG, another Rho family GTPase central to *Salmonella* invasion (117). Recently, it has been discovered that SopB possesses the ability to rearrange actin even in the absence of its phosphatase activity. A

SopB mutant lacking phosphatase activity was still capable of disrupting normal actin dynamics in both yeast and mammalian cells (118). However, the impact of SopB's phosphatase independent actin modulation on *Salmonella* invasion has not been determined.

b. The role of specific Rho family GTPases in actin remodeling

Although it has long been held that activation of Cdc42 is crucial for *Salmonella* mediated invasion, these original results were obtained from experiments using dominant negative Rho family GTPases and are now disputed (109, 117, 119). In a recent study using RNAi to knockdown Cdc42, RhoG, and Rac1 expression, it was found that only RhoG and Rac1 activation are needed for *Salmonella* mediated actin rearrangements, whereas Cdc42 activation primarily leads to nuclear changes in infected cells (Figure 1-4) (117). (The nuclear changes associated with Cdc42 activation will be discussed in the section describing T3S-1's role in enhancing inflammatory responses.) The ability of SopE, SopE2, and SopB to activate Rho family GTPases is at least partially redundant as any single one of the three proteins is sufficient to induce actin remodeling and promote cell invasion by *Salmonella* (105, 120, 121). A *Salmonella* strain carrying mutations in all three of these genes is severely defective in its ability to invade cultured epithelial cells.

c. Recruitment of the Arp2/3 complex to promote actin nucleation

Salmonella T3S-1 mediated activation of Rho family GTPases appears to promote actin polymerization through either direct or indirect recruitment and activation of the Arp2/3 complex (110, 122-124). Cdc42 activation and phosphatidylinositol 4,5-

bisphosphate (a product of SopB activity) directly interact with the Wiskott-Aldrich syndrome protein (WASP) family protein, N-WASP, causing a conformational change in

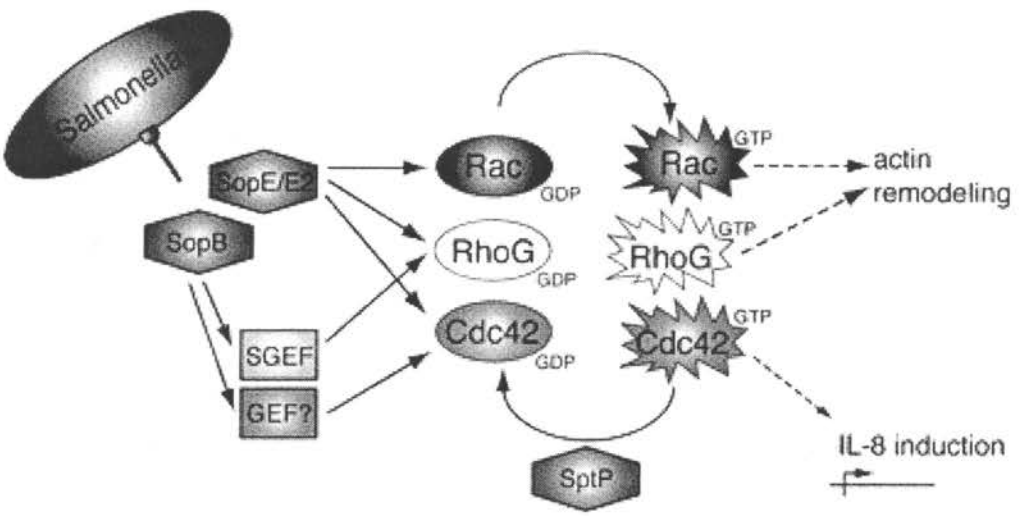

Figure 1-4. Model for *Salmonella* signaling to Rho family GTPases. Through T3S-1, *Salmonella* delivers SopE, SopE2, and SopB, which differentially activate different Rho family GTPase members either directly (SopE and SopE2) or indirectly via the stimulation of the endogenous exchange factor SGEF (SopB). Activation of the different Rho family GTPases leads to specific cellular responses, such as actin remodeling, and the stimulation of nuclear responses, such as the production of IL-8. Responses are subsequently reversed by the action of the T3S GTPase-activating protein effector protein SptP. [Reproduced from (117)]

N-WASP that promotes actin recruitment and activation of the Arp2/3 complex (125-127). Activation of Rac1 in *Salmonella* infected cells leads to an indirect interaction with suppressor of cAMP receptor/WASP family verprolin-homologous (SCAR/WAVE) family proteins (122, 123). Specifically, PIR121/Sra-1 is recruited by activated Rac1 and forms a large multi-protein complex with WAVE2. This PIR121/WAVE2 complex is known to stimulate Arp2/3 activity (128-130) and disruption of the PIR121/WAVE2 complex leads to a blockage to *Salmonella* invasion (123). Arp2/3 in turn promotes actin polymerization, or the organization of monomeric globular actin (G-actin) into filamentous actin (F-actin) (124). Presumably localized Arp2/3 recruitment and activation by T3S-1 secreted effectors leads to actin polymerization that contributes to membrane ruffle formation and *Salmonella* internalization.

d. Direct actin modulation by T3S-1 effectors

Despite Arp2/3's central role in mediating SopE, SopE2, and SopB actin rearrangements, blockage of Arp2/3 activity only results in a partial defect in *Salmonella* invasion (122). This is likely due to other T3S-1 secreted effector proteins that directly interact with actin: SipA and SipC. SipA lowers the critical concentration for F-actin formation by promoting F-actin stabilization by acting as a molecular staple that cross-links G-actin monomers (131, 132). Although a mutation in SipA does not appear to affect membrane ruffling, it significantly reduces the rate of epithelial cell invasion by *Salmonella* indicating that SipA appears to improve the efficiency of the internalization process (133). A second protein SipC is a component of the T3S-1 translocon that is associated with the host-cell membrane and has an additional function as an effector protein that binds to actin (134, 135). SipC has actin nucleating activity and may help

localize actin polymerization to sites of *Salmonella* contact. This nucleating activity is distinct from its translocase activity since a *Salmonella* strain containing a SipC mutation that retains its translocase activity but is impaired in actin nucleation still has a defect in invading epithelial cells (134).

e. SptP mediated reversal of membrane ruffling

Along with the initial observation of membrane ruffles on intestinal epithelial cells following *in vivo Salmonella* infection, Takeuchi found that the ruffling appeared to be reversible (21). Ruffles on *Salmonella* infected cells normally recover after around 2-3 hours of infection (95). However, infected cells fail to recover their actin cytoskeleton when infected with a *Salmonella* strain carrying a mutation in the gene encoding the secreted effector protein, SptP (136). SptP is a tyrosine phosphatase that has GAP (GTPase activating protein) activity for Cdc42 and Rac1 that reverses the GEF activities of SopE, SopE2 and SopB. Interestingly, the antagonistic affect of SptP on the activities of SopE, SopE2 and SopB still allows the activation of Rho family GTPases and actin polymerization at early stages of infection yet quickly reverses the membrane ruffling. The sequential activation and deactivation of Rho family GTPases following *Salmonella* infection is at least partially mediated by the rate of protein-turnover. SptP was found to have a significantly longer half-life than SopE following infection, indicating that delayed proteosome mediated degradation of SptP allows it to persist longer in the host cell (137). Additionally, SptP is secreted at low levels via T3S-2, indicating that it can continue to be secreted into cells during later stages of infection when T3S-1 is no longer active (138).

Many aspects of *Salmonella* invasion of epithelial cells have been characterized and the molecular mechanisms of several of the T3S-1 effectors responsible for promoting invasion have been elucidated. However, the way in which some T3S-1 secreted effectors contribute to *Salmonella* invasion of epithelial cells has not been determined. Recently two other effectors SopA and SopD have been shown to contribute to Salmonella infection of epithelial cells (139). However, the mechanism by which SopA and SopD contribute to invasion is unknown. Clearly, there remain aspects of *Salmonella* invasion that are not fully understood and warrant further investigation.

3. T3S-1 mediated intestinal inflammation

T3S-1 is involved in activating inflammatory responses that elicit intestinal neutrophil infiltration in animals infected with non-typhoid *Salmonella enterica* serovars (22, 30, 140). The T3S-1 effectors that are involved in the invasion process also contribute to *in vivo* inflammatory responses (141-145) and in cell culture experiments, *Salmonella* invasion results in secretion of pro-inflammatory cytokines (146, 147). In particular, epithelial cells secrete the CXC chemokine IL-8 during *Salmonella* infection, a chemoattractant that plays a central role in neutrophil recruitment. However, it is not clear whether these inflammatory responses are directly stimulated by effector-mediated mechanisms or indirectly through the invasion process itself.

a. Direct activation of inflammatory responses by T3S-1 effectors

Several effectors directly activate inflammatory pathways in *Salmonella* infected cells. For example, activation of Cdc42 and Rac-1 by SopE, SopE2, and SopB leads to activation of the mitogen activated protein (MAP) kinases Erk, Jnk, and p38 (119, 147).

Erk, Jnk, and p38 in turn activate transcription by Nf-κB and AP-1 and thereby induce production of cytokines such as IL-8 (146, 147). In addition, SopB activates Akt, another kinase that promotes Nf-κB activity (148). Furthermore, T3S-1 mediated activation of Rho family GTPases leads to disruption of tight junction integrity and may facilitate neutrophil transmigration across epithelial barriers (149, 150). Tight junction disruption may also expose basolateral surfaces of epithelial cells to bacterial products and help amplify innate inflammatory pathways. SopB also mediates production of D-myo-inositol 1,4,5,6–tetrakisphosphate (Ins(1,4,5,6)P4) that directly stimulates chloride secretion, another important factor in mediating the inflammation and diarrhea associated with gastroenteritis (112, 151, 152). Additionally, the production of Ins(1,4,5,6)P4 also contributes to loss of tight junction integrity possibly through activation of protein kinase C (PKC) (151).

In transepithelial neutrophil migration models, SipA has been shown to play a key role in mediating neutrophil migration across an infected polarized epithelial layer (153, 154). SipA activates PKC and pathogen elicited epithelial chemoattractant (PEEC) secretion, another chemokine involved in neutrophil transmigration (155).

b. Indirect activation of inflammatory responses through innate immune receptors

Salmonella invasion of epithelial cells also induces inflammatory responses through innate immune signaling pathways. Invasion leads to exposure of *Salmonella* pathogen associated molecular patterns (PAMPs), such as lipopolysaccharide, flagellin, fimbriae, and peptidoglycan, to innate immune receptors such as nucleotide binding oligomerization domain (NOD) receptors and toll like receptors (TLR) (156-159).

Recognition of these PAMPs by their cognate immune receptors leads to activation of inflammatory pathways and secretion of cytokines including IL-8. Although *Salmonella* has many PAMPs that are likely to activate NOD receptors and TLRs, the contributions of innate immune signaling mediated inflammation during *in vivo* infection has been difficult to ascertain because mutations in genes encoding PAMPs often have multiple virulence associated phenotypes (22). Therefore, I will only mention two of *Salmonella*'s PAMPs that clearly play a prominent role in stimulating inflammatory responses from intestinal epithelial cells. *Salmonella* flagellin and thin curled fimbriae are potent activators of innate signaling pathways that lead to IL-8 secretion (160-162). Polarized epithelial cells respond to *Salmonella* flagellin via TLR5 (161, 162). However, the contribution of flagellin to *in vivo* inflammation is difficult to determine because non-flagellated *Salmonella* mutants are defective for invasion (163-165). *Salmonella*'s thin curled fimbriae are recognized by TLR2 and a mutation in the genes encoding the thin curled fimbria, *csgA* and *csgB*, results in reduced inflammation during calf infection (160). In this case, *csgA* and *csgB* did not have any affect on *Salmonella* invasion of epithelial cells either *in vivo* or in cell cultures, indicating that the reduced inflammation is likely to be entirely due to the loss of TLR2 activation.

Inactivation of individual genes encoding SopE, SopE2, SopD, SopA, and SopB all lead to decreased inflammation in the calf infection model (141-145), indicating that these effectors may play a direct role in initiating inflammatory responses. However, mutations in each of these genes only results in partial loss of inflammation in infected calves when compared to a *sipB* mutant that is completely deficient in T3S-1 mediated secretion and invasion (141). This partial loss of inflammation is parallel to the partial

loss of *Salmonella* invasion of epithelial cells that occurs when these genes are individually mutated. This indicates that the loss of inflammation could be due to a loss of invasion and consequently a lack of activation of innate immune signaling pathways. However, both direct and indirect activation of inflammatory responses by effectors are probably significant and both directly and indirectly eliciting inflammatory responses likely make contributions that influence the outcome of *Salmonella* infections.

4. T3S-1 mediated cell death

The outcome of T3S-1 mediated cell invasion varies depending on the cell type *Salmonella* infects. Infected epithelial cells are resistant to apoptosis, whereas infected dendritic cells and macrophages undergo rapid death (within 1-2 hours) via a mechanism involving caspase-1 activation (69, 70, 166, 167). In infected epithelial cells, SopB mediated activation of Akt leads to resistance to induction of a caspase-3 mediated apoptotic pathway (166). This resistance to apoptosis may enable *Salmonella* to persist within epithelial cells for longer periods of time and allow more time for replication within a protected niche.

a. T3S-1 mediated caspase-1 dependent macrophage killing

In contrast to infected epithelial cells, macrophages and dendritic cells infected with logarithmic-phase cultures of *Salmonella* that are expressing T3S-1 undergo a rapid cell death triggered in part by activation of caspase-1 (92, 168-172). Caspase-1 activation is mediated by direct binding of a caspase-1 containing complex by SipB and SipB microinjection into macrophages is sufficient to induce caspase-1 dependent cell death (172). These findings indicate that SipB dependent cell death was not an indirect affect due to its role as a translocase. However, since SipB deficient *Salmonella* mutants are

incapable of translocating other effectors, it has not been possible to determine whether other T3S-1 secreted effectors contribute to the killing of infected cells.

There has been much controversy over the nature of the cell death induced by SipB activation of caspase-1. It is clear that T3S-1 mediated cell death is different from both apoptosis and necrosis, and instead occurs via a unique process called pyroptosis (173, 174). Pyroptosis is characterized by caspase-1 activation, DNA fragmentation, and formation of pores in the host cell membrane that cause osmotic lysis of the cell. Caspase-1 activates the inflammatory cytokines IL-1β and IL-18, both of which are secreted by macrophages infected with *Salmonella* (175-177).

Infection experiments using caspase-1 knock out mice have yielded contradictory results. In the first study, *Salmonella* was found to spread less efficiently to systemic sites following oral inoculation of caspase-1 deficient mice (40). This was interpreted as meaning that inflammatory responses mediated by caspase-1 are required for dissemination to systemic sites, most likely through recruitment of phagocytic cells via IL-1β and IL-18 secreted by dying macrophages. However, new studies have found that caspase-1 deficient mice are actually more susceptible to *Salmonella* infection with more inflammation observed in histological sections (178, 179). The reason for the disparity between these studies is unknown, but it is clear that caspase-1 activation plays a pivotal role in mediating cell death and inflammation in *Salmonella* infected animals.

b. SipB mediated caspase-1 independent macrophage killing

Interestingly, SipB is also capable of mediating a caspase-1 independent cell death. In one study, SipB was responsible for a caspase-2 and caspase-3 dependent delayed cell death (~4 hours) during *Salmonella* infection of macrophages isolated from

caspase-1 knockout mice (180). In another study, transfection of SipB into macrophages from caspase-1 knockout mice resulted in cell death via autophagy (181). In this study, SipB localized to mitochondria in transfected cells and induced mitochondrial damage. Evidence for authophagy was also found in *Salmonella* infected caspase-1 knockout macrophages and was completely independent of any caspase activation. Therefore, T3S-1 is capable of engaging both caspase-1 dependent and caspase-1 independent cell death pathways. Although the significance of these caspase-1 independent death mechanisms is not clear, it appears that co-evolution of *Salmonella* with its hosts has generated multiple pathways to promote cell death.

C. The role of the SPI-2 encoded type III secretion system

The SPI-2 locus was first identified as a *Salmonella* virulence factor required for mouse virulence and for survival within macrophages (182, 183). Subsequently, T3S-2 itself was shown to be required for replication at systemic sites in mice and replication in cultured macrophages (184, 185). Since this initial discovery, intense research has been undertaken to determine the mechanisms by which T3S-2 mediates *Salmonella*'s intracellular survival and virulence in mice. Several phenotypes in *Salmonella* infected cells and animals have been associated with T3S-2, including modulation of host cell processes in order to promote intracellular survival, inducing cell death in infected cells, and mediating inflammatory responses (reviewed in (58, 186)). New research is emerging that is just beginning to shed light on the molecular mechanisms by which T3S-2 effector proteins manipulate host cells (see Figure 1-5).

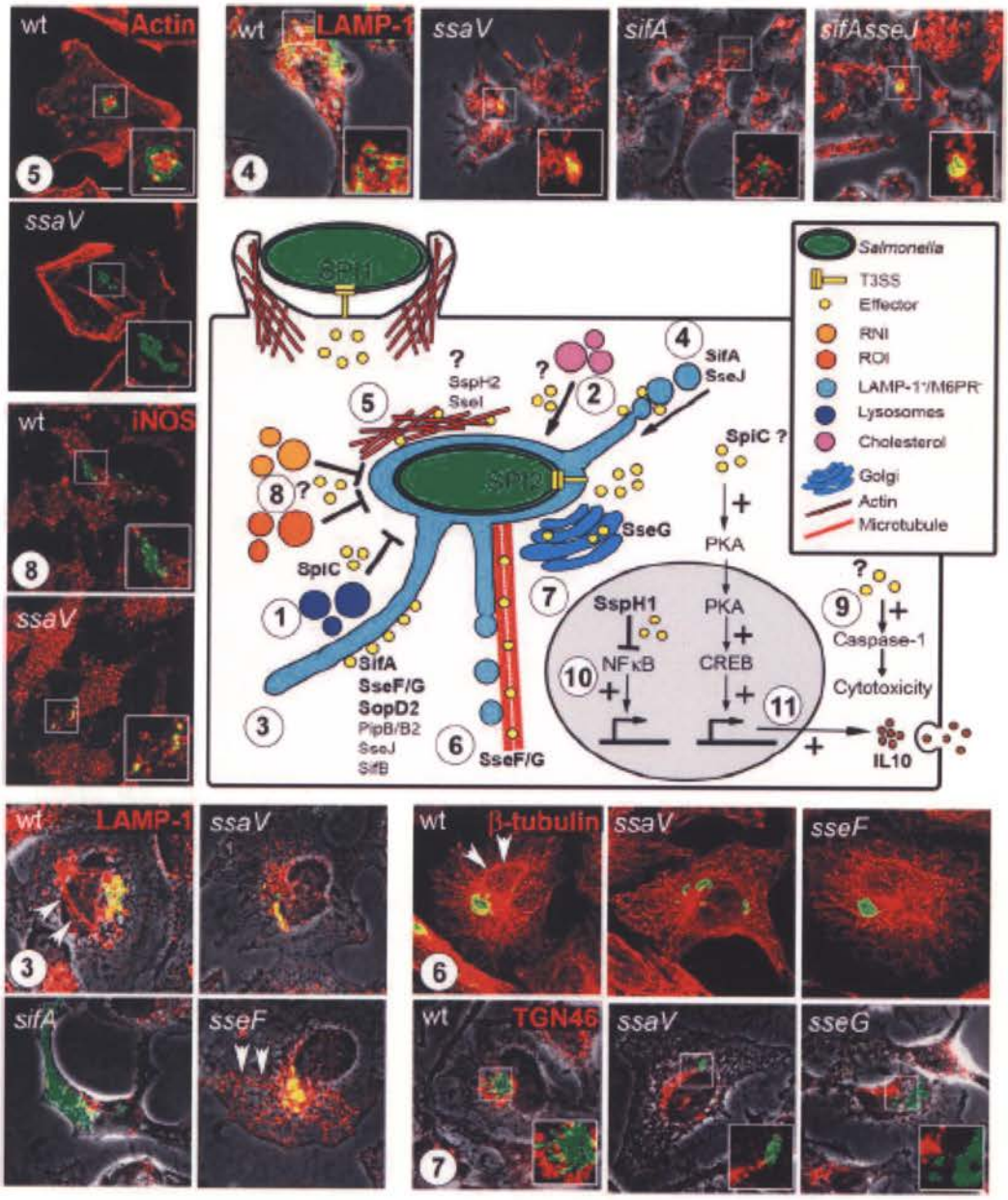

Figure 1-5. Cellular phenotypes associated with the function of T3S-2. The interactions of intracellular *S. enterica* with host cell functions via T3S-2 are shown in a model, and examples for representative host cell phenotypes after infection with *Salmonella* wildtype (wt) or relevant mutant strains are shown in micrographs. T3S-2 function interferes with a variety of different host cell processes: (1) modification of cellular trafficking and alteration of *Salmonella* containing vacuole (SCV) maturation; (2) recruitment of cholesterol (lipid rafts) to the SCV; (3) formation of *Salmonella*-induced filaments (SIF)

(indicated by arrows). Infection with an *ssaV* strain (lacking T3S-2 secretion) or *sifA* strain did not induce SIF in HeLa cells, while infection with the *sseF* strain results in 'pseudo-SIF' formation; (4) maintenance of SCV integrity by combined fusion and scission events. In the presence of SseJ, a *sifA*-deficient strain escapes the SCV and is killed or replicates in the cytoplasm of macrophages or HeLa cells, respectively; (5) actin accumulation in the vicinity of the SCV; (6) bundling of microtubules and associated SIF-formation; (7) association of the trans-Golgi network (TGN) with the SCV; (8) inhibition of delivery of reactive oxygen (ROI) and nitrogen intermediates (RNI) to the SCV; (9) delayed cell death; (10) inhibition of Nf-κB dependent gene expression and (11) induction of IL10 expression. Bold typeface indicates involvement of SPI2 effectors in the respective phenotype; regular typeface indicates localization of the effector to the respective compartment. For infection experiments shown in micrographs, RAW264.7 (4, 8) and HeLa cells (3, 5, 6, 7) were infected at a multiplicity of infection of 5 with the indicated strains expressing green fluorescent protein. The cells were fixed 16 h after infection and stained with the indicated primary antibodies and Cy3-coupled secondary antibodies. Actin was stained by Phalloidin-Texas-Red. Scale bars represent 8 *m*m. [Reproduced from (58)]

1. Regulation of T3S-2

a. Regulators involved in T3S-2 expression

The regulation of T3S-2 is not fully understood. A two-component regulatory system, SsrA/B, encoded within SPI-2 is essential for expression of T3S-2 genes, as well as effectors and other virulence factors encoded elsewhere on the chromosome (187-189) (Figure 1-2). However, the environmental signals detected by SsrA, the sensor kinase, are unknown. In addition, several global regulators regulate *ssrAB* expression in response to various environmental signals, including OmpR/EnvZ, PhoP/PhoQ, and SlyA (190-197). There is much debate as the nature of the signal to which PhoP, OmpR and SlyA respond. OmpR/EnvZ induces T3S-2 expression in low osmolarity, and low pH minimal media (195). Phosphorylated OmpR activates *ssrA/B* expression through direct binding to DNA in the *ssrA/B* promoter region (194). The PhoP/PhoQ system responds to low concentrations of magnesium and/or to the presence of antimicrobial cationic peptides (198-200). PhoP regulates SsrA expression post-transcriptionally by binding to *ssrA* mRNA (197). The mechanism of SlyA regulation of *ssrA/B* expression is poorly understood and is complicated by the fact that SlyA has functional overlap with both PhoP and OmpR (191, 192). *Salmonella* carrying a mutation in *slyA* is not as attenuated for virulence in a mouse infection as a mutant lacking T3S-2, indicating that SlyA is only partially required for T3S-2 gene expression (191). The mechanism by which environmental signals such as low pH, low inorganic phosphate, and low calcium activate T3S-2 expression is still under investigation.

b. Regulation of T3S-2 in response to environmental signals

All the environmental signals that activate T3S-2 expression have one thing in common; low pH, low magnesium, low calcium, low inorganic phosphate, low osmolarity and the presence of anti-microbial peptides are all conditions that are present in the intracellular environment of macrophages. Therefore, the complex network of signal transduction proteins that regulate T3S-2 work in concert to activate T3S-2 expression when *Salmonella* encounters the intracellular environment. However, a recent paper has disputed this strict dependency on an intracellular environment for T3S-2 expression. It was discovered that during mouse infections, a significant population of *Salmonella* expresses T3S-2 at very early stages of infection in the extracellular environment prior to penetrating the intestinal epithelium (201). However, it is unlikely that T3S-2 secretion takes place in the neutral to slightly alkaline pH environment of the intestinal lumen. This is because assembly of the T3S-2 secretion apparatus and secretion via T3S-2 requires an acidic environment such as the low pH environment of an intracellular vacuole (188, 202, 203). Therefore, it is likely that low level T3S-2 expression prior to entering cells enables immediate functional assembly of T3S-2 and rapid delivery of effectors once *Salmonella* is internalized by a phagocyte. In support of this hypothesis, our laboratory has recently identified a T3S-2 dependent phenotype that occurs in mice only 30 minutes after oral inoculation (this phenotype will be described in more detail in the discussion in Chapter 4).

Stationary phase cultures of *S. typhimurium* are used to perform T3S-2 dependent macrophage infections. Using stationary phase cultures for infecting macrophages, SsrB regulated gene expression is activated very rapidly (Heffron and Gustin, unpublished

results). However, we only detect T3S-2 dependent secretion after 8-10 hours of infection (138). Interestingly, one T3S-2 secreted effector, SseK2, has been identified that is only secreted into macrophages after 21 hours of infection (204). The significance of this late secretion phenotype and the mechanisms that regulate it are unknown.

2. Intracellular survival

Mediation of *Salmonella* survival in the *Salmonella* containing vacuoles (SCV) of infected cells is thought to be the primary function of the effectors secreted via T3S-2 (205). It has been shown that T3S-2 prevents SCV maturation into phagolysosomes thus maintaining a favorable intracellular environment for *Salmonella* survival (206). In addition, *Salmonella* evades the major oxygen dependent killing mechanisms of macrophages by T3S-2 mediated disruption of NADPH oxidase and iNOS trafficking to SCVs (207-210). Although the precise mechanism underlying these observations has yet to be determined, it is becoming apparent that they are likely to be a consequence of another interesting T3S-2 dependent feature in *Salmonella* infected cells called *Salmonella* induced filaments (Sifs) (Figure 1-5) (211-214).

a. The role of SifA in *Salmonella* induced filaments

T3S-2 controls SCV maturation in part by altering cellular trafficking and preventing SCV fusion with endosomal vesicles. A central component to mediating intracellular vesicle transport and fusion events appear to be the filamentous, tubular structures called Sifs that develop in *Salmonella* infected cells within hours of infection (213). The formation of Sifs requires the T3S-2 secreted effector, SifA (211, 214, 215). SifA deficient *Salmonella* strains are attenuated in mice and survive poorly in macrophages (212, 214). In addition, SifA mutants fail to form Sifs in infected cells, and

escape from the SCV into the host cell cytoplasm due to loss of vacuolar integrity (211, 214). Interestingly, SifA mutants actually replicate at a faster rate than wild-type *Salmonella* in epithelial cells, presumably because the cytoplasmic environment of epithelial cells is not as harsh as that of a macrophage (216, 217).

SifA on its own is sufficient to induce structures resembling Sifs in transfected cells (218). SifA also causes aggregation of lysosome associated membrane protein-1 (LAMP-1) containing compartments when transfected into host cells and mediates the fusion of LAMP-1 containing vesicles with the SCV during infection (218, 219). The method by which SifA mediates LAMP-1 vesicle fusion is unknown but it may be related to its interaction with microtubules.

SifA directs the formation Sifs along microtubules in infected cells (220). This association with microtubules is mediated in part by SifA dependent displacement of dynein and kinesin from SCVs. Dynein and kinesin are microtubule motor proteins that are required for accumulation of microtubules around SCVs and for Sif formation (221). SifA modulation of vesicle trafficking along microtubules also involves Rab7 activity, another protein that accumulates on SCVs (222). Rab7 interacts with Rab7-interacting lysosomal protein (RILP), which in turn recruits dynein to SCVs during early stages of infection. However as Sif formation progresses, SifA prevents the association of Rab7 with RILP and prevents the recruitment of dynein to SCVs. Additionally, SifA was found to interact directly with a host cell protein called SKIP (SifA and kinesin-interaction protein) which resulted in displacement of kinesin from the SCV (223). Displacement of kinesin and dynein by SifA may help maintain the integrity of the SCV by preventing

loss of vacuolar membrane since these proteins have been implicated in regulating membrane dynamics.

b. SseJ and SopD2 regulation of Sif dynamics

SseJ is another T3S-2 secreted effector protein that works in conjunction with SifA to regulate Sif formation (219). SseJ is required for full virulence in cell cultures as well as in mouse infections (219, 224). In infected cells, SseJ accumulates on SCVs and Sifs (224). Transfection of SseJ results in formation of globular membranous compartments (GMC) and can prevent Sif formation in *Salmonella* infected cells (219). This is consistent with the observation that SseJ is required to reverse Sif formation during later stages of epithelial cell infection (225, 226). Intriguingly, a *Salmonella* strain lacking both SseJ and SifA no longer escapes from the vacuole during infection, indicating that SseJ and SifA have complementary functions in maintaining the integrity of the SCV (226). SseJ has been shown to have deacyclase activity that is required for GMC formation and virulence (219, 226). However, the mechanism by which SseJ deacyclase activity regulates Sif formation is not known.

The T3S-2 secreted effector SopD2 enhances Sif formation and is required for full virulence in mice (227, 228). Infection of cells with a *Salmonella* strain lacking SopD2 results in impaired Sif formation (228). SopD2 is targeted to late endocytic compartments in transfected cells and also has a Golgi targeting domain (227, 229). In addition, it causes swelling of endocytic compartments when transfected into cells on its own (227). Interestingly, co-transfection of SopD2 with SifA results in enhanced formation of pseudo-Sifs. Currently the biological function of SopD2 remains poorly defined, and the mechanism by which it cooperates with SifA to enhance Sif formation is not known.

c. SseF and SseG couple SCV dynamics with the Golgi network

Two other T3S-2 secreted effectors, SseF and SseG, are both required for normal Sif formation in infected cells (230, 231). SseF and SseG deficient strains of *Salmonella* are weakly attenuated in the mouse infection model and grow poorly in cultured macrophages (232). SseF and SseG are both targeted to endosomal membranes and microtubules in host cells (230, 233) and they both function to position the SCV in proximity to the Golgi apparatus (234-236). This in an interesting observation considering that the Golgi network is closely associated with the microtubule organizing center and is intricately involved in regulating intracellular vesicle trafficking. SseF's ability to position SCVs near the Golgi is not well understood but it is dependent on its ability to recruit dynein to the SCV (234). The mechanism by which SseG positions the SCV by the Golgi is unknown, however, SseG was shown to localize to the Golgi network when transfected into cells (236).

d. The role of PipB2 in altering trafficking of endosomal vesicles

Two more T3S-2 effectors that share homology with each other, PipB and PipB2, contribute to Sif formation and localize to detergent resistant micro-domains (cholesterol enriched plasma membranes also known as lipid rafts) found in SCVs and Sifs (237). PipB is not required for mouse infections; however, it contributes to intestinal inflammation in calf infections (238, 239). A *Salmonella* strain lacking PipB2, on the other hand, was slightly attenuated in the mouse infection model (237). The results from these virulence studies suggest that PipB2 plays a more prominent role in systemic

survival and intracellular survival in macrophages than PipB. In addition to localization on the SCV and Sifs, PipB2 localizes to late endosomal/lysosomal compartments that traffic away from the SCV along microtubules and accumulate in the periphery of infected cells. Subsequently, it was shown that PipB2 transfection on its own could cause accumulation of late endosomal/lysosomal compartments in the periphery of cells (240). This PipB2 dependent process acts through Rab7 and Rab34 by an unknown mechanism. The biological function of PipB has yet to be characterized, but it is clear that its behavior is distinct from that of PipB2, as it does not accumulate in late endosomal/lysosomal vesicles.

e. The role of SpiC in mediating vesicle trafficking and fusion

Another T3S-2 translocated protein, SpiC, prevents normal endosomal vesicle trafficking and fusion, and disrupts normal Golgi morphology when transfected into cells (206, 241). SpiC interacts with the host protein Hook3, a protein implicated in vesicular trafficking events due to its interactions with microtubules and the Golgi network (242). SpiC also interacts with another host protein called TassC, another protein implicated in vesicle trafficking (243). Interestingly, depletion of TassC from host cells allows a SpiC deficient strain of *Salmonella* to survive within macrophages at levels comparable to wild type *Salmonella*. However, the functions of Hook3 and TassC remain poorly defined and it is not clear how SpiC interaction with these proteins prevents vesicle trafficking and fusion events. Despite the fact that a SpiC mutant strain of *Salmonella* is highly attenuated in mice, fails to form Sifs, and fails to disrupt vesicular trafficking in infected cells, it is not clear whether this is a direct affect of SpiC, or an indirect affect because

SpiC is required for translocon assembly and therefore is required for secretion of other T3S-2 effectors (244, 245).

f. T3S-2 mediated vacuole associated actin polymerization

In addition to modulating host cell microtubules, T3S-2 mediates actin rearrangements in *Salmonella* infected cells. In *Salmonella* infected cells, vacuole-associated actin polymerization (VAP) occurs in the vicinity of the SCV and is thought to help maintain the stability of the SCV (246). Development of these structures is completely independent of T3S-1 mediated actin rearrangements and require the presence of T3S-2. T3S-2 mediated VAP formation also requires a secreted protein, SpvB, encoded on the *Salmonella* virulence plasmid (247). Although it has been proposed that SpvB is a substrate for T3S-2 secretion (183, 232), one study found that *in vitro* SpvB secretion is independent of both T3S-1 and T3S-2 (248). However, it seems likely that SpvB is in fact a substrate for T3S-2 since VAP formation in infected cells is absolutely dependent the presence of both T3S-2 and SpvB. T3S-1 and T3S-2 independent secretion of SpvB *in vitro* could be an artifact resulting from secretion via the flagellar export mechanism. The flagellar export system is a specialized type III secretion system and some substrates for T3S-1 and T3S-2 are capable of secretion through the flagellar export pathway (and vice versa). However, effector proteins secreted via the flagellar pathway presumably do not have access to the host cell cytosol because the translocon proteins are not present. Therefore, more work is needed to conclusively determine whether SpvB is a genuine substrate for T3S-2. That being said, SpvB is an attractive candidate for mediating T3S-2 dependent VAP formation since it ADP ribosylates actin and disrupts the actin cytoskeleton when transfected into cells (249). Furthermore, the ADP

ribosylation activity of SpvB is required for mouse virulence. SpvB activity mediates F-action depolymerization through actin degradation and is required for reducing Sif formation during later stages of host cell infection (225, 250).

Another T3S-2 secreted protein, SspH2, has also been implicated in VAP formation (247). SspH2 is not involved in mouse virulence, but enhances the formation of intestinal lesions in calves (247, 251). Although SspH2 was not essential for VAP formation, its characteristics suggest that it is likely to play an important role in VAP formation because SspH2 binds filamin, an actin cross-linking protein, and profilin, a molecule that enhances actin polymerization (247). Further studies showed that SspH2 and filamin localize to VAPs in infected cells. Additionally, it was shown that SspH2 inhibits actin polymerization *in vitro*. The search for other proteins that might have overlapping function with SspH2 identified the T3S-2 effector SrfH (also called SseI) as another protein that interacts with filamen. However, SrfH does not have an affect on overall mouse virulence and does not affect VAP formation (219, 247). It is not clear whether SrfH is involved VAP formation, and new research from our laboratory indicates that it is involved in a completely different aspect of virulence (as will be discussed in Chapter 4).

Great progress has been made in understanding the mechanisms that underlie T3S-2 mediated intracellular survival. Although the precise pathways by which T3S-2 alters NADPH oxidase and iNOS localization has not been determined, it appears that these phenotypes are due to disruption of vesicle trafficking and fusion events. Sif formation, involving rearrangements of microtubules and actin, seems to be essential for mediating these vesicle trafficking and fusion events. Manipulation of the Golgi network

also appears to be involved in regulating microtubule organization and vesicle trafficking. Overall, much work remains to be performed in order to further elucidate the complex mechanisms involved in intracellular *Salmonella* survival.

3. T3S-2 mediated cell death

Infecting macrophages with stationary-phase cultures of *Salmonella* that are not expressing T3S-1 results in a delayed T3S-2 mediated cell death (after ~24 hours) (176, 252, 253). Although T3S-2 dependent macrophage killing is not as well characterized as T3S-1 mediated cytotoxicity, T3S-2 mediated killing requires caspase-1 activation and involves secretion of IL-1β (176), and therefore it is likely to involve a similar mechanism as the pyroptosis that has been described for T3S-1 mediated cell killing.

One study has found that triggering innate immune responses through TLR4 signaling is required for *Salmonella* mediated delayed macrophage killing (254). However, T3S-2 meditated cell death does not simply result from activation of innate death pathways as a consequence of intracellular bacterial growth, since *Salmonella* mutants that have reduced intracellular growth but express T3S-2 are still capable of killing macrophages (252). Rather, T3S-2 secreted effector(s) most likely influence the outcome of innate signaling events in such a way as to favor the activation of a caspase-1 mediated death process.

The identity of T3S-2 effectors involved in this process has remained elusive. However, the virulence plasmid-encoded virulence factor, SpvB, is a secreted protein that is involved in T3S-2 induced macrophage cytotoxicity (255). Micro-injection of SpvB into macrophages is sufficient to induce cell death (256). The mechanism by which SpvB induces cytotoxicity is not known, however it appears to be linked to its ability to

depolymerize actin. As mentioned earlier it is disputed whether or not SpvB is a substrate for T3S-2 secretion, but once again it has been implicated in a T3S-2 dependent phenotype in *Salmonella* infected cells.

4. T3S-2 mediated inflammatory responses

a. Contribution of T3S-2 to *in vivo* inflammatory responses

T3S-2 contributes to *Salmonella* induced inflammatory responses in both the calf model and streptomycin pre-treated mouse model (31, 257, 258). It is not clear whether T3S-2 directly mediates inflammatory responses or whether the inflammation is a consequence of intracellular replication and the resulting production of bacterial products capable of stimulating innate immune receptors (such as NOD receptors and TLRs). It was recently reported that T3S-2 mediated inflammation in streptomycin pretreated mice requires the presence of MyD88, a protein required for innate signaling through TLRs (257). This suggests that T3S-2's role in mediating inflammation is primarily to prolong PAMP production and trigger innate immune responses.

T3S-2 does not contribute to the T3S-1 mediated intestinal inflammation seen after 24 hours of *S. typhimurium* infection in calves (258). Instead, T3S-2 mediated inflammation was evident in calves only after 5 days of infection, suggesting the need for a prolonged exposure to bacterial products in order to trigger inflammation. Interestingly, the level of inflammation seen after 5 days was not affected in a T3S-1 mutant *Salmonella* strain, indicating that T3S-2 on its own appears to be capable of inducing late inflammatory responses in cows (258).

b. T3S-2 mediated inhibition of inflammatory pathways

T3S-2 mediates changes in gene expression in infected cells that are likely to have anti-inflammatory effects (259, 260). At least two effectors secreted via T3S-2, are capable of mediating changes in infected cells that are anti-inflammatory: SspH1, and AvrA. SspH1 is secreted via both T3S-1 and T3S-2; however, it is not involved in T3S-1 mediated intestinal inflammation (141, 251). Instead, SspH1 has been implicated in T3S-2 mediated inhibition of Nf-κB dependent gene expression (260). SspH1 is targeted to the host cell nucleus and interacts with a host cell protein called PKN1. This interaction leads to inhibition of Nf-κB dependent gene expression via an unknown mechanism (261). AvrA is an effector encoded within SPI-1 that is secreted by both T3S-1 and T3S-2 (138, 141). Like SspH1, AvrA is not involved in T3S-1 mediated inflammation in calves (138, 141). Coincidentally, AvrA also has been shown to inhibit Nf-κB dependent gene expression via an unknown mechanism (262). Interestingly, infecting macrophages with *Salmonella* induces T3S-2 dependent expression of host cell proteins including the cytokine IL-10, cyclooxygenase-2 (COX-2), and suppressor of cytokine signaling 3 (SOCS-3), all of which are thought to have anti-inflammatory properties (259, 263, 264). However, the effectors involved in inducing IL-10, COX-2 and SOCS-3 expression have not been identified.

Intiguingly, T3S-2 is involved in attenuating inflammatory pathways in cultured cells yet is required for triggering inflammation *in vivo*. It is not clear that these two phenotypes are directly linked. However, one possibility is that T3S-2 mediated inhibition of inflammatory pathways can allow the *Salmonella* to penetrate deeper tissues and reach higher titers before triggering inflammation. Then when inflammatory

responses are eventually activated they are amplified because of the high levels of PAMPs that were generated. Another important consideration is the T3S-2 mediated activation of caspase-1 mediated cell death that leads to production of inflammatory cytokines. However, it is not known whether T3S-2 mediated activation of caspase-1 is involved in the delayed inflammation that is observed in calves.

V. Research objectives

When my project began in the spring of 2001, very little was known about T3S-2 secreted effectors. Although a few effectors had been identified, none of them had been characterized to any significant extent. In addition, several T3S-2 mediated phenotypes in *Salmonella* infected cells, such as inhibition of iNOS and NADPH oxidase function, had been described but the effectors responsible for these phenotypes had not been identified.

We hypothesized that effectors responsible for T3S-2 dependent phenotypes had yet to be identified and devised a new strategy to screen for secreted effector proteins. The manuscript in Chapter 2 (138) describes the strategy we used to successfully identify three new effector proteins. In this study we also found that effectors have different secretion patterns. Some are secreted only by T3S-1, some are secreted only by T3S-2, and some are secreted by both T3S-1 and T3S-2. We wished to explore this differential secretion phenotype further, because we felt it could shed light on the roles of T3S-1 and T3S-2 during *Salmonella* infecion.

Although the existing paradigm is that T3S-1 is only involved in the intestinal phase of Salmonellosis, and T3S-2 is primarily involved in the systemic phase of disease, one recent study found that T3S-1 was required for systemic survival in a chronic mouse infection model (265). We decided to investigate the *in vivo* secretion patterns of

effectors that are differentially secreted by T3S-1 and T3S-2 by analyzing spleen cells from an infected mouse. We wished to determine if T3S-1 was active at a systemic site and whether T3S-1 and T3S-2 target different cell types. We hypothesized that effectors secreted by T3S-2 would target mostly phagocytic spleen cells and if T3S-1 is active in the spleen, it could target both phagocytic and non-phagocytic cells. The manuscript in Chapter 3 describes the findings from our efforts to identify the *in vivo* targets of *Salmonella*'s T3Ss.

Chapter 2

Identification of New Secreted Effectors in *Salmonella enterica* Serovar Typhimurium

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Abstract

A common theme in bacterial pathogenesis is the secretion of bacterial products that modify cellular functions to overcome host defenses. Gram-negative bacterial pathogens use type III secretion systems (T3Ss) to inject effector proteins into host cells. The genes encoding the structural components of the type III secretion apparatus are conserved among bacterial species and can be identified by sequence homology. In contrast, the sequences of secreted effector proteins are less conserved and are therefore difficult to identify. A strategy was developed to identify virulence factors secreted by *Salmonella enterica* serovar Typhimurium into the host cell cytoplasm. We constructed a transposon, which we refer to as mini-Tn*5*-cycler, to generate translational fusions between *Salmonella* chromosomal genes and a fragment of the calmodulin-dependent adenylate cyclase gene derived from *Bordetella pertussis* (*cyaA'*). In-frame fusions to bacterial proteins that are secreted into the eukaryotic cell cytoplasm were identified by high levels of cyclic AMP in infected cells. The assay was sufficiently sensitive that a single secreted fusion could be identified among several hundred that were not secreted. This approach identified three new effectors as well as seven that have been previously characterized. A deletion of one of the new effectors, *steA* (*Salmonella* translocated effector A), attenuated virulence. In addition, SteA localizes to the *trans*-Golgi network in both transfected and infected cells. This approach has identified new secreted effector proteins in *Salmonella* and will likely be useful for other organisms, even those in which genetic manipulation is more difficult.

Introduction

Pathogenic bacteria interact with host cells to create unique niches for replication and dissemination. Bacterial pathogens modify their host cells via the expression of exotoxins, proteases, and several other factors that are required for virulence. To alter the host cell, bacterial virulence factors must reach a host target. The ability of bacterial proteins to gain access to the host cell cytoplasm is often a critical step in pathogenesis. There are several defined mechanisms by which this secretion and subsequent uptake can take place. Bacterial proteins can be auto-transported, they can pass through the general secretory pathway, or most important from the standpoint of virulence, they can be secreted by one of several specialized mechanisms found in pathogenic bacteria. Many gram-negative bacterial pathogens encode type III secretion systems (T3Ss), syringe-like macromolecular complexes, to directly inject proteins into the host cell (57, 266-268). The structural genes encoding the T3S "needle complex" are conserved among bacterial pathogens and appear to have been acquired through horizontal gene transfer. This high degree of homology has facilitated their identification through genome sequencing and analysis. In contrast, the secreted effector proteins (EPs) are often species specific, lack a consensus secretion signal, and have been difficult to identify.

Salmonella enterica serovar Typhimurium encodes two T3Ss on separate pathogenicity islands. *Salmonella* pathogenicity island 1 (SPI-1) encodes a T3S (T3S-1) that is responsible for mediating the intestinal phase of *Salmonella* infection (54, 97). T3S-1 is highly expressed during late log phase in media that are relatively rich and contain high levels of salt, conditions that are thought to simulate the environment in the

small intestine (84). SopE, SipA, SptP, and AvrA are effector proteins secreted via T3S-2, and they promote the invasion of epithelial cells and enhance inflammation (54, 97, 141, 262, 269, 270).

A second T3S, encoded by *Salmonella* pathogenicity island 2 (SPI-2), is essential for the systemic phase of infection (271). This secretion system is expressed under nutrient-starved conditions (including low magnesium and low pH) that may mimic the intracellular environment encountered by *Salmonella* (184, 187, 193, 272). The expression of the structural components of the secretion apparatus and many of its secreted proteins is controlled by a two-component regulatory system encoded within SPI-2 by the *ssrA/B* genes (53, 182, 183, 189, 215). Many phenotypes in infected cells have been associated with this T3S. These phenotypes include delayed macrophage cytotoxicity, avoidance of oxidative burst, and altered inducible nitric oxide synthase (iNOS) localization (208, 210, 252, 271). However, the secreted virulence factors responsible for producing these phenotypes have yet to be identified. Further elucidation of EPs in *S. enterica* serovar Typhimurium may reveal the mechanisms responsible for these and other phenotypes.

An extremely useful technique has been developed to investigate the secretion of EPs. Sory et al. used the amino-terminal adenylate cyclase domain of the hemolysin/adenylate cyclase toxin (CyaA) from *Bordetella pertussis* as a tool to demonstrate type III secretion of EPs in *Yersinia enterocolitica* (273, 274). The adenylate cyclase domain is contained within the first 400 amino acids of CyaA and is called CyaA'. CyaA' activity is entirely dependent on host cell calmodulin and is thus inactive within the bacterial cell. Adenylate cyclase activity is therefore only observed when

CyaA' is translocated into host cells as part of a translational fusion to a secreted EP. The secretion of fusion proteins can thereby be easily monitored by measuring the levels of cyclic AMP (cAMP) in infected cells.

For this study, we adapted the reporter system developed by Sory et al. (274) for use in the construction of a EZ::TN (Epicenter) (275) derived transposon called mini-Tn*5*-cycler. Mini-Tn*5*-cycler mutagenesis was used to introduce translational fusions to CyaA', thereby identifying secreted effectors by assaying cAMP levels in infected cells. The technique is sensitive because the assay detects secreted fusions even if they constitute <0.5% of the bacteria used to infect cells. The method is versatile, requiring only electroporation of a transposon/transposase complex into the target organism and no other genetic manipulation. Using this method, we identified three previously uncharacterized *S. enterica* serovar Typhimurium secreted effectors. One of these localizes to the *trans*-Golgi network (TGN) and is required for the colonization of mouse spleens following intraperitoneal infection.

Materials and methods

Bacterial strains, tissue culture, and growth conditions.

The strains and plasmids used for this study are listed in Table 2-1. *Salmonella enterica* serovar Typhimurium strain 14028s was used as the wild-type (WT) strain. Bacteria were grown at 37°C in Luria-Bertani broth (LB). Kanamycin was used at 60 μg ml–1. Chloramphenicol was used at 30 μg ml^{-1}. Carbenicillin was used at 100 μg ml^{-1}. Tetracycline was used at 20 μg ml^{-1}. HeLa cells and J774 macrophages were obtained from the American Type Culture Collection. Cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum, sodium

Table 2-1. Strains and plasmids used for this study

Strain or plasmid	Genotype	Source or reference
Strains		
14028s	WT *S. typhimurium*	ATCC
HH100	14028s *steC*::mini-Tn5-cycler fusion at amino acid 447	This work
HH101, 104	14028s *steA*::mini-Tn5-cycler fusion at amino acid 174	This work
HH102, 105, 106, 109, 110, 111-114, 116-123, 126	14028s *slrP*::mini-Tn5-cycler fusion at amino acid 123	This work
HH107	14028s *steC*::mini-Tn5-cycler fusion at amino acid 428	This work
HH108	14028s *pipB2*::mini-Tn5-cycler fusion at amino acid 176	This work
HH115	14028s *sptP*::mini-Tn5-cycler fusion at amino acid 307	This work
HH124, 125	14028s *pipB2*::mini-Tn5-cycler fusion at amino acid 305	This work
HH127	14028s *pipB2*::mini-Tn5-cycler fusion at amino acid 165	This work
HH128, 131	14028s *srfH*::mini-Tn5-cycler fusion at amino acid 149	This work
HH129	14028s *steC*::mini-Tn5-cycler fusion at amino acid 448	This work
HH130	14028s *steC*::mini-Tn5-cycler fusion at amino acid 369	This work

HH132	14028s *steB*::mini-Tn5-cycler fusion at amino acid 137	This work
HH133, 134	14028s *pipB2*::mini-Tn5-cycler fusion at amino acid 241	This work
HH135, 138	14028s *pipB2*::mini-Tn5-cycler fusion at amino acid 145	This work
HH136	14028s *sseJ*::mini-Tn5-cycler fusion at amino acid 168	This work
HH137	14028s *avrA*::mini-Tn5-cycler fusion at amino acid 252	This work
HH139	14028s *sseJ*::mini-Tn5-cycler fusion at amino acid 368	This work
HH140, 141, 142	14028s *sipA*::mini-Tn5-cycler fusion at amino acid 511	This work
HH200	MJW1835 *steC*::mini-Tn5-cycler fusion at amino acid 447	This work
HH201	MJW1835 *steA*::mini-Tn5-cycler fusion at amino acid 174	This work
HH202	MJW1835 *slrP*::mini-Tn5-cycler fusion at amino acid 123	This work
HH208	MJW1835 *pipB2*::mini-Tn5-cycler fusion at amino acid 176	This work
HH215	MJW1835 *sptP*::mini-Tn5-cycler fusion at amino acid 307	This work
HH228	MJW1835 *srfH*::mini-Tn5-cycler fusion at amino acid 149	This work
HH232	MJW1835 *steB*::mini-Tn5-cycler fusion at amino acid 137	This work
HH237	MJW1835 *avrA*::mini-Tn5-cycler fusion at amino acid 252	This work
HH239	MJW1835 *sseJ*::mini-Tn5-cycler fusion at amino acid 368	This work
HH240	MJW1835 *sipA*::mini-Tn5-cycler fusion at amino acid 511	This work
HH300	MJW1301 *steC*::mini-Tn5-cycler fusion at amino acid 447	This work
HH301	MJW1301 *steA*::mini-Tn5-cycler fusion at amino acid 174	This work
HH302	MJW1301 *slrP*::mini-Tn5-cycler fusion at amino acid 123	This work
HH308	MJW1301 *pipB2*::mini-Tn5-cycler fusion at amino acid 176	This work
HH315	MJW1301 *sptP*::mini-Tn5-cycler fusion at amino acid 307	This work
HH328	MJW1301 *srfH*::mini-Tn5-cycler fusion at amino acid 149	This work
HH332	MJW1301 *steB*::mini-Tn5-cycler fusion at amino acid 137	This work

HH337	MJW1301 *avrA*::mini-Tn5-cycler fusion at amino acid 252	This work
HH339	MJW1301 *sseJ*::mini-Tn5-cycler fusion at amino acid 368	This work
HH340	MJW1301 *sipA*::mini-Tn5-cycler fusion at amino acid 511	This work
MA6054	14028s *ara-907 araD 901*::MudJ	(276)
MJW1883	14028s *srfH::mini-Tn5-cycler* chromosomal *srfH-cyaA'* fusion at amino acid 145	This work
MJW1301	14028s *ssaK::cat*	This work
MJW1835	14028s *invA::cat*	This work
Δ*slrP*	14028s Δ*slrP*	This work
ΔsteA	14028s Δ*steA::kan*	This work
ΔsteB	14028s Δ*steB::kan*	This work
ΔsteC	14028s Δ*steC::kan*	This work
SteA-HA/14028s	14028s with chromosomal 2X HA tagged SteA	This work
Plasmids		
pMJW1753	pWSK29 + *cyaA'*	This work
pMJW1791	pMJW1753 + *lacZ* (pLacZ-CyaA')	This work
pMJW1810	pMJW1753 +*srfH* (pSrfH-Cycler)	This work
pMini-Tn5-cycler	pCRScript + mini-Tn5-cycler	This work
pEGFP-N1	EGFP transfection vector	Clontech
pSteA-EGFP	pEGFP-N1 + *steA*	This work
pSteA	pWKS30 + *steA*	This work
pNFB15	Template vector for making 2X HA tag with a kanamycin resistance cassette	Lionello Bossi

pyruvate, and nonessential amino acids and grown at 37°C with 5% CO2. All P22 transductions were performed as previously described (277). Transductants were streaked for isolation on LB agar containing 10 mM EGTA and then confirmed for smooth lipopolysaccharide and lack of pseudolysogeny by cross-streaking transductants against P22 on Evan's blue uranine plates.

Construction of *S. enterica* serovar Typhimurium mutant strains.

An *ssaK*::*cat* (MJW1301) strain was constructed by first cloning the *ssaK* open reading frame (ORF) using PCR and then introducing a chloramphenicol resistance cassette into the SepI site of the gene. This construct was moved into the suicide vector pKAS32 (278), and then the disrupted *ssaK* gene was reintroduced into strain 14028s as previously described (189). The construction of *invA*::*cat* is described elsewhere (252) and was transduced from SR-11 x 3014 into 14028s using P22 phage, resulting in strain MJW1835. *slrP*, *steA*, *steB*, and *steC* strains were constructed using the -red PCR-based gene deletion method (279) and were verified by PCR. All PCR primer sequences can be obtained upon request.

Construction of mini-Tn*5*-cycler transposon and mutagenesis using mini-Tn*5*-cycler transposomes.

The mini-Tn*5*-cycler transposon was constructed from the DICE II transposon (5). pDICE II was digested with EcoRI and XbaI and religated. A BamHI site downstream of the kanamycin cassette was then removed using a Quick Change site-directed mutagenesis kit (Stratagene). The *cyaA'* gene was PCR amplified from pMJW1753 and then cloned into the NdeI and BamHI sites. The resulting plasmid, pCycler, contains the completed mini-Tn*5*-cycler transposon. Mini-Tn*5*-cycler transposon/transposase

complexes were prepared as previously described (275). Transposon/transposase complexes were electroporated into *Salmonella* using the following electroporation conditions. Overnight cultures of *Salmonella* were diluted 1:100 in LB and grown at 37°C for 3 h with aeration. The culture was then pelleted and washed three times with ice-cold deionized water. Following the washes, the pellet was resuspended in 1/500 the original culture volume in ice-cold 10% glycerol. One to 3 µl of transposon/transposase complex was added to 70 µl of electrocompetent cells, which were transferred into 1-mm-gap electroporation cuvettes (BTX). For electroporation, an Electro Cell manipulator 600 (BTX) was used with the following settings: resistance, 2.5 kV; capacitance timing, 25 µF; resistance timing, 129 ; and charging voltage, 1.70 kV.

Creation of *srfH-cyaA'*, *steA-cyaA'*, *steB-cyaA'*, and *steC-cyaA'* fusions.

The *srfH* ORF was PCR amplified and cloned into pBluescript. This construct was mutagenized with the transposon in vitro, and in-frame fusions to *srfH* were identified by PCR and sequencing. In vitro mutagenesis of *srfH* was performed using an EZ::TN kit (Epicenter). The in-frame fusion was then cloned into the suicide vector pKAS32 and used for allelic exchange, as previously described (278), to generate the chrom-*srfH-cyaA'* strain MJW1883. To generate pMJW1753, *cyaA'* (bp 4 to 1233) was PCR amplified from a clinical isolate of *B. pertussis* and cloned into pWSK29 (280) under *lacp* control, with a GGG 5' extension to recreate the SmaI site. Cloning into this site creates a glycine linker. The *srfH* ORF and promoter were PCR amplified and cloned into the SmaI site of pMJW1753 to generate p*srfH-cyaA'*. As a control, *cyaA'* was fused to the carboxy-terminal end of the ß-galactosidase alpha peptide, generating p*lacZ-cyaA'*. The full-length *steA-cyaA'*, *steB-cyaA'*, and *steC-cyaA'* fusions were generated using the -red

recombination system (279). To generate PCR products for recombination, forward primers contained 40 bp from the carboxy terminus of the gene being targeted at the 5' end plus the sequence 5'-CTGTCTCTTATACACATCTCA-3', and reverse primers contained 40 bp downstream of the gene being targeted plus the sequence 5'-CTGTCTCTTATACACATCTGGT-3'. Primers containing overhanging 5' sequences specific for *steA*, *steB*, and *steC* were then used to amplify the mini-Tn*5*-cycler transposon using PCR. The PCR products were digested with DpnI, dialyzed, and then electroporated into 14028s/pKD46.

Screening for translocated proteins.

Libraries of 5,000 mini-Tn*5*-cycler insertions were made. Libraries were diluted in LB to approximately 500 to 1,000 CFU/ml based on optical density readings at 600 nm. One hundred microliters of diluted library was grown in each well of a 96-well plate. Each well was then used to infect J774 cells (using SPI-2 conditions) or HeLa cells (using SPI-1 conditions) seeded in 96-well plates. If infection resulted in at least a 10-fold increase in cAMP levels, then the pool of mini-Tn*5*-cycler insertions from the 96-well plate was diluted and plated to isolate individual colonies. One hundred fifty to 300 colonies (three times the original pool size) were isolated using toothpicks, patched, and numbered. Numbered colonies were grouped into pools of 10 and then used to reinfect J774 or HeLa cells. If infection resulted in increased cAMP, then the colonies from that group of 10 mini-Tn*5*-cycler insertions were retested individually. Individual colonies with adenylate cyclase activity were transduced using P22 and then retested. Isolates that maintained adenylate cyclase activity following transduction were processed for sequencing.

Bacterial infection of cultured cells and ELISAs.

Unless otherwise stated, J774 or HeLa cells were plated in 96-well plates at 2 x 104 cells/well and incubated overnight at 37°C with 5% CO2. For the infection of J774 cells under SPI-2 conditions, stationary-phase bacteria were added at a multiplicity of infection (MOI) of 250. Bacteria were centrifuged onto the cell monolayer at 200 x *g* for 5 min and then incubated at 37°C with 5% CO2 for 1 h. The cell culture was then washed twice with phosphate-buffered saline (PBS), DMEM supplemented with 100 μg ml−1 gentamicin was added, and the culture was incubated for another hour. After 1 h, the culture was washed twice with PBS, overlaid with DMEM containing 10 μg ml−1 gentamicin, and incubated for another 7 to 9 h. For SPI-1-dependent infections of J774 and HeLa cells, stationary-phase cultures of 14028s were diluted 1:33 in LB and grown with aeration at 37°C for 3 h. Bacteria were then added to J774 cells at an MOI of 50, centrifuged onto the monolayer at 200 x *g* for 5 min, and incubated for 1 h. HeLa cells were infected at an MOI of 150, centrifuged at 200 x *g* for 5 min, and incubated for 1.5 to 2 h. Following infections, cells were washed once with PBS and then lysed with 0.1 M HCl. The level of cAMP in the lysates was determined using a direct cAMP enzyme-linked immunosorbent assay (ELISA) kit (Assay Designs) according to the manufacturer's instructions. In all cases, the MOI refers to the amount of bacteria initially added to host cells. The actual number of bacteria entering host cells was between 1 and 5% of the initial inoculum.

Sequencing of mini-Tn*5*-cycler insertion sites and sequence analysis.

Chromosomal DNA was prepared from isolated mini-Tn*5*-cycler mutants as previously described (281). Chromosomal DNA was digested with EcoRI and cloned into

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Unless otherwise stated, J774 or HeLa cells were plated in 96-well plates at 2 x 104 cells/well and incubated overnight at 37°C with 5% CO2. For the infection of J774 cells under SPI-2 conditions, stationary-phase bacteria were added at a multiplicity of infection (MOI) of 250. Bacteria were centrifuged onto the cell monolayer at 200 x *g* for 5 min and then incubated at 37°C with 5% CO2 for 1 h. The cell culture was then washed twice with phosphate-buffered saline (PBS), DMEM supplemented with 100 μg ml−1 gentamicin was added, and the culture was incubated for another hour. After 1 h, the culture was washed twice with PBS, overlaid with DMEM containing 10 μg ml−1 gentamicin, and incubated for another 7 to 9 h. For SPI-1-dependent infections of J774 and HeLa cells, stationary-phase cultures of 14028s were diluted 1:33 in LB and grown with aeration at 37°C for 3 h. Bacteria were then added to J774 cells at an MOI of 50, centrifuged onto the monolayer at 200 x *g* for 5 min, and incubated for 1 h. HeLa cells were infected at an MOI of 150, centrifuged at 200 x *g* for 5 min, and incubated for 1.5 to 2 h. Following infections, cells were washed once with PBS and then lysed with 0.1 M HCl. The level of cAMP in the lysates was determined using a direct cAMP enzyme-linked immunosorbent assay (ELISA) kit (Assay Designs) according to the manufacturer's instructions. In all cases, the MOI refers to the amount of bacteria initially added to host cells. The actual number of bacteria entering host cells was between 1 and 5% of the initial inoculum.

Sequencing of mini-Tn*5*-cycler insertion sites and sequence analysis.

Chromosomal DNA was prepared from isolated mini-Tn*5*-cycler mutants as previously described (281). Chromosomal DNA was digested with EcoRI and cloned into

the EcoRI site of pACYC184. Plasmids containing chromosomal inserts were electroporated into GeneHogs competent cells (Invitrogen), and insertions harboring chromosomal fragments with mini-Tn*5*-cycler were selected on LB agar supplemented with kanamycin. Plasmids from kanamycin-resistant colonies were then purified using a QIAprep spin miniprep kit (QIAGEN). The DNA sequence of the fusion junction was obtained using the primer 5' GTTGACCAGGCGGAACATCAATGTG 3', which is complementary to bp 166 to 190 of the 5' end of mini-Tn*5*-cycler. Sequence analysis was performed using MacVector 7.1.1 software and the NCBI BLAST server at http://www.ncbi.nlm.nih.gov/BLAST/.

Competitive infection studies.

Competitive infections were based on a protocol described by Ho et al. (276). Each strain was grown overnight in LB at 37°C with aeration. The bacteria were pelleted, resuspended in PBS, and diluted in PBS to approximately 2,000 to 20,000 CFU/ml. Each test strain was mixed 1:1 with the reference strain MA6054, and 100 µl of the mixture was injected intraperitoneally into 6- to 8-week-old female BALB/c mice. Three days after injection, the mice were sacrificed, and their spleens were harvested and homogenized. Spleen suspensions were diluted and plated on LB plates containing X-Gal (5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside; 40 µg/ml) and arabinose (1 mM). The reference strain MA6054 has arabinose-inducible ß-galactosidase activity and can be easily distinguished from the test strains when plated on LB agar with X-Gal and arabinose. The competitive index (CI) was then calculated using the following equation: (percentage of test strain recovered/percentage of reference strain recovered)/(percentage of test strain inoculated/percentage of reference strain inoculated). Student's *t* test was

performed to analyze the CIs. Complementation of *steA* was achieved by cloning the entire *steA* ORF and 62 bp upstream of the start codon into the low-copy-number expression vector pWKS30. The resulting plasmid, p*steA*, was electroporated into the *steA* strain.

Expression of SteA-EGFP and SteA-HA in HeLa cells and visualization by microscopy.

To make SteA-enhanced green fluorescent protein (SteA-EGFP), *steA* was PCR amplified and cloned into pEGFP-N1 (Clontech). The resulting plasmid, pSteA-EGFP, and pEGFP-N1 were purified using a QIAGEN EndoFree Maxi kit. HeLa cells were grown to 25 to 50% confluency on Lab-Tek II chambered cover glass (Nalge Nunc International) and were transfected for 24 h using FuGENE 6 transfection reagent (Roche). Bodipy-TR-ceramide (Molecular Probes) was used to stain the Golgi network in live cells following the manufacturer's recommendations. A chromosomal SteA-hemagglutinin (SteA-HA) fusion was constructed using the -red recombination system as described by Uzzau et al. (282). To make a double-HA-tagged SteA, the plasmid pNFB15 (received from Lionello Bossi) was used as a template for PCR using the following primer pair: 5' CGACATAAAAGCTCGCTACCATAACTATTTGGACAAT-TATTATCCGTATGATGTGCCGGA 3' and 5' CTGATTTCTAACAAAACTGGCTAA-ACATAAACGCTTTTTACACCTGCAGATCATCGAGCT3'. The PCR product generated from these primers was introduced into 14028s/pKD46 via electroporation, and transformants were selected on LB agar containing kanamycin. The SteA-HA fusion was verified by PCR and Western blotting. SPI-1 conditions (described above) were used to infect confluent HeLa cells on cover glass in six-well plates with SteA-HA-expressing

14028s and WT 14028s, using an MOI of 100. Bacteria were centrifuged onto the cell monolayer, and the infection was allowed to proceed at 37°C for 20 min. After this incubation, the cells were washed three times with PBS, and DMEM supplemented with 100 μg ml^{-1} gentamicin was added for 1 hour and then replaced with DMEM supplemented with 10 μg ml^{-1} gentamicin for the remainder of the 4-hour infection. Bodipy-TR-ceramide (Molecular Probes) was used to stain the TGN, and then the cells were fixed in 4% paraformaldehyde for 20 min. A mouse anti-HA monoclonal antibody (Covance) was used at a 1:100 dilution, and an Alexa Fluor 488-conjugated goat anti-mouse (Molecular Probes) secondary antibody was used at a 1:1,000 dilution. The DNA stain DRAQ5 (Alexis Biochemicals) was used at a 1:1,000 dilution to visualize host cell nuclei and bacteria. A 60x oil-immersion, 1.4-numerical-aperture objective lens was used along with standard filter sets for EGFP and Alexa Fluor 488 (488 nm), Texas Red (568 nm), and DRAQ5 (685 nm) visualization. *z* sections (0.2 μm) were captured at a resolution of 1,024 by 1,024 pixels. Images were acquired by Aurelie Snyder of the OHSU-MMI Research Core Facility (http://www.ohsu.edu/core) with an Applied Precision DeltaVision image restoration system. This includes an API chassis with a precision motorized XYZ stage, a Nikon TE200 inverted fluorescence microscope with standard filter sets, halogen illumination with an API light homogenizer, a CH350L camera (500 kHz, 12-bit, 2 Mp, KAF 1400 GL, 1,317 x 1,035, liquid cooled), and DeltaVision software. Deconvolution using the iterative constrained algorithm of Sedat and Agard and additional image processing were performed on an SGI Octane workstation. Images were processed for deconvolution using Softworx (Applied Precision) image processing software.

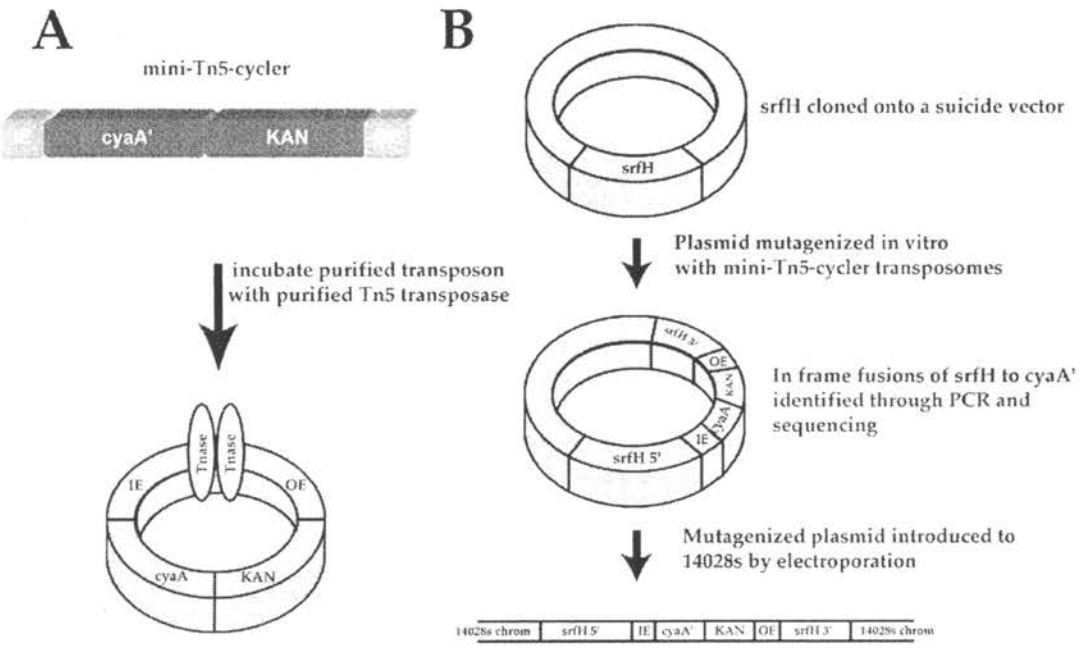
Results

Construction of mini-Tn*5*-cycler transposon.

The mini-Tn*5*-cycler transposon (shown in Figure 2-1A) is a modified EZ::TN (Epicenter)-based transposon. One advantage of this transposon is that stable transposon/transposase complexes can be prepared that can then be introduced to recipient bacteria by direct transformation of chemically competent or electrocompetent bacteria (275). The transposition reaction requires magnesium ions supplied from the recipient cell cytoplasm to complete the reaction, resulting in insertions in the recipient DNA. Alternatively, the complete reaction may be carried out in vitro, and the recombinant DNA can then be introduced directly into the desired bacterium. This last method of transposition allows for the generation of DNA insertions within genes of bacteria that are not usually amenable to such genetic manipulation, and this procedure can be further extended to yeast and mammalian cells. Thus, this construct can be utilized in many pathogenic organisms, making it an important tool for the identification of secreted virulence factors. The basis for the identification of secreted *Salmonella* virulence factors is that the mini-Tn*5*-cycler transposon contains a promoterless *cyaA'* gene, oriented to allow the construction of translational fusions with external genes.

Functional analysis of mini-Tn*5*-cycler mutagenesis.

To confirm that mini-Tn*5*-cycler transposition could result in functional *cyaA'* gene fusions, *srfH* (also called *sseI*), an *S. enterica* serovar Typhimurium gene encoding an effector secreted by T3S-2 (189, 215, 283), was cloned into a suicide vector and


A
mini-Tn5-cycler
IE
cyaA'
KAN
incubate purified transposon
with purified Tn5 transposase
Tnase
Tnase
IE
OE
cyaA
KAN
mini-Tn5-cycler transposome
B
srfH cloned onto a suicide vector
srfH
Plasmid mutagenized in vitro
with mini-Tn5-cycler transposomes
srfH 3'
OE
KAN
cyaA
IE
srfH 5'
In frame fusions of srfH to cyaA'
identified through PCR and
sequencing
Mutagenized plasmid introduced to
14028s by electroporation
14028s chrom
srfH 5'
IE
cyaA'
KAN
OE
srfH 3'
14028s chrom
Kanamycin resistant colonies following electroporation either have an
integrated plasmid with two copies of srfH after a single crossover
or have a single copy of srfH with a cyaA' fusion after a double cross
over, as shown above. Double crossovers were identified by PCR

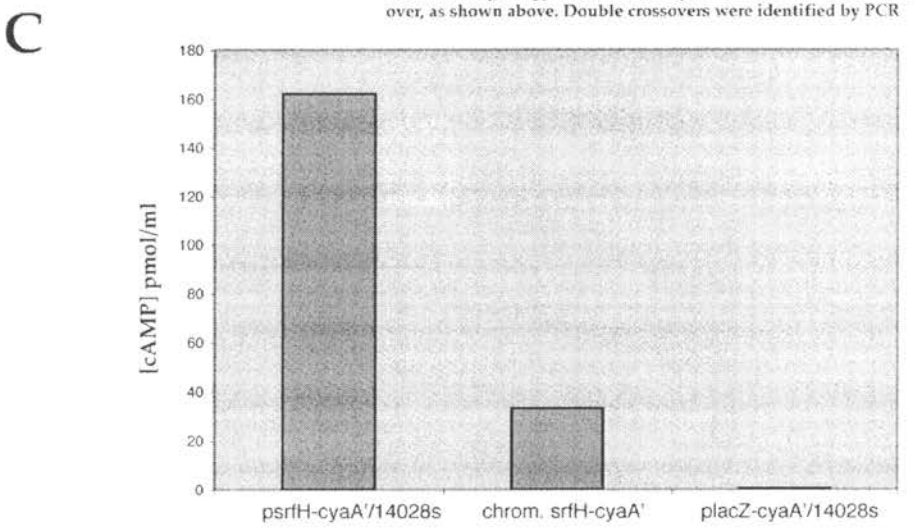

C
[cAMP] pmol/ml
0
20
40
60
80
100
120
140
160
180
psrfH-cyaA'/14028s
chrom. srfH-cyaA'
placZ-cyaA'/14028s

Figure 2-1. Schematic representation of the mini-Tn*5*-cycler transposon (A) and mutagenesis of *srfH* (B). IE and OE are the modified Tn*5* transposon ends (also called mosaic ends (275)). *cyaA'* is the promoterless 400 amino acids of the amino terminus of *cyaA* from *Bordetella bronchiseptica* (273, 274). KAN represents the Tn*903* aminoglycoside phosphotransferase. To test our system, *srfH* was cloned into pBluescript (B). The purified plasmid was mutagenized in vitro using mini-Tn*5*-cycler transposon/transposase complexes (transposons) by the addition of 5 mM magnesium. Plasmids containing insertions were selected on LB-kanamycin plates, and in-frame insertions in *srfH* were identified by PCR and sequencing. An in-frame fusion of *srfH-cyaA'* at the codon encoding amino acid 145 was then cloned into the suicide vector pKas32. The suicide vector containing an in-frame insertion of mini-Tn*5*-cycler was then electroporated into 14028s. Crossover events were selected on LB-kanamycin, and a double crossover was identified by PCR. (C) Secretion by *srfH::mini-Tn5-cycler*. Stationary-phase cultures of *srfH::mini-Tn5-cycler*, a mutant expressing a fusion from a low-copy-number plasmid (p*srfH-cyaA'*), or a mutant expressing a fusion of CyaA' to the ß-galactosidase alpha peptide (p*lacZ-cyaA'*) were used to infect J774 macrophages for 8 h at an MOI of 1. Infected cells were lysed with 0.1 M HCl, and the concentration of cAMP (pmol/ml) in the lysates was measured by ELISA.

mutagenized with mini-Tn*5*-cycler in vitro (Figure 2-1B). An in-frame chromosomal *srfH::mini-Tn5-cycler* allele was created. This strain was used to infect J774 macrophages under growth conditions in which T3S-1 is repressed and T3S-2 is induced (252). The level of cAMP in the infected cells was then measured by ELISA. Using an input MOI of 1, which results in <5% of cells being infected, we observed a >30-fold increase in host cell cAMP over the background levels when J774 macrophages were infected with *srfH::mini-Tn5-cycler* (Figure 2-1C). Background levels of cAMP were detected in cells infected with either WT 14028s or a strain expressing a ß-galactosidase-*cyaA'* (p*lacZ-cyaA'*) in-frame fusion from a low-copy-number vector (Figure 2-1C). Approximately 160-fold higher levels of cAMP were observed if a *srfH-cyaA'* fusion was expressed from a low-copy-number plasmid vector (p*srfH-cyaA'*) (Figure 2-1C). Secretion of the SrfH-CyaA' fusion protein did not appear to significantly increase the level of macrophage cell death during the course of an 8-h assay (data not shown). We wished to establish if a mixed infection containing a minority of the hybrid fusion-expressing bacteria and a majority of bacteria that do not express *cyaA'* could be used. This would allow us to screen large pools of mutagenized bacteria rather than having to screen the bacteria one by one, which is an impossible task. For control experiments, we used a mixed infection containing *srfH::mini-Tn5-cycler* at various ratios with the parent strain. The dilution of *srfH::mini-Tn5-cycler* with a 200-fold excess of wild-type 14028s cells still resulted in a 10-fold increase in cAMP levels in infected J774 cells (data not shown). These results demonstrate that a single in-frame fusion to a secreted EP can be detected among 200 proteins that do not express *cyaA'*. To make the assay even more sensitive, we tried varying the input MOI and found that even an MOI of 500 bacteria per cell was tolerated

and further increased the detected cAMP levels.

Library construction and analysis.

The strategy used to identify secreted effectors is shown in Figure 2-2. Mini-Tn*5*-cycler transposon/transposase complexes were electroporated into *S. enterica* serovar Typhimurium strain 14028s to create libraries containing approximately 5,000 independent insertions. These bacteria were mixed together, the number of bacteria was determined by measuring the optical density, and the bacteria were then diluted into wells of a 96-well microtiter dish so that the wells contained pools of 50 to 100 bacteria. These pools were either grown overnight to stationary phase and used to infect J774 macrophages for 8 to 10 h at an input MOI of 250 or grown to logarithmic phase and used to infect HeLa cells for 2 h at an input MOI of 150. Following infection, cells were lysed with 0.1 M HCl, and the concentration of intracellular cAMP was determined. The bacteria corresponding to any well showing at least a 10-fold increase in cAMP above background levels were replated for the isolation of individual colonies. From these colonies, smaller and smaller pools were constructed until individual positive clones were obtained. The transposon in each positive clone was P22 transduced to a new background, retested, and processed for DNA sequencing to identify the transposon-*Salmonella*-chromosome junction.

Six libraries were generated from independent electroporation reactions containing a total of 30,000 insertions. The majority of these were screened for *cyaA'* secretion in infected J774 macrophages. After screening these insertions, we identified a total of 23 positive signals, of which 17 were fusions to the known secreted effector *slrP*. Sequence analysis demonstrated that all *slrP* insertions had occurred at the same nucleotide

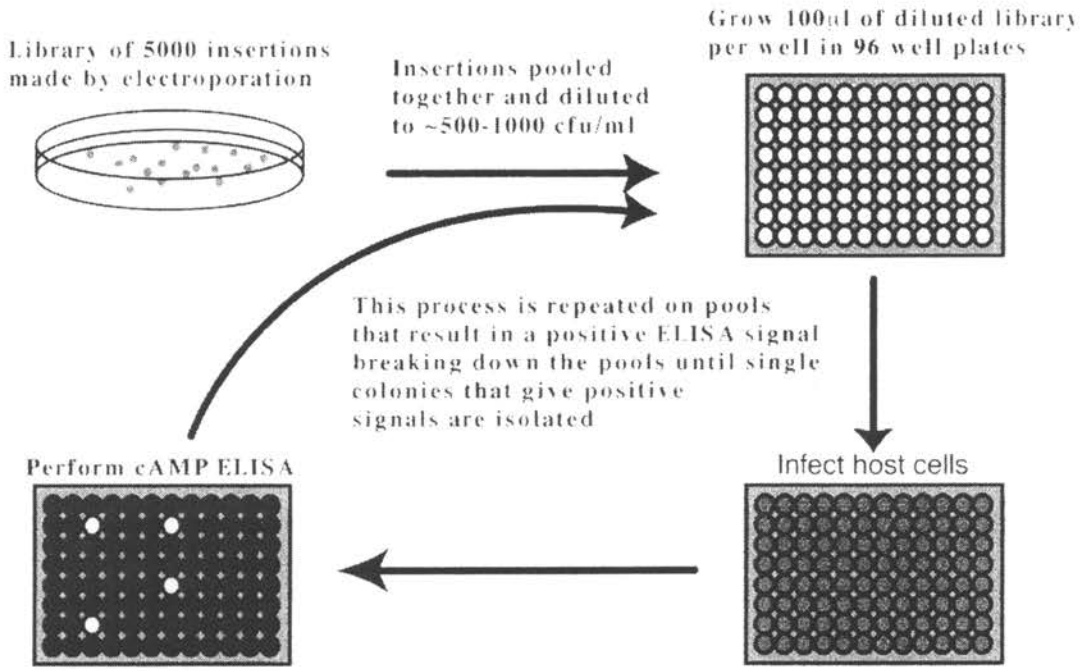


Figure 2-2. Strategy for identifying effectors. Libraries of approximately 5,000 insertions were generated by electroporating mini-Tn*5*-cycler transposon/transposase complexes into either 14028s or a *slrP* derivative. These colonies were diluted in LB broth to approximately 500 to 1,000 bacteria/ml (based on optical density readings at 600 nm), and 100-µl aliquots of the diluted library were grown to either stationary phase or late log phase. These cultures were then used to infect J774 cells or HeLa cells seeded in 96-well plates. After 8 to 10 h of infection for J774 cells or 2 h for HeLa cells, the medium was removed, and 0.1 M HCl was added to lyse the cells. The level of cAMP in each well was then measured using an ELISA. Any pool from which infected cells showed at least 10 times higher levels of cAMP than the background level was isolated and screened further by repooling into smaller groups (10 or 1) until individual bacteria containing the cognate CyaA' fusion were obtained.

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position, although at least five of these were independent isolates. This suggested the presence of a Tn*5* transpositional "hot spot." To avoid this hot spot, six additional libraries, each containing approximately 5,000 insertions, were constructed in a *slrP* background. Sixteen positive fusions were identified from a screen of 25,000 insertions in this *slrP* background. In addition to our screens with the J774 macrophage cell line, a single library of 5,000 insertions in the *slrP* background was screened in HeLa cells. Three clones were identified from this pool. Each contained a *cyaA'* fusion to *sipA*, which encodes a previously characterized effector (284). Sequence analysis of each of these *sipA* insertions demonstrated that they were identical and likely to be siblings. In summary, for every 5,000 mini-Tn*5*-cycler insertions screened, three or four positive fusions were identified.

In total, we isolated 42 positive clones, each of which contained an in-frame insertion in either a gene encoding a known EP or an ORF encoding a protein of unknown function. Following DNA sequencing of all 42 clones, we found fusions to 10 different ORFs, of which 7 had been previously identified to encode secreted effectors. Three of the fusions were to unknown ORFs that presumably encode new effectors. Table 2-2 lists the genes isolated in our screen, along with a short description of each gene's reported function, the number of times each gene was isolated, and the number of unique insertion sites and independent isolates. The genes identified were *sipA* (284), *slrP* (215, 285), *pipB2* (237), *sptP* (286), *sseJ* (215), *srfH* (189, 215, 283), *avrA* (262) and *Salmonella enterica* serovar Typhimurium LT2 reference numbers STM1583, STM1629, and STM1698 (287). We refer to these last genes as *Salmonella* translocated effectors (*ste*) *steA* (STM1583), *steB* (STM1629), and *steC* (STM1698). Interestingly, there were

Table 2-2. List of genes isolated in screens[a]

Fusion	# of times isolated	# of unique insertion-sites	# of independent isolates	Known/predicted function of gene	References
slrP	17	1	5	Type III secreted protein involved in murine virulence	(285)
pipB2	8	5	5	Type III secreted protein-SsrB regulated	(237)
steC (STM1698)	5	4	5	Putative inner membrane protein-SsrB regulated	(287), Rue and Heffron (unpublished)
sipA[b]	3	1	1	Type III secreted protein-neutrophil migration, inflammation, actin polymerization	(141, 269, 270)
sseJ	2	2	2	Type III secreted protein-SsrB regulated	(215)
steA (STM1583)	2	1	1	Putative cytoplasmic protein	(287)
srfH	2	1	1	Type III secreted protein-affects macrophage motility-SsrB regulated	(189, 215), Worley and Heffron (in press)
sptP	1	1	1	Type III secreted protein with phosphatase activity	(286)
steB (STM1629)	1	1	1	Putative dipicolinate reductase	(287)
avrA	1	1	1	Type III secreted protein- Nf-κB localization	(262)

[a] Genes were isolated by mini-Tn*5*-cycler mutagenesis and ELISA of J774 cells or HeLa cells.
[b] Identified from infection of HeLa cells.

five unique insertions in *pipB2* and four unique insertions in *steC* (Table 2-2).

An intact T3S is required for secretion of the newly identified EP.

The fact that seven of the identified genes encode known effectors strongly suggested that our approach was working, but it was necessary to confirm that the newly identified ORFs were also secreted via a type III secretion apparatus. For these experiments, we utilized both genetic mutants defective in needle complex assembly and growth conditions that either induce or repress expression of the two *Salmonella* type III secretion systems. Each fusion was transduced into both an *invA*::*cat* mutation that renders the bacteria defective for T3S-1-dependent secretion and an *ssaK*::*cat* mutant defective for SPI-2 T3S-dependent secretion. The 10 unique mini-Tn*5*-cycler fusions were tested under conditions that allow expression of T3S-1 (288). Strains harboring *cyaA'* fusions were grown to late log phase and used to infect J774 macrophage-like cells for 1 h. As shown in Figure 2-3A, there was a significant increase in cAMP for J774 cells infected with the SipA-, SptP-, AvrA-, SlrP-, SteA-, and SteB-CyaA' fusions. The secretion of these fusions was dependent on an intact SPI-1- but not SPI-2-encoded T3S. Secretion of the remaining four fusions (SseJ, SrfH, PipB2, and SteC) could not be detected under SPI-1-inducing conditions (Figure 2-3A). Similar results were observed following infection of HeLa cells (data not shown).

Next, strains harboring each *cyaA'* fusion in either a WT, *invA*::*cat*, or *ssaK*::*cat* background were grown to stationary phase in order to repress SPI-1 and induce SPI-2 expression. These cultures were used to infect J774 macrophages for 8 h at an input MOI of 250. As shown in Figure 2-3B, with the exception of SipA-CyaA', every fusion that we tested resulted in a significant increase in host cell cAMP which was dependent on an

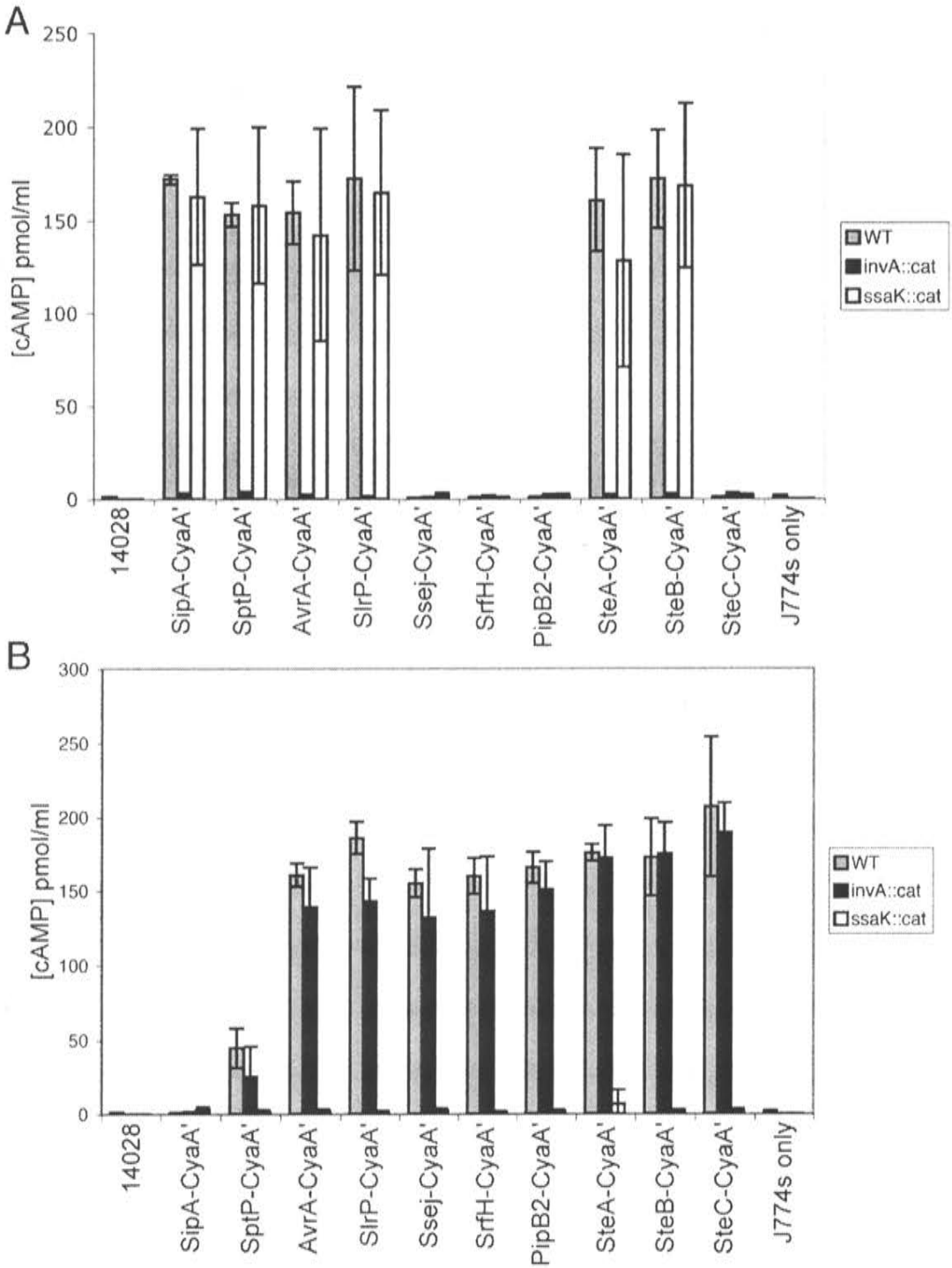
A
250
200
150
100
50
0
[cAMP] pmol/ml
WT
invA::cat
ssaK::cat
14028
SipA-CyaA'
SptP-CyaA'
AvrA-CyaA'
SlrP-CyaA'
Ssej-CyaA'
SrfH-CyaA'
PipB2-CyaA'
SteA-CyaA'
SteB-CyaA'
SteC-CyaA'
J774s only
B
300
250
200
150
100
50
0
[cAMP] pmol/ml
WT
invA::cat
ssaK::cat
14028
SipA-CyaA'
SptP-CyaA'
AvrA-CyaA'
SlrP-CyaA'
Ssej-CyaA'
SrfH-CyaA'
PipB2-CyaA'
SteA-CyaA'
SteB-CyaA'
SteC-CyaA'
J774s only

Figure 2-3. T3S-1 (A)- and SPI-2 T3S (B)-dependent secretion of *cyaA'* fusions into J774 cells. Levels of cAMP were measured within the macrophage-like cell line J774 following infection with the 10 *cyaA'* fusions listed in Table 2-2. Three different backgrounds were used for this experiment. These were WT (gray bars), *invA*::*cat* (black bars), and *ssaK*::*cat* (white bars). Bacteria were grown to late log phase and used to infect J774 cells at an input MOI of 50 for 1 hour (A) or were grown to stationary phase and used to infect J774 cells for 8 h at an input MOI of 250 (B). The cells were then lysed with 0.1 M HCl, and an ELISA (Assay Designs) was used to quantitate the cAMP levels. The cAMP concentration (in pmol/ml) was measured in triplicate samples, and the error bars represent 1 standard error of the mean.

intact SPI-2 T3S. Similar results were found when we infected the dendritic cell line JAWS II (data not shown).

We focused on the characterization of the three newly identified secreted effectors. We constructed *cyaA'* fusions to full-length copies of *steA*, *steB*, and *steC* to rule out aberrant secretion by the flagella or some as yet uncharacterized mechanism. As before, we tested the full-length CyaA' fusions to SteA, SteB, and SteC in either the WT, *invA*::*cat*, or *ssaK*::*cat* background for secretion into infected host cells. The same conditions were used as before to induce either T3S-1 or T3S-2, and the secretion profiles of the full-length fusion proteins were found to be identical to those of the original fusions (Figure 2-4).

***steA* is required for efficient colonization of mouse spleens.**

To determine if *steA*, *steB*, or *steC* plays a role in a mouse infection model, competitive infections were performed. Deletions of *steA*, *steB*, and *steC* were generated using the -red recombination system (279), and the competitive index of each strain was determined (Table 2-3). Neither the *steB* nor *steC* strain had a competitive index statistically different from that of the control wild-type strain. However, the *steA* strain had an approximately threefold competitive disadvantage for mouse spleen colonization. Expressing *steA* from its native promoter in a low-copy-number vector (p*steA*) complemented this competitive defect.

SteA localizes to the Golgi network in host epithelial cells.

Because of its potential role as a virulence factor, we further characterized *steA*. HeLa cells were transfected with an expression vector expressing either EGFP alone or a translational fusion of SteA to EGFP. As shown in Figure 2-5, cells transfected with the

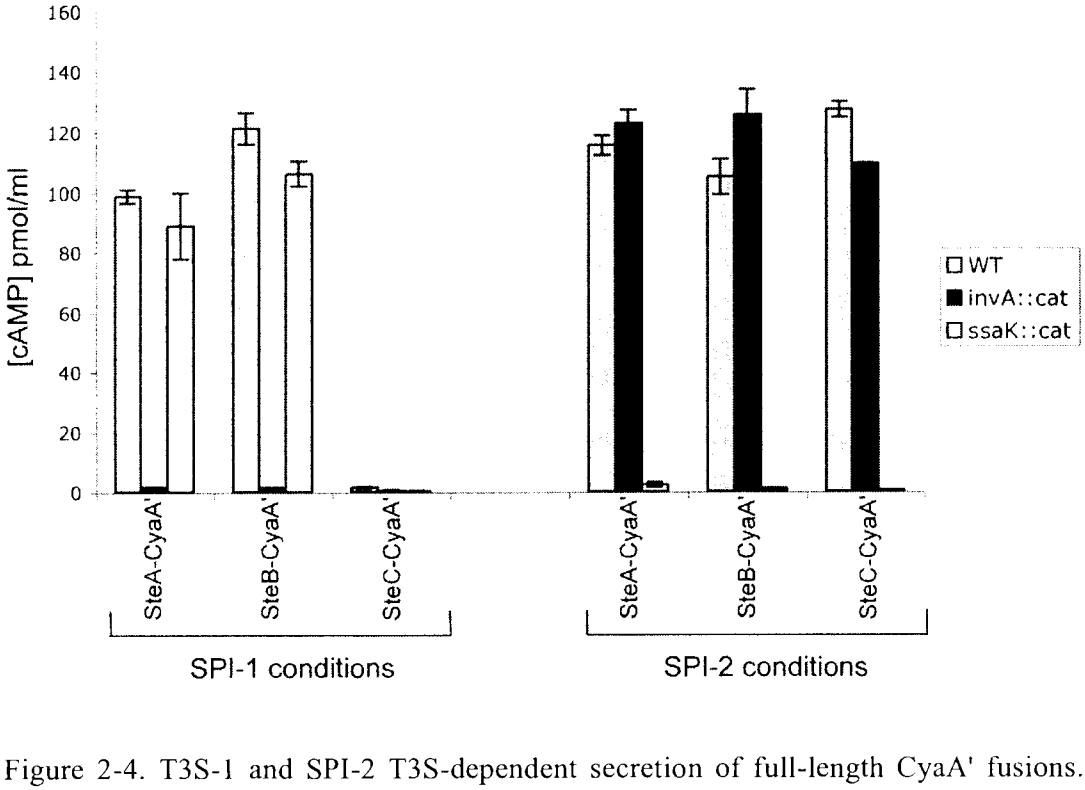

Figure 2-4. T3S-1 and SPI-2 T3S-dependent secretion of full-length CyaA' fusions. Levels of cAMP were measured within the macrophage-like cell line J774 following infection with SteA-CyaA', SteB-CyaA', and SteC-CyaA'. Three different backgrounds were used for this experiment. These were WT (gray bars), *invA*::*cat* (black bars), and *ssaK*::*cat* (white bars). Bacteria were grown to late log phase and used to infect J774 cells at an input MOI of 50 for 1 hour (SPI-1 conditions) or were grown to stationary phase and used to infect J774 cells for 8 h at an input MOI of 250 (SPI-2 conditions). The cells were then lysed with 0.1 M HCl, and an ELISA (Assay Designs) was used to quantitate the cAMP levels. The cAMP concentration (in pmol/ml) was measured in triplicate samples, and the error bars represent I standard error of the mean.

Table 2-3. Competitive infections using Δ*steA*, Δ*steB*, Δ*steC*, and *steA* complemented strains[a]

Genotype	Median CI	No. of mice	P value
14028s	1.026	10	>0.05
Δ*steA*	0.306	10	<0.05
p*steA*/Δ*steA*	1.261	10	>0.05
Δ*steC*	2.497	10	>0.05
Δ*steB*	1.071	10	>0.05

[a]All strains were competed against MA6054. BALB/c mice were injected intraperitoneally, and then spleens were harvested and recovered CFUs were used to determine the CI as described in Materials and Methods. Student's *t* test was used for statistical analysis of the data, and the resulting *P* values are shown.

EGFP expression vector alone displayed uniform fluorescence throughout the cell. In contrast, EGFP fluorescence was concentrated in perinuclear regions in cells transfected with a plasmid expressing the SteA-EGFP fusion protein. To further define this perinuclear compartment, transfected cells were costained with Bodipy-TR-ceramide, a dye that targets the Golgi network. In Figure 2-5C, SteA-EGFP is shown to extensively colocalize with Bodipy-TR-ceramide. This suggests that SteA localizes to the TGN when it is expressed in host cells.

The subcellular localization of SteA translocated by the bacteria was also investigated. SteA-HA/14028s, a double-HA-tagged SteA fusion-expressing strain, was used to infect HeLa cells for 4 hours under SPI-1-inducing conditions. Alexa Fluor 488-conjugated antibodies were used to visualize SteA-HA by fluorescence microscopy, and Bodipy-TR-ceramide was again used to visualize the TGN. In many infected cells, little to no SteA-HA-specific fluorescence was seen, possibly due to low expression levels of SteA. In addition, most of the SteA-HA-specific fluorescence that was observed was found only in proximity to bacteria in infected cells. However, in a few isolated cells containing large numbers of bacteria, broader SteA-HA-specific staining could be seen (Figure 2-6B). In these cases, it was possible to see SteA-specific staining that was not directly adjacent to bacteria. As shown in Figure 2-6D, in a cell with extensive SteA-HA-specific staining, SteA-HA colocalized with Bodipy-TR-ceramide. This staining was specific, as it was never observed in cells infected with WT 14028s (Figure 2-6F). These data, along with the data from transfected cells, strongly suggest that secreted SteA localizes to the TGN.

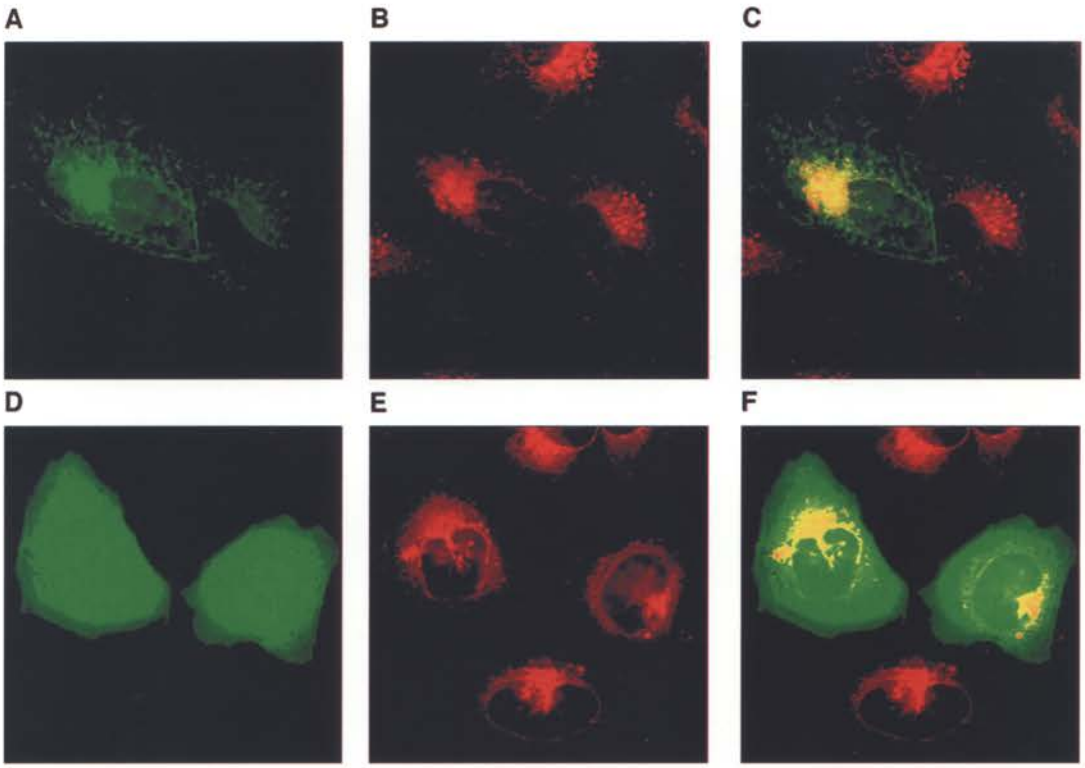

Figure 2-5. A SteA-EGFP fusion expressed in HeLa cells colocalizes with the TGN. HeLa cells were transfected for 24 h with pEGFP (bottom panels) or pSteA-EGFP (top panels), and Bodipy-TR-ceramide (red) was used to stain the TGN. The images shown are 0.2-μm *z* sections captured using deconvolution microscopy. EGFP fluorescence images are shown in panels A and D, and Bodipy-TR-ceramide fluorescence images are shown in panels B and E. Panels C and F show mergers of panels A and B and panels D and E, respectively. In panels C and F, the areas where EGFP and Bodipy-TR-ceramide fluorescence overlap are yellow.

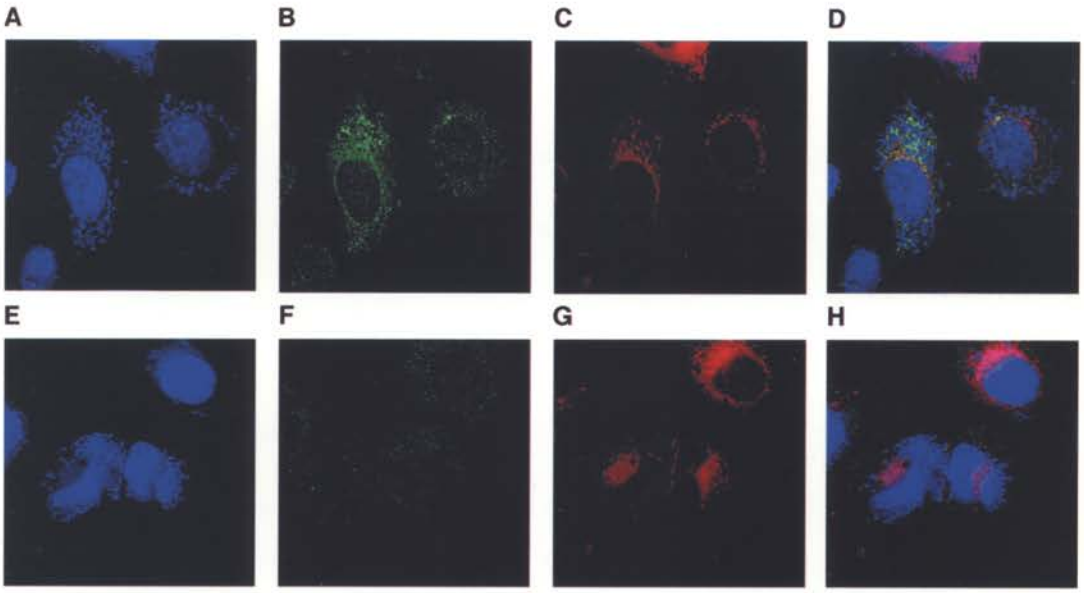

Figure 2-6. Secreted SteA colocalizes with the TGN in infected HeLa cells. HeLa cells were infected with SteA-HA/14028s (top panels) or with WT 14028s (bottom panels) for 4 hours at an MOI of 100 under SPI-1-inducing conditions. HA-specific antibodies were used to visualize HA-tagged SteA (green). Bodipy-TR-ceramide (red) was used to stain the TGN, and the DNA stain DRAQ5 (blue) was used to visualize host cell nuclei and bacteria. DRAQ5 fluorescence images are shown in panels A and E, HA tag-specific fluorescence images are shown in panels B and F, and Bodipy-TR-ceramide fluorescence images are shown in panels C and G. Panels D and H show mergers of the images from panels A to C and E to G, respectively.

Discussion

This report describes a novel strategy for the identification of secreted effector proteins. In this work, three previously unidentified effectors, SteA, SteB, and SteC, were found. Using a competitive infection model, we show that one of these effectors, SteA, is required for *Salmonella* to colonize the mouse spleen. SteA was also shown to localize to the *trans*-Golgi network within both transfected and infected epithelial cells. Evidence of the power of this approach is demonstrated by the identification of seven known secreted effectors in the same screen.

At least four strategies have been used to identify secreted EPs in *Salmonella* and other pathogens. Guttman et al. described a de novo method of screening using wilting of plant leaves as an easily observed phenotype. However, their method is limited to certain plant pathogens such as *Pseudomonas syringae* (289). Luo and Isberg used selection and screening to identify type IV secreted proteins in *Legionella pneumophila* (290). Their method requires the identification of secreted proteins based on interbacterial transfer and thus could not be applied to the type III secreted effectors we have found. Tu et al. constructed a mini-Tn*5cyaA'* transposon similar to ours but identified only surface-exposed proteins in *Bordetella bronchiseptica* (291). Our mini-Tn*5*-cycler screen employed a more sensitive enzymatic assay and relied on the infected host cell to supply calmodulin. In our assay, we only identified translocated effectors, as evidenced by the fact that an intact secretion apparatus was required for each of the 10 EPs found. Of the 60,000 mutants we screened, 42 produced detectable adenylate cyclase activity in infected cells, and each encoded an in-frame fusion to a secreted effector protein.

We wondered if it is possible to calculate the total number of effectors encoded by

Salmonella based on the sample we examined. Assuming that insertion is random, there are several other factors that will reduce the chance of identifying any given effector. First, there is a one-in-six chance of an insertion occurring in the correct orientation and reading frame of any given gene. Second, the target area must be only a portion of a given gene because sequences that are essential for secretion or binding to a chaperone will be excluded. Third, however sensitive the assay is, the level of expression must be above a given threshold of detection. These caveats make it difficult to extrapolate from the number of effectors identified in our screen but do imply that there are many as yet undetected effectors. In addition, we have only examined specific conditions and cell types. More EPs might be identified if other cell types are used and if the infection time is varied. For example, SseK2, a recently identified effector in *S. enterica* serovar Typhimurium, is secreted only after 21 h of infection (204). SseK2 and possibly other effectors secreted at later time points would only have been detected if we had lengthened the infection time. One additional limitation that we observed stemmed from the existence of transpositional hot spots resulting in the repeated isolation of mini-Tn*5*-cycler fusions to *slrP*. In fact, many of the identified genes were only found after the deletion of *slrP*. Presumably, a systematic deletion of effectors that are uncovered in the screen could be used to detect additional new genes. Additionally, some genes encoding EPs are simply not amenable to mini-Tn*5*-cycler mutagenesis, including any that are targeted to vesicles that do not contain calmodulin as well as those with extremely small targets for transposition.

Our technique can be used to identify secreted type III EPs from a wide range of pathogens and possibly proteins secreted by other mechanisms. CyaA' has been used to

demonstrate type IV secretion (292), and in *B. pertussis*, CyaA is secreted via a type I secretion system (293). Finally, there are many genetically intractable organisms for which the isolation of a large number of transposon insertions is simply not possible, even by electroporation of transposon complexes. In these cases, it may be possible to express a gene library from a plasmid in a genetically tractable host that also expresses the complete structural apparatus for secretion, thereby making it amenable to mini-Tn*5*-cycler mutagenesis.

Three new secreted EPs were identified in the screen, namely, SteA, SteB, and SteC. The genes encoding all three of these proteins have low GC contents (*steA* GC content, 43%; *steB* GC content, 41.9%; and *steC* GC content, 38%), suggesting horizontal acquisition, which is common for virulence-associated genes. The *steA* strain was found to have a competitive defect in colonization of the mouse spleen, whereas *steB* and *steC* did not appear to play a significant role in this model. This competitive defect suggests that *steA* is required either for passage of the bacteria from the peritoneal cavity into the spleen, for survival and replication within host cells, or for avoiding host immune defenses. Interestingly, SteA localizes to the Golgi network in transfected and infected HeLa cells. SseG, another EP in *S. enterica* serovar Typhimurium, has also been shown to localize to the Golgi network (236). The presence of SseG was found to be important for the association of *Salmonella*-containing vacuoles with the Golgi network. Furthermore, the association of *Salmonella*-containing vacuoles with the Golgi network was required for normal bacterial replication within HeLa cells. We are investigating whether SteA plays a similar role to that of SseG in infected cells. The coding sequence of *steA* is 94% conserved in *Salmonella enterica* serovar Typhi strains TY2 and CT18

and 95% conserved in *Salmonella enterica* serovar Paratyphi strain ATCC 9150. This conservation suggests that *steA* may be important for virulence in human infections as well. In a recent paper by Morgan et al., STM1698 (the ORF encoding SteC) was identified as the gene for a colonization factor specific for the chick infection model (294). The coding sequence of *steC* is 93% conserved in *Salmonella enterica* serovar Paratyphi strain ATCC 9150 and *Salmonella enterica* serovar Typhi strains TY2 and CT18, again suggesting a possible role in human infection. Of the three newly described proteins, only SteB has significant homology to a bacterial protein from a different species: it shares 40% amino acid identity to a protein in the tropical pathogen *Chromobacterium violaceum*. This pathogen is found in water and soil throughout tropical South America and causes septicemia with metastatic abscesses with a 64% fatality rate. *C. violaceum* contains genes encoding a T3S, suggesting that the homology may be meaningful (295). SteB (STM1629) is encoded in a genetic island in close proximity to the gene for another secreted protein, SseJ (STM1631). STM1630, the ORF immediately downstream of *steB*, is required for virulence in both the calf and chick infection models (294).

Interestingly, five of the EPs identified were secreted by both the SPI-1 and SPI-2 T3Ss (SptP, SlrP, AvrA, SteA, and SteB), whereas SipA was observed to be secreted only via T3S-1. Since these five proteins are secreted by both T3Ss, they may function in both the intestinal and systemic phases of infection. Four of the identified proteins, SseJ, SrfH, PipB2, and SteC, were only secreted via T3S-2. These results raise two possibilities that are not exclusive, either that these effectors are only expressed under one condition or that they cannot be secreted through the alternative needle complex. The expression of

all four of these genes is regulated by SsrB ((189, 215); J. Rue and F. Heffron, unpublished data). These data suggest that proteins secreted exclusively by T3S-2 are regulated by SsrB, while proteins secreted by both the SPI-1 and SPI-2 T3Ss are regulated by an unknown mechanism. The observed secretion patterns may be a result of a SPI-1 or SPI-2 T3S-specific signal in the RNA messages or amino acid sequences of these proteins. Alternatively, T3S specificity may be determined by either the regulation of expression of the EPs themselves or the regulation of expression of the chaperones required for their secretion.

While the mini-Tn*5*-cycler transposon may allow the identification of a large number of new EPs, identifying these proteins is only the first step in the further study of EPs. Many years have been spent studying secreted bacterial EPs, but the functions of only a few have been fully elucidated. Several more *S. enterica* serovar Typhimurium EPs are thought to exist because the cognate EPs for many observed pathogenic phenotypes remain a mystery. This report provides the initial step in expanding our knowledge of the repertoire of secreted EPs in *Salmonella* and potentially many other bacterial pathogens.

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Chapter 3

In vivo analysis of *Salmonella* type III secretion in an acute mouse infection model

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Abstract

The type III secretion systems (T3S) encoded in *Salmonella* pathogenicity island-1 and 2 (T3S-1 and T3S-2) are virulence factors required for specific phases of *Salmonella* infection in animal hosts. However, the host cell types targeted by *Salmonella*'s T3Ss *in vivo* have not been identified. *Salmonella* strains expressing six different effector protein-β-lactamase fusions were generated and injection of these fusions into host cells was detected by cleavage of a fluorescent substrate. Secretion of all six effectors could be detected following infection of cultured cells. Following infection of mice only effectors secreted via T3S-2 were detected in spleen cells. Secretion of these effectors was detected in all spleen cell types except for mature macrophages. *Salmonella* secreted effectors had a surprisingly specific tropism for infiltrating monocytes and neutrophils. The majority of the secretion was detected in neutrophils, which are potently bactericidal innate immune cells. Despite the bactericidal properties of neutrophils, most of the viable intracellular *Salmonella* were associated with these cells and cells that were positive for secretion were also found to contain intracellular bacteria. These findings suggest that *Salmonella* targets neutrophils utilizing T3S effectors to attenuate neutrophil function and promote bacterial survival in the host.

Introduction

Bacterial pathogens can bombard host cells with an arsenal of proteins, lipids and other factors that disarm and leave the cell vulnerable to infection. However, in the complex internal environment of mammalian organisms, different cell types communicate with each other and coordinate the multi-faceted defenses of the innate and adaptive immune systems. Microbial pathogens have therefore evolved increasingly complex strategies that enable them to counter the immune system's defenses. One of these adaptations is the type III secretion system (T3S) found in several Gram-negative bacterial pathogens. These T3Ss are sophisticated secretion machines that inject effector proteins directly into the host cell cytoplasm. Although many secreted effector proteins have been discovered, the study of their function has been largely restricted to cell culture models. Consequently, the *in vivo* targets of *Salmonella*'s T3S have not been identified and are the focus of this study. Identifying the targets of T3S will be crucial in furthering our understanding of the infectious process of this and other important human pathogens.

Salmonella enterica serovar typhimurium has two T3Ss that are expressed under different conditions and required for distinct aspects of infection (54, 186, 271). Effectors secreted by the *Salmonella* pathogenicity island-1 T3S (T3S-1) are associated with the invasion of intestinal epithelial cells and enhanced intestinal inflammation in infected hosts and (30, 97, 269). The *Salmonella* pathogenicity island-2 T3S (T3S-2) is required for intracellular survival during the systemic phase of infection (182, 183, 185, 232, 296) and enhances inflammation during the enteric phase of infection (31, 258). In this preliminary investigation, we investigated the spleen cell types targeted by *Salmonella*'s T3S in an acute mouse infection. During the systemic phase of infection in mice,

macrophages, neutrophils, and dendritic cells contain *Salmonella* (36, 42, 43, 45). However, the specific cells targeted by T3S-1 and T3S-2 *in vivo* have not been identified.

Cell culture studies have demonstrated that T3S-1 can inject proteins into both phagocytic and non-phagocytic cells whereas T3S-2 is primarily active inside phagocytic cells (138). One method commonly used to investigate T3S mediated protein translocation is the use of effector fusions to the calmodulin dependent adenylate cyclase, (CyaA), from *Bordetella pertussis*. We used CyaA fusions in a previous study to monitor the secretion of several T3S secreted effectors in *S. typhimurium* (138). We found that T3S secreted effectors could be placed into three categories based on their secretion properties: those secreted via T3S-1, those secreted by T3S-2, or those secreted by both secretion systems. We wished to elaborate on this previous study to determine if different secretion patterns in cultured cell lines correlated with different secretion patterns *in vivo*. A reporter enzyme strategy involving ß-lactamase (Bla) fusions was used to identify the spleen cell types targeted by the T3S of *Yersinia pestis* (297, 298). We combined this strategy with advanced FACS analysis to identify the *in vivo* targets of the *S. typhimurium* T3Ss.

In this study, mice were infected by i.p. inoculation with strains of *S. typhimurium* expressing different effector-Bla fusions. Using FACS analysis we identified the *in vivo* targets of *S. typhimurium*'s T3S and cells containing viable bacteria. The use of advanced FACS analysis technology allowed us to identify T3S targeted cells based on cleavage of a Bla substrate and simultaneous staining of four cell surface markers. Each sample was divided into two and a total of eight cell surface markers were used to analyze cell types. This approach provided extremely high resolution, allowing for the identification of cell

types involved in *Salmonella* infection. Microscopic visualization of spleen cells from mice infected with a *Salmonella* strain expressing Tomato fluorescent protein (299) indicated that T3S targeted cells contain intact bacteria. Using these methods, we discovered that *Salmonella* mainly targets a very specific subset of infiltrating monocytes and neutrophils, and that mature macrophages are not targeted. We identified neutrophils as the primary target for the T3S-2 in the murine spleen and unexpectedly, these cells also contain most of the viable intracellular *Salmonella*.

Materials and Methods

Bacterial strains and cell culture

ATCC *S. typhimurium* 14028 was the parental strain used in all assays. LB media supplemented with antibiotics was used unless otherwise stated. Antibiotics were used at the following concentrations: kanamycin 60 μg/ml, carbenicillin 100 μg/ml. 14028 derivatives and plasmids used in this study are listed in Table 3-1. J774s and HeLa cells were obtained from ATCC and grown in DMEM supplemented with 10% FBS. HeLa cells and J774s were infected using T3S-1 and T3S-2 conditions, respectively, as previously described (138). To prepare samples for FACS analysis, infected HeLa cells were trypsonized, and infected J774 cells were scraped to prepare cell suspensions, then 10^7 cells/ml were loaded with .125X solution of CCF2-AM (Invitrogen) for 2 hours at room temperature following the manufacturer's recommendations. To prepare samples of J774 or HeLa cells for microscopy, cells were seeded in Lab-Tek II chamber coverglass slides (Nalge Nunc International), infected, and then 10^7 adherent cells were loaded with 1 ml of 1X CCF2-AM solution for 2 hours at room temperature.

Generation of effector-Bla fusions and protein expression analysis

The mini-Tn5-*bla* transposon was created by replacing the *cyaA'* gene in mini-Tn5-cycler (138) with codons 70-792 of the ß-lactamase gene from pBluescriptSK. This transposon was then used as a template for PCR using primers with extensions specific for the target effector. PCR products were then used to transform 14028 using the λ-red PCR based recombination technique (279) (See Table 3-2 for a list of primers used). Constructs were confirmed by PCR and by sequencing the PCR products. Each fusion was then

Table 3-1. Strains and plasmids used in this study.

Strain or plasmid	Genotype	Source or reference
Strains		
14028s	Wild type *Salmonella enterica serovar typhimurium*	ATCC
JKG100	Full length chromosomal SseJ::Mini-TN5-BLAM (SseJ-BLAM) in 14028s	This work
JKG101	Full length chromosomal SseJ::Mini-TN5-BLAM (SseJ-BLAM) in MJW1835	This work
JKG102	Full length chromosomal SseJ::Mini-TN5-BLAM (SseJ-BLAM) in MJW1301	This work
JKG200	Full length chromosomal SlrP::Mini-Tn5-BLAM (SlrP-BLAM) in 14028s	This work
JKG201	Full length chromosomal SlrP::Mini-Tn5-BLAM (SlrP-BLAM) in MJW1835	This work
JKG202	Full length chromosomal SlrP::Mini-Tn5-BLAM (SlrP-BLAM) in MJW1301	This work
JKG300	Full length chromosomal SptP::Mini-Tn5-BLAM (SptP-BLAM) in 14028s	This work
JKG301	Full length chromosomal SptP::Mini-Tn5-BLAM (SptP-BLAM) in MJW1835	This work
JKG302	Full length chromosomal SptP::Mini-Tn5-BLAM (SptP-BLAM) in MJW1301	This work
JKG400	Full length chromosomal SipA::Mini-Tn5-BLAM (SipA-BLAM) in 14028s	This work
JKG401	Full length chromosomal SipA::Mini-Tn5-BLAM (SipA-BLAM) in MJW1835	This work
JKG402	Full length chromosomal SipA::Mini-Tn5-BLAM (SipA-BLAM) in MJW1301	This work
JKG500	Full length chromosomal SteA::Mini-Tn5-BLAM (SteA-BLAM) in 14028s	This work
JKG501	Full length chromosomal SteA::Mini-Tn5-BLAM (SteA-BLAM) in MJW1835	This work
JKG502	Full length chromosomal SteA::Mini-Tn5-BLAM (SteA-BLAM) in MJW1301	This work
JKG600	Full length chromosomal SteC-BLAM (SteC-BLAM) in 14028s	This work
JKG601	Full length chromosomal SteC-BLAM (SteC-BLAM) in MJW1835	This work
JKG602	Full length chromosomal SteC-BLAM (SteC-BLAM) in MJW1301	This work
MA6054	14028s *ara-907 araD 901*::MudJ	(63)
MJW1301	14028s *ssaK::cat*	(138)
MJW1835	14028s *invA::cat*	(138)
Plasmids		
pMini-Tn5-BLAM	pCRScript + mini-Tn5-BLAM	This work
pWKS30-Tomato	pWKS30 + Tomato (fluorescent protein)	This work

Table 3-2. Primers used in this study.

Primer name	Sequence	Purpose
Tn5-*bla* frwd (NdeI)	CATATGGGCACCCAGAAACGCTGGTGAAAGTA	Cloning codons 70-792 of the ß-lactamase gene from pBluescriptSK
Tn5-*bla* reverse (BamHI)	GGATCCTTACCAATGCTTAATCAGTGAGGCA	Cloning codons 70-792 of the ß-lactamase gene from pBluescriptSK
LacZ-a fusion verification primer	GCTTTTGTTCCCTTTAGTGAGG	Verifying ß-lactamase insertions (in combination with verification primers for specific fusions)
Full length SseJ-Bla forward	TGTTAGAAAGTTTTATAGCTCATCATTATTCCACTGAA CTGTCTCTTATACACATCTCA	For amplifying Mini-Tn5-*bla* to make chromosomal full length SseJ-Bla fusion
Full length SseJ-Bla reverse	GAGCTGTGTTTTGCTCAAGGCGTACCGCAGCCGATG GAACTCTGTCTCTTATACACATCTGGT	For amplifying Mini-Tn5-*bla* to make chromosomal full length SseJ-Bla fusion
SseJ fusion verification primer	CTGTCTCTTATACACATCTGGT	Verifying the full length SseJ-Bla fusion
Full length SlrP-Bla forward	AAAAAGAGGTGAGCTCGCTCATGAGCGCCTACTGGC GA CTGTCTCTTATACACATCTCA	For amplifying Mini-Tn5-*bla* to make chromosomal full length SlrP-Bla fusion
Full length SlrP-Bla reverse	GGTAAACAGGCTCTCTCCCTCTTCTGATAAACTGCGT TCAGCTGTCTCTTATACACATCTGGT	For amplifying Mini-Tn5-*bla* to make chromosomal full length SlrP-Bla fusion

SlrP fusion verification primer	AGAATCAGTTACGAGAGTCGCT	Verifying the full length SlrP-Bla fusion
Full length SptP-Bla forward	AAGCAATGCAAGCCCAGTTGCTTATGACGACGGCAA GC CTGTCTCTTATACACATCTCA	For amplifying Mini-Tn5-*bla* to make chromosomal full length SptP-Bla fusion
Full length SptP-Bla reverse	AGCTTACTTTCAGATAGTTCTAAAAGTAAGCTATGTT TTTACTGTCTCTTATACACATCTGGT	For amplifying Mini-Tn5-*bla* to make chromosomal full length SptP-Bla fusion
SptP fusion verification primer	CTGTCTCTTATACACATCTGGT	Verifying the full length SptP-Bla fusion
Full length SipA-Bla forward	GGGTTATTACTACCGTTGATGGCTTGCACATGCAGCG T CTGTCTCTTATACACATCTCA	For amplifying Mini-Tn5-*bla* to make chromosomal full length SipA-Bla fusion
Full length SipA-Bla reverse	CTTGCTTCAATATCCATATTCATCGCATCTTTCCCGGT TAACTGTCTCTTATACACATCTGGT	For amplifying Mini-Tn5-*bla* to make chromosomal full length SipA-Bla fusion
SipA fusion verification primer	CTGTCTCTTATACACATCTGGT	Verifying the full length SipA-Bla fusion
Full length SteA-Bla forward	GCCGACATAAAAGCTCGCTACCATAACTATTTGGAC AATTAT CTGTCTCTTATACACATCTCA	For amplifying Mini-Tn5-*bla* to make chromosomal full length SteA-Bla fusion
Full length SteA-Bla reverse	TCTGATTTCTAACAAAACTGGCTAAACATAAACGCTT TTTA CTGTCTCTTATACACATCTGGT	For amplifying Mini-Tn5-*bla* to make chromosomal full length SteA-Bla fusion
SteA fusion verification primer	GAAAGGCATCTTGTATGTGCT	Verifying the full length SteA-Bla fusion

Full length SteC-Bla forward	GGACTCTTGTGGCTAAGGTATTAAAGGATGAATTAA AAAAA CTGTCTCTTATACACATCTCA	For amplifying Mini-Tn5-*bla* to make chromosomal full length SteC-Bla fusion
Full length SteC-Bla reverse	TGCCCCCGGCGATTCGCAGAAAAGAACGGAACTAAA TGCTA CTGTCTCTTATACACATCTGGT	For amplifying Mini-Tn5-*bla* to make chromosomal full length SteC-Bla fusion
SteC fusion verification primer	TGAATATGGCCAATGACGACA	Verifying the full length SteC-Bla fusion
fpTOMATO forward (KpnI + ribosomal binding site)	GGTACCTAAGGAGGATATTCATATGGTGAGCAAGGG CGAGGAGGTCATCA	For cloning Tomato fluorescent protein with an added Shine-Dalgarno sequence into pWKS30
fpTOMATO reverse (NotI)	GCGGCCGCTTACTTGTACAGCTCGTCCATGCCGTACA GGAACA	For cloning Tomato fluorescent protein with an added Shine-Dalgarno sequence into pWKS30

transduced into WT 14028, MJW1301 (*ssak::cat*) and MJW1835 (*invA::cat*) backgrounds using P22 phage as previously described (277).

Mouse infection studies

All mouse studies were approved by the Oregon Health & Science University institutional animal care and use committee (animal protocol #A085). All C57BL/6 mice were inoculated via i.p. injection with ~2 X 10^5 CFU of the desired *S. typhimurium* strain using an inoculum of 100 μl. To determine the number of bacteria present in the spleen, mice were euthanized at the indicated time points and spleens were removed and homogenized. Spleen cells were lysed with PBS containing 1% triton and serial dilutions of this solution were plated on LB agar and colonies were counted the next day. The competitive infections were performed as previously described (138, 276). Each test strain was mixed 1:1 with the reference strain MA6054. 2 days after injection the mice were sacrificed and their spleens harvested and homogenized. Spleen suspensions were diluted and plated on LB plates containing X-gal (40 μg/ml) and arabinose (1mM). The reference strain MA6054 has arabinose inducible β-galactosidase activity and can be easily distinguished from the test strains when plated on LB with X-gal and arabinose. The competitive index (CI) was then calculated using the following equation: (percentage of test strain recovered / percentage of reference strain recovered) / (percentage of test strain inoculated / percentage of reference strain inoculated).

Preparation of spleen cells

C57BL/6 mice were inoculated via i.p. injection with ~2 X 10^5 CFU of the desired *S. typhimurium* strain. For microscopy, mice were infected with 14028 carrying plasmid pWKS30-Tomato. Tomato fluorescent protein was a kind gift from the Tsien laboratory

(299). pWKS30-Tomato was generated by cloning a Shine-Dalgarno sequence and the first half of the tdTomato fluorescent protein (299) into pWKS30. After 2 days of infection, spleens were removed and homogenized using sterile glass slides. The spleen cell suspensions were then digested with collagenase 0.5 mg/ml and DNAse 50 μg/ml in RPMI at 37° C for 25 minutes. Red blood cells in the spleen cell suspensions were lysed by incubation in a hypotonic red blood cell lysis buffer on ice for 5 minutes. Debris and clumps were removed by straining though a 70 µm cell strainer (BD Falcon). The procedure described above for cultured cells was used to load CCF2-AM. To prepare samples for microscopy, CCF2-AM loaded spleen cells were stained with DRAQ5 (Alexis Biochemicals) at a 1:1000 dilution then seeded in Lab-Tek II chamber coverglass slides (Nalge Nunc International).

FACS analysis and sorting

To prepare samples for FACS, spleen cells loaded with CCF2-AM as described above, were first treated with Fc receptor blocking antibodies were (see Table 3-3 for a list of anti-bodies used) for 15 minutes at 4° C, followed by staining with fluorescently conjugated antibodies at 4° C for 15 minutes. FACS analysis was performed using an LSRII (Becton Dickinson) FACS analysis machine equipped with 488, 633, and 405nm lasers. Four cell-surface markers could be simultaneously analyzed along with CCF2-AM fluorescence. Cells were simultaneously stained for four lymphoid specific markers or four myeloid specific markers. The lymphoid specific markers that were analyzed are: CD3, CD4, CD8, and CD19. Myeloid specific markers were: CD11c, CD11b, GR-1, and F4/80 (Table 3-3). Appropriate isotype control antibodies were used to determine the levels of background staining. Parallel samples were stained with propidium iodide to

Table 3-3. Antibodies used for FACS analysis.

Antibody	Clone	Source
Affinity Purified Rat IgG2a, l anti-mouse CD16/32, blocks Fc	93	eBioscience (Cat. #14-0161)
APC conjugated Armenian hamster IgG1,l1 monoclonal immunoglobulin isotype control	G235-2356	BD Bioscience (Cat. #553956)
APC conjugated Armenian hamster IgG1, l2 anti-mouse CD11c monoclonal antibody	HL3	BD Bioscience (Cat. #550261)
APC conjugated rat IgG2a, k anti-mouse CD4	RM4-5	BD Bioscience (Cat. #553051)
APC conjugated rat IgG2a, k monoclonal immunoglobulin isotype control	R35-95	BD Bioscience (Cat. #553932)
APC-Cy7 conjugated rat IgG2b, k anti-mouse GR-1 monoclonal antibody	RB6-8C5	BD Bioscience (Cat. #557661)
APC-Cy7 conjugated rat IgG2b, k monoclonal immunoglobulin isotype control	A95-1	BD Bioscience (Cat. #552773)
APC-Cy7 conjugated rat IgG2a, k anti-mouse CD19 monoclonal antibody	1D3	BD Bioscience (Cat. #557655)
APC-Cy7 conjugated rat IgG2a, k monoclonal immunoglobulin isotype control	R35-95	BD Bioscience (Cat. #552770)
PerCP-Cy5.5 conjugated rat IgG2a, k anti-mouse CD8a	53-6.7	BD Bioscience (Cat. #551162)
PerCP-Cy5.5 conjugated rat IgG2a, k monoclonal immunogobulin isotype control	R35-95	BD Bioscience (Cat. #550765)
PerCP-Cy5.5 conjugated rat IgG2b, k anti-mouse CD11b monoclonal antibody	M1/70	BD Bioscience (Cat. #550993)
PerCP-Cy5.5 conjugated rat IgG2b, k monoclonal immunogobulin isotype control	A95-1	BD Bioscience (Cat. #550764)
PE-Cy7 conjugated Rat IgG2a, k anti-mouse F4/80 monoclonal antibody	BM8	eBioscience (Cat. #25-4801)
PE-Cy7 conjugated rat IgG2a, k monoclonal immunoglobulin isotype control	Not designated	eBioscience (Cat. #25-4321)
PE-Cy7 conjugated Armenian hamster IgG anti-mouse CD3e monoclonal antibody	145-2C11	eBioscience (Cat. #25-0031)
PE-Cy7 conjugated Armenian hamster IgG monoclonal immunoglobulin isotype control	eBio299Arm	eBioscience (Cat. #25-4888)

determine the settings for a live cell gate based on light scatter properties. FlowJo (Tree Star) software was used to analyze the FACS data.

FACS sorting was performed using a FACSVantage SE (Becton Dickinson), with the Digital Vantage option, using the 633, and 488 nm lasers. Spleen cells from infected mice were prepared as described above, but were not loaded with CCF2-AM. Then the cells were stained with myeloid specific antibodies (CD11c, CD11b, GR-1, and F4/80). Cells of the desired populations, as described in the results section, were sorted into RPMI media containing gentamicin 100 μg/ml. Reanalysis of sorted cells determined that >99% purity was achieved.

Cell pathology

Spleen samples from infected mice were prepared for Wright-Giemsa staining as follows. 1 ml of RPMI containing ~1 X 10^4–5 X 10^4 cells were cytospun onto slides using a Cytospin 2 (Shandon) at 1500 RPM for 5 minutes. After air-dry fixing, samples were processed for Wright-Giemsa staining (300, 301). Samples were visualized by microscopy and scored for differentials, counting at least 300 cells per sample. Microscopy pictures of Wright-Giemsa stained slides were captured at 100X magnification using a Microphot-FX (Nikon) microscope equipped with a Magnafire (Optronics) camera and using Magnafire (Optronics) software.

Microscopy on infected cells

Cells prepared as described above were visualized using 60X or 40X oil-immersion lenses along with emission filter sets for blue (457nm) and green (528 nm) fluorescence by CCF2-AM, Tomato (617 nm), and DRAQ5 (685 nm). A UV laser with a

DAPI excitation filter was used for CCF2-AM excitation, a 568 nm laser was used for Tomato excitation, and a 647 nm laser was used for DRAQ5 excitation. *z*-sections (0.2 μm) were captured at a resolution of 1,024 by 1,024 pixels. Images were acquired by Aurelie Snyder of the OHSU-MMI Research Core Facility (http://www.ohsu.edu/core) with an Applied Precision DeltaVision image restoration system. This includes an API chassis with a precision motorized XYZ stage, a Nikon TE200 inverted fluorescence microscope with standard filter sets, halogen illumination with an API light homogenizer, a CH350L camera (500 kHz, 12-bit, 2 Mp, KAF 1400 GL, 1,317 X 1,035, liquid cooled), and DeltaVision software. Deconvolution using the iterative constrained algorithm of Sedat and Agard, and additional image processing were performed using Softworx Explorer Suite (Applied Precision) image processing software.

Determining percentage of intracellular bacteria in various cell populations

To determine intracellular CFUs, non-specifically sorted spleen cells (viable cell gate based on light scatter properties) or specifically sorted spleen cell populations were incubated in RPMI containing gentamicin 100 µg/ml ~2 hours. The cells were then lysed in PBS with 1% Triton X-100 (Sigma) and serial dilutions were plated on LB agar to determine the number of CFU of *S. typhimurium* present per 10^5 host cells. To determine the percentage of intracellular bacteria present in different cell populations, the following formula was used: [(CFU/10^5 specifically sorted spleen cells) X F1] / [(CFU/10^5 nonspecifically sorted spleen cells) X F2] X 100. Where F1 and F2 are the fractions that a specifically sorted cell population or a non-specifically sorted cell population, respectively, represent out of the total spleen cell population. For example, if the CD11b+/GR-1 Hi/CD11c Lo (neutrophils) cell population represented 6% (F1 = 0.06) of

the total spleen cells and contained ~40 CFU / 10^5 cells and if in the same mouse, the non-specifically sorted spleen cells contain ~10 CFU / 10^5 cells and represent 33.4% (F2 = 0.334) of the total spleen cells. Then using the formula above we would calculate: (40 X 0.06) / (10 X 0.334) X 100 = 72%.

Results

Detecting secretion of effector-Bla fusions in cell cultures

We wished to investigate the differences between T3S-1 and T3S-2 secretion *in vivo*. Six effector proteins were selected that display different secretion patterns in cultured cell lines and are directly involved in virulence (65, 97, 138, 141, 219, 265, 294). These secreted proteins were classified into three groups based on their secretion patterns in cultured cells that we had previously reported (138): the T3S-1 dependent group (S1), the T3S-1 and T3S-2 dependent group (S1/2), and the T3S-2 dependent group (S2). S1 includes two proteins, both encoded within *Salmonella* pathogenicity island 1: SipA, secreted exclusively via T3S-1, and SptP, a protein secreted primarily by T3S-1 and at low levels through T3S-2. S1/2 consists of SteA and SlrP secreted via both T3S-1 and T3S-2. S2 consists of SteC and SseJ, proteins secreted exclusively via the T3S-2.

Using effector-Bla fusions, we visualized cells targeted by T3S. We generated chromosomally encoded effector-Bla fusions in *S. typhimurium* strain 14028 to the six T3S effectors listed above and tested them to verify the appropriate secretion patterns in cultured cells. Full-length Bla fusions to each of the effectors were expressed from their natural location on the chromosome in order to maintain their native expression patterns. For these experiments, we used infection conditions under which only T3S-1 or T3S-2 are induced and expressed. The six Bla fusion strains were tested following infection of HeLa cells for 2 hours and the J774 macrophage-like cell line for 10 hours using either T3S-1 inducing or T3S-2 inducing conditions, respectively. Following the infection, the cells were loaded with CCF2-AM (Invitrogen), a Bla substrate that remains localized within the host cell cytoplasm and whose emission spectrum shows a strong shift in

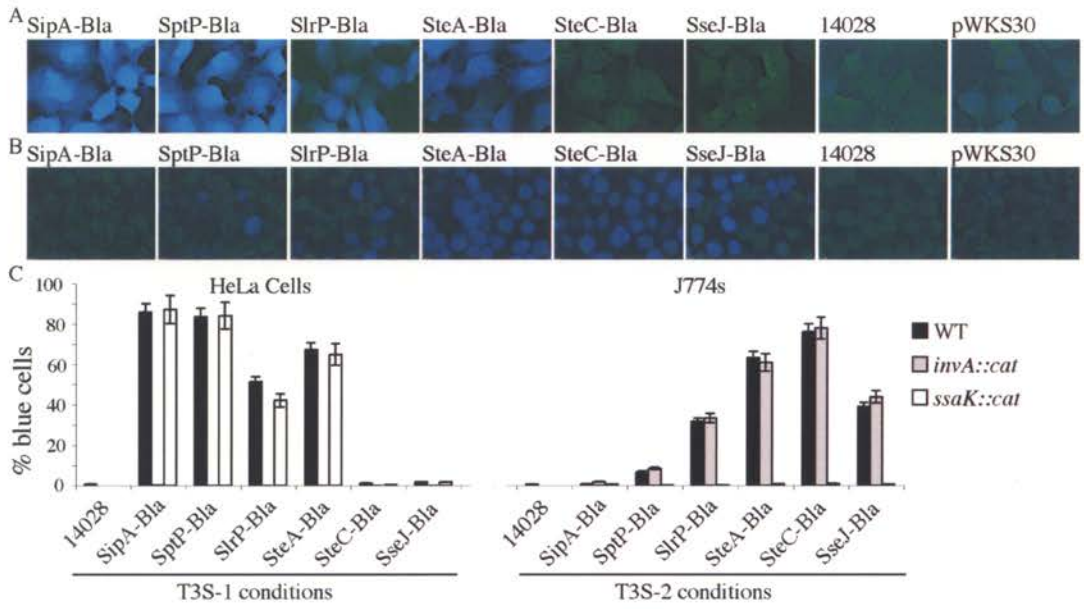


Figure 3-1. Visualization of secretion in HeLa cells (A) and J774s (B) by microscopy (60X magnification). *S. typhimurium* 14028 strains expressing Bla fusions to SipA, SptP, SteA, SteC and SseJ, WT 14028, or 14028 harboring pWKS30 were used to infect HeLa cells for 2 hours using T3S-1 inducing conditions and J774s for 10 hours using T3S-2 inducing conditions. Following the infections, cells were loaded with CCF2-AM and visualized for green and blue fluorescence by microscopy. Green fluorescence indicates CCF2-AM was loaded and the presence of blue cells is evidence of secretion. T3S-1 and T3S-2 dependent secretion detected by FACS analysis (C). HeLa cells and J774s were infected as described above with the six effector-Bla fusions that were expressed in WT 14028, *invA*::*cat* and *ssaK*::*cat* backgrounds. FACS analysis was performed on CCF2-AM loaded cells to determine the percentage of blue cells (positive for secretion). Background percentage of blue cells was set using WT 14028 infected cells. At least 10 000 cells were analyzed for each sample. Each bar represents the mean percentage of blue cells from triplicate samples and the error bars are ± one standard error of the mean.

fluorescence upon cleavage (from green to blue) (302). Therefore, infected cells containing effector-Bla fusion protein in their cytosol will turn blue when loaded with CCF2-AM. Microscopic analysis revealed blue cells among the HeLa cells infected with the S1 and S1/2 fusion strains (Figure 3-1A), indicating CCF2-AM cleavage by Bla under T3S-1 inducing conditions. No blue cells were observed in HeLa cells infected with the S2 fusions or in control infections using the WT parental 14028 strain or 14028 harboring pWKS30, a plasmid expressing a non-secreted Bla protein. In J774s infected with the S1/2 and S2 Bla fusions, blue cells could be seen by microscopy (Figure 3-1B). For the S1 fusions, a low level of secretion was observed in J774s infected with the SptP-Bla strain and no secretion of SipA-Bla was detected in J774s. No blue cells were observed in J774s infected with WT 14028 or 14028 carrying pWKS30. These results confirmed that secretion by the six Bla fusion strains could be detected in cultured cells under the appropriate infection conditions.

Demonstrating the requirement of a functional T3S-1 or T3S-2 for secretion into cultured cells

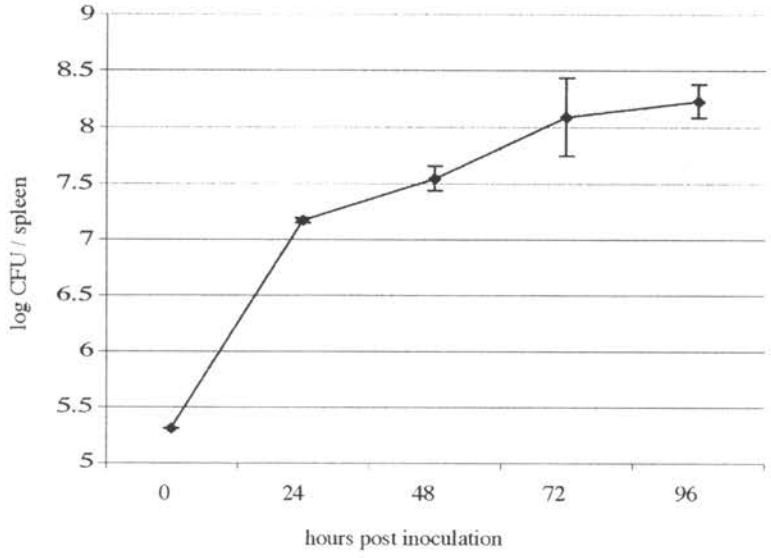
To verify the T3S-1 or T3S-2 dependence of secretion into HeLa cells or J774s, respectively, each of the six Bla fusions was expressed in genetic backgrounds lacking essential structural components of T3S-1 (*invA::cat*), or T3S-2 (*ssaK::cat*). FACS analysis was performed to determine the percentage of CCF2-AM-loaded cells that turned blue when they were infected by strains expressing the Bla fusions (Figure 3-1C). Using T3S-1 inducing conditions for infection, a high percentage of blue cells was detected in HeLa cells infected with the S1 or S1/2 fusion strains. As expected, the secretion in HeLa cells was abrogated in an *invA::cat* background. Similarly, using T3S-2

inducing conditions for infection, FACS analysis detected a high percentage of blue cells in J774s infected with S1/2 and S2 fusion strains and a low percentage of blue cells among J774s infected with SptP-Bla. No secretion was observed in J774s when they were infected with Bla fusions expressed in an *ssaK::cat* background. This preliminary analysis of the six Bla fusion expression strains confirmed that secretion could be detected in cultured cells under the appropriate conditions in the predicted T3S-1 or T3S-2 dependent manner.

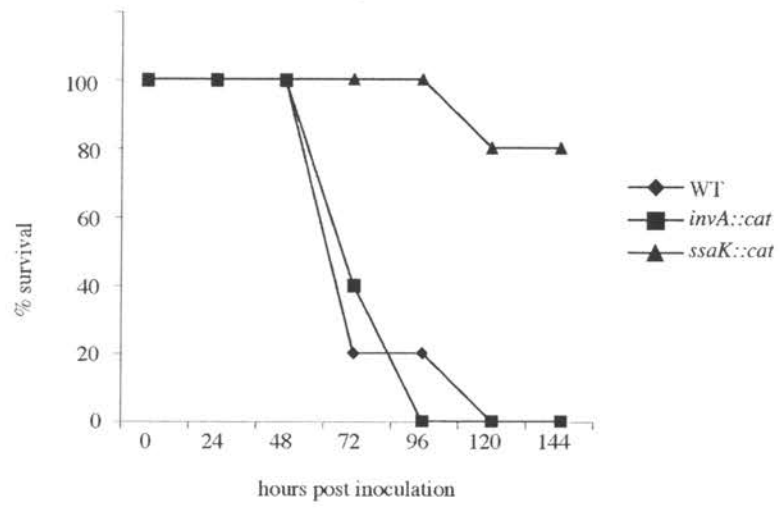
Optimization of infection conditions for detection of secretion *in vivo*

To test secretion in an *in vivo* model, C57BL/6 mice were inoculated via i.p. injection with *S. typhimurium* strains expressing the effector-Bla fusions. Spleens from infected mice were then removed at 24-hour intervals, homogenized, and loaded with CCF2-AM. By counting viable bacteria recovered from the spleen of infected mice, we confirmed that none of the effector-Bla fusion strains exhibited any defect in their ability to colonize and replicate within the spleen. FACS analysis was performed to detect blue spleen cells, or CCF2-AM cleavage. We were only able to detect secretion in mice when the bacterial load in the spleen was greater than ~3 X 10^7 and therefore could not detect secretion within the first 24 hours of infection. However, we observed that if the bacterial load within the spleen exceeded 1 X 10^8, the mice had overt indications of sepsis, such as lethargy, loss of hair, shivering, and ocular discharge. We could reproducibly recover between 3 X 10^7 and 1 X 10^8 bacteria from the spleen 2 days following infection using an i.p. inoculum of 2 X 10^5 (Figure 3-2A) and therefore performed our studies using these conditions.

A


9
8.5
8
7.5
7
6.5
6
5.5
5
log CFU / spleen
0
24
48
72
96
hours post inoculation

B


100
80
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% survival
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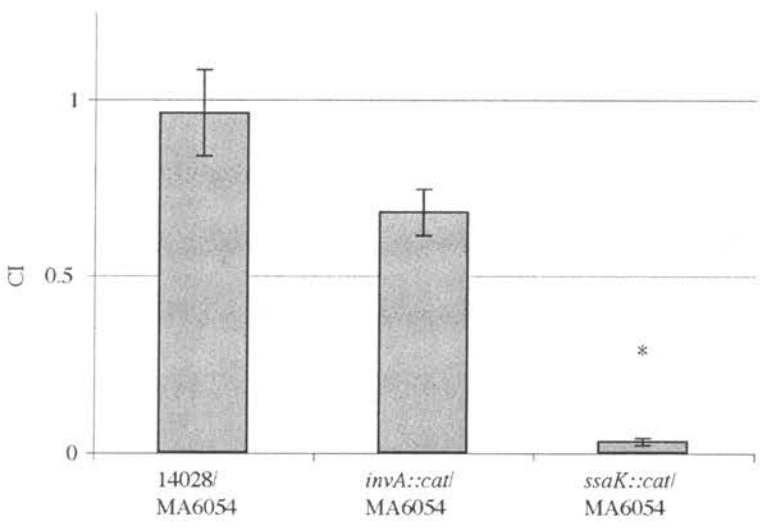
C


1
0.5
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14028/
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MA6054

Figure 3-2. Analysis of mice infected i.p. with an inoculum of 2 X 10^5. The number of bacteria present in the spleen at 24-hour intervals following *S. typhimurium* 14028 infection was determined using at least 3 mice per time-point (A). At 72 hours only 9 of 14 mice survived and at 96 hours only 3 out of 7 mice survived. The graph shows the log of the number of bacteria present ± one standard error of the mean. Mouse survival assays comparing WT 14028, *invA::cat* and *ssaK::cat* were performed using 5 mice per group (B). The percentage of mice surviving at 24-hour intervals following inoculation is shown. 4/5 mice infected with *ssaK::cat* survived for 28 days (not shown). Competitive infections were performed mixing WT 14028, *invA::cat* or *ssaK::cat* with MA6054 at a 1:1 ratio (C). 2 days following infection, the number of both strains of bacteria present in the spleen was determined and used to calculate the competitive index (CI) (see materials and methods). The graph shows the mean CI from 5 mice ± one standard error of the mean. The * indicates that the Student's t-test returned a value of $p < 0.0001$ when compared to the control infection.

Determining the contributions of T3S-1 and T3S-2 in our infection model

Using an i.p. inoculum of 2 X 10^5, we found that mice generally died 3-4 days post infection (Figure 3-2B) and an *invA::cat* strain, defective for T3S-1 function, killed mice with similar timing as the wild type strain indicating that T3S-1 is dispensable for overall killing. However, 4 out of 5 mice infected with an *ssaK::cat* strain, lacking T3S-2 secretion, survived the entire duration of the experiment (28 days), demonstrating the importance of this secretion system for virulence in this model of infection.

We also performed competitive infections to determine the contributions of T3S-1 and T3S-2 to spleen colonization by day 2 of infection using these same infection conditions (Figure 3-2C). For these experiments equal numbers of a mutant strain, *invA::cat* or *ssaK::cat*, were mixed with a reference strain, MA6054, and injected i.p.. Two days following infection, the numbers of each strain recovered from the spleen were determined and used to calculate the competitive index (CI), a ratio of the test strain to the reference strain. The expected CI value for a non-attenuated strain is 1, where equal numbers of the test and reference strains are recovered following infection. However, if the test strain is attenuated then the CI will be less than one because a greater quantity of the reference strain will be recovered. In control infections competing 14028 against MA6054, we calculated a mean CI of 0.96. When *invA::cat* was competed against MA6054 the mean CI dropped to 0.68, not statistically significant by Student's t-test when compared to the control infections ($p = 0.096$). In contrast, *ssaK::cat* was dramatically out-competed by MA6054 yielding a significantly low mean CI value of 0.03 ($p < 0.0001$). This demonstrates that by day 2 using our infection conditions, T3S-2 plays a significant role in spleen colonization, but T3S-1 may not play any role.

Detection of secretion in mice infected with effector-Bla fusion expression strains

At 48, 72, and 96 hours post i.p. injection of Salmonella, varying degrees of secretion could be detected by FACS analysis of CCF2-AM loaded spleen cells. However, due to the fact that many mice were dead 72 or 96 hours post inoculation, we performed the majority of our analysis after 48 hours. Secretion was detected only in mice infected with strains harboring S1/2 and S2 effector fusions (Figure 3-3A). Infections with strains expressing fusions from S1/2 and S2 resulted in significant increase in the average percentage of total spleens cells that turned blue (Figure 3-3B). No secretion was detected in mice infected with the S1 effector fusions. Therefore, the *in vivo* secretion we observed is likely due to the expression of the T3S-2. This finding corresponds well with the virulence data, indicating that T3S-2 but not T3S-1 is required for spleen colonization and morbidity in this acute model of mouse infection.

Identification of spleen cells targeted by the S1/2 and S2 effectors

To identify the cell types that are targeted by T3S-2, spleen cells from mice infected with *S. typhimurium* expressing S1/2 and S2 fusions were loaded with CCF2-AM, then subsequently stained with several fluorescently conjugated antibodies and analyzed by FACS. Using three-laser FACS analysis technology, we were able to simultaneously detect four cell-surface markers, and blue and green CCF2-AM fluorescence. Each sample was split into two groups and stained with a total of eight antibodies. One group was stained with antibodies that recognize cell surface markers specific for T cells and B cells (CD4, CD8, CD3, and CD19) and the second group was stained with antibodies to distinguish macrophages, neutrophils, monocytes and dendritic cells (CD11b, CD11c, GR-1 and F4/80). Table 3-4 summarizes the percentage of each

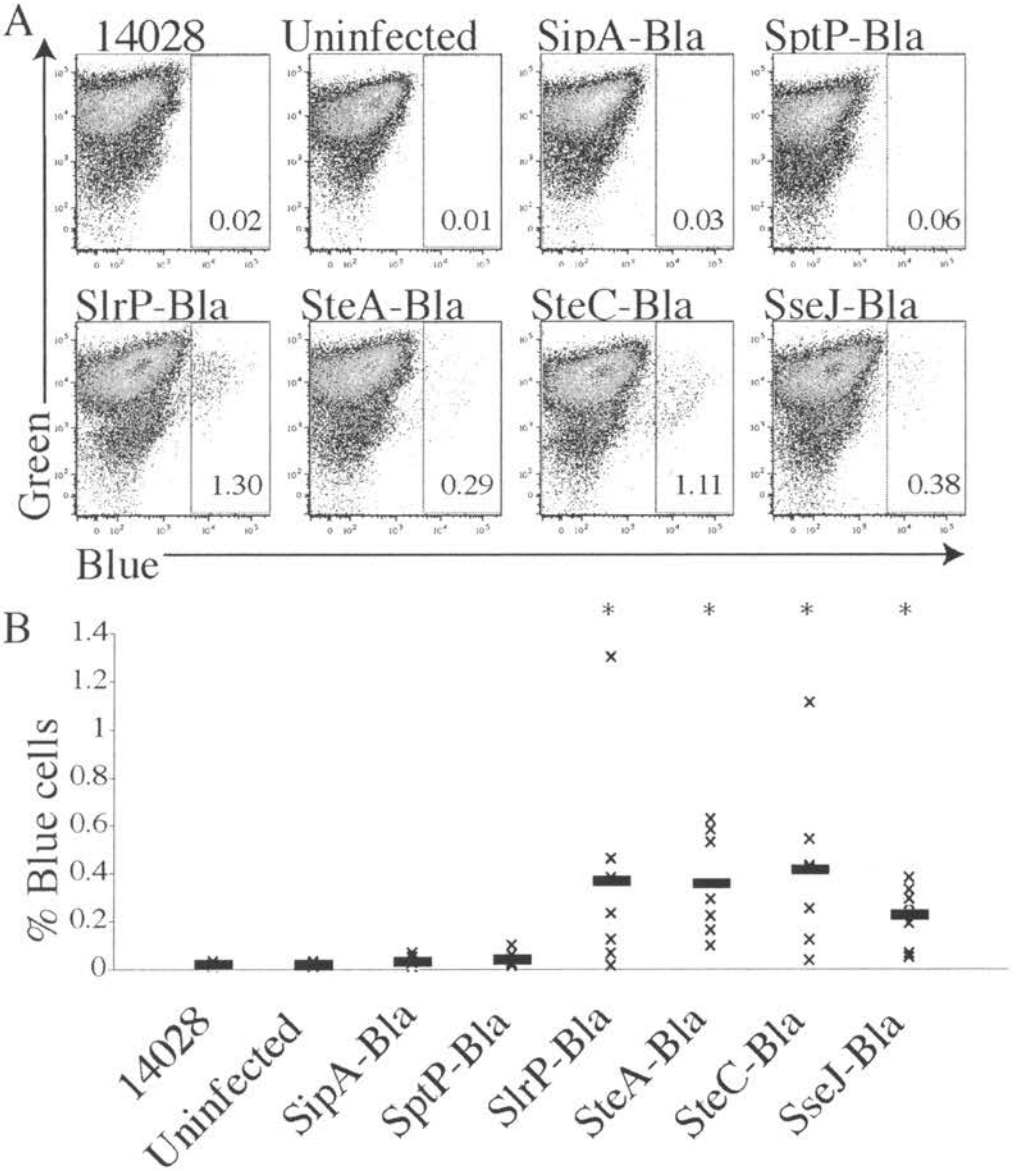

Figure 3-3. Detection of secretion in C57BL/6 spleen cells. FACS data with levels of green and blue CCF2-AM fluorescence in spleen cells from mice infected with each fusion strain (A). Green fluorescence indicates CCF2-AM is present within the cells. The percentage of blue cells, positive for secretion, is shown in the lower right corner of each dot plot. The graph below shows the percentage of total spleen cells emitting blue fluorescence as detected by FACS analysis (B). Each X represents the value for one mouse infected with the indicated strain and horizontal bars represent the average value of 7 mice. The * denotes samples for which the Student's t-test returned a value where $p < 0.05$ when compared to 14028 infected mice.

Table 3-4. FACS analysis of spleen cell from uninfected and infected mice.

			% spleen cells[b]	
		Cell-surface markers	Uninfected	Infected
Cell type	CD4 T cells	CD4+/CD3+	19.1±2.3	14.7±0.8
	CD8 T cells	CD8+/CD3+	8.7±2.2	7.9±0.5
	B cells	CD19+/CD3-	35.9±3.7	40.5±1.4
	DCs[a]	excluded Figure 3A (R1) GR-1+/CD11b+ cells, CD11c+	3.4±0.5	3.8±0.3
	Macrophages[a]	excluded Figure 3A (R1) GR-1+/CD11b+ cells, CD11c-/CD11b+ or F4/80+	2.1±0.7	1.3±0.2
	GR-1+/CD11b+	GR-1+/CD11b+	2.0±0.5	7.6±0.6
	Monocytes[c]	CD11b+/GR-1 Int /CD11c Hi	0.18±0.1	1.5±0.1
	Neutrophils[c]	CD11b+/GR-1 Hi /CD11c Lo	1.8±0.4	6.1±0.5

[a]GR-1+/CD11b+ double positive cells (cells falling in the R1 gate in Figure 4A) were excluded from these populations.
[b]Values shown are averages of at least 5 mice ± one standard error of the mean.
[c]Monocytes and neutrophils are subsets of GR-1+/CD11b+ cells

Table 3-5. Percentage of blue spleen cells representaed by specific cells types from mice infected with effector-Bla fusion expressing *Salmonella* strains.

		% of blue cells represented by specific cell types[a]				
		Salmonella strain				
		SlrP-Bla	SteA-Bla	SteC-Bla	SseJ-Bla	Total[b]
Cell type	CD4 T cells	4.6	5.6	6.4	7.1	6.6±0.4
	CD8 T cells	6.5	7.8	5.8	6.4	7.6±0.7
	B cells	9.8	10.3	6.5	9.9	9.1±0.9
	DCs	1.8	1.2	2.6	2.6	2.1±0.3
	Macrophages	ND[c]	ND[c]	ND[c]	ND[c]	ND[c]
	GR-1+/CD11b+	76	73.9	71.8	71.4	73.2±1.1
	Monocytes[d]	15.1	18.2	20.3	19.7	18.3±1.2
	Neutrophils[d]	60.9	55.7	51.5	51.5	54.9±2.2

[a]The infections were performed as in Figure 2 and the values represent the average percentages of blue cells that were of each cell type from at least three mice.
[b]The Total column is the average value of the four different fusions ± one standard error of the mean.
[c]ND, not detected.
[d]Monocytes and neutrophils are subsets of GR-1+/CD11b+ cells

spleen cell type that was observed in infected and uninfected mice as well as the marker designations used to define each cell type. The percentage of lymphocytes, macrophages, monocytes and neutrophils in infected mice, as determined by FACS, corresponded well with the percentages observed by analyzing Wright-Giemsa stained slides. Table 3-5 shows the percentage of blue cells represented by various spleen cell types in mice infected with the S1/2 or S2 fusions. There was no statistically significant difference in the cell types targeted by the S1/2 and S2 effectors. Most surprisingly, no secretion was detected in mature macrophages. Varying degrees of secretion could be detected in all other cell populations analyzed including CD4 T-cells, CD8 T-cells, B-cells and dendritic cells (DCs). However, most of the secretion (~73% of blue cells) was found in GR-1+/CD11b+ cells.

Analyzing the GR-1+/CD11b+ cell population

Wright-Giemsa staining of FACS sorted GR-1+/CD11b+ cells from infected mice revealed that these cells consist of neutrophils (~80%) and monocytes (~20%) (Figure 3-4 A and C). FACS analysis also revealed that GR-1+/CD11b+ cells could be further subdivided into two clearly distinct populations based on GR-1 and CD11c levels: GR-1 Hi/CD11c Lo and GR-1 Int/CD11c Hi (Figure 3-4A right panel, gates R2 and R3, respectively). Wright-Giemsa staining of FACS sorted cells from infected mice demonstrated that GR-1 Int/CD11c Hi cells are mostly monocytes (>72%) and GR-1 Hi/CD11c Lo cells are mostly neutrophils (>90%) (Figure 3-4C). The FACS data indicated that the GR-1 Int/CD11c Hi cells (mostly monocytes) represent ~18% of the secretion positive cells while the GR-1 Hi/CD11c Lo cells (mostly neutrophils) represent the greatest percentage of the targeted cells (~55% of blue cells) (Table 3-5).

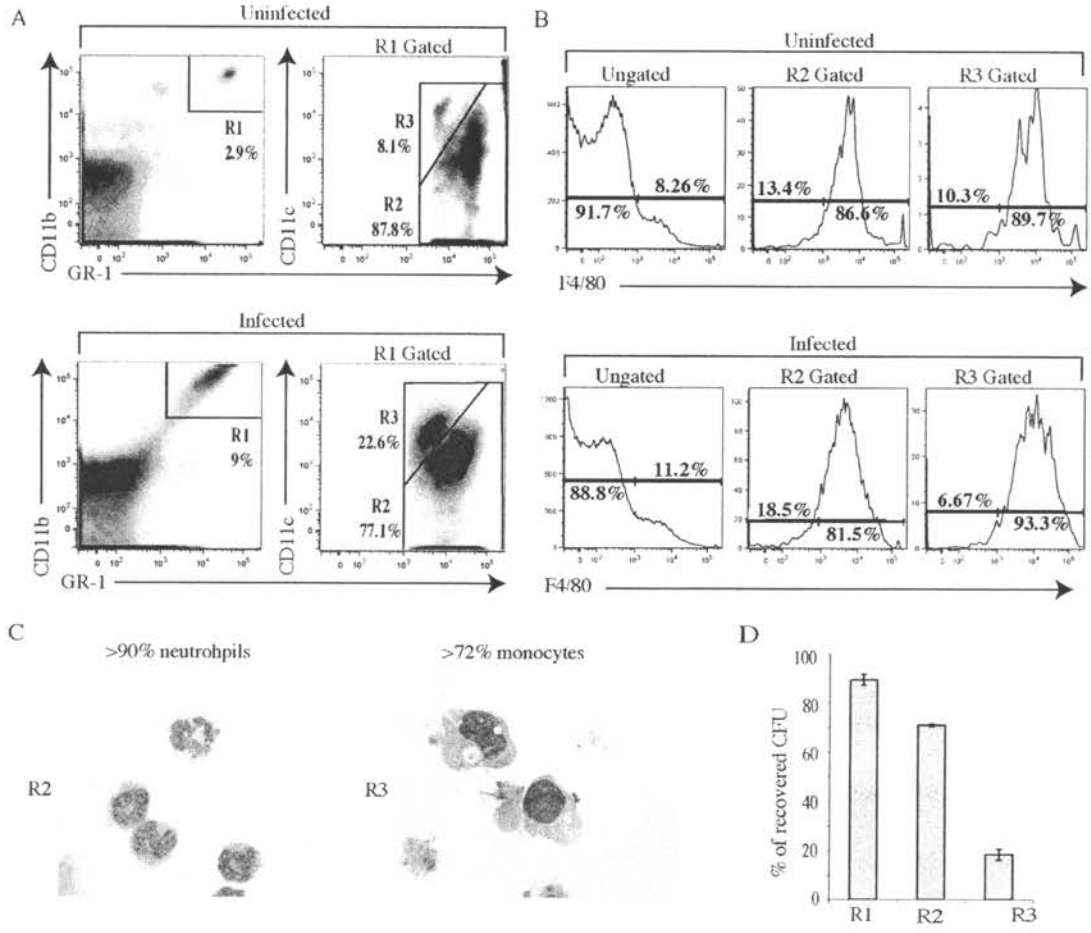

Figure 3-4. GR-1+/CD11b+ cells consist of neutrophils and monocytes. FACS analysis was performed on spleen cells from uninfected C57BL/6 mice or mice infected with 14028 for 2 days (A). The level of GR-1 and CD11b in total viable spleen cells is shown in the density plots on the left. GR-1+/CD11b+ cells (R1 gate) were analyzed for CD11c and GR-1 expression levels in the density plots to the right. The level of F4/80 expression for ungated cells (all analyzed cells), R2 and R3 gated cells are shown in the histograms (B). GR-1 Hi/CD11c Lo (R2) cells and GR-1 Int/CD11c Hi (R3) cells from infected mice were FACS sorted, then cytospun onto slides, and stained with Wright-Giemsa stain and visualized by microscopy (C). 300 cells from Wright-Giemsa stained slides were analyzed and the percentage of neutrophils and monocytes was determined. The percentage of total intracellular *Salmonella* in GR-1+/CD11b+ cells was estimated (D). GR-1+/CD11b+ cells (R1), GR-1 Hi / CD11c Lo (R2), GR-1 Int / CD11c Hi (R3) populations were FACS sorted, lysed, then plated on LB to determine the number of intracellular CFU. The percentage of total recovered CFU was then calculated for each FACS sorted population (see materials and methods). The graph shows the average percentage total recovered CFU present in each FACS sorted population from three mice and the error bars represent one standard error of the mean.

It is important to note that in a previous study, F4/80 positive spleen cells containing *Salmonella* 2 days post i.p. inoculation were defined as macrophages (42). However, we find that F4/80 is expressed on most GR-1+/CD11b+ cells regardless of CD11c expression (Figure 3-4B) indicating that F4/80 on its own cannot distinguish monocytes from neutrophils. In uninfected mice, the majority of the spleen cells expressing F4/80 are monocytes and macrophages, however, following infection we find that most of the spleen cells expressing F4/80 are neutrophils (Table 3-4 and Figure 3-4A).

Determining whether GR-1+/CD11b+ cells contain viable bacteria

The GR-1+/CD11b+ neutrophils and monocytes are the cell types whose frequencies increase the most dramatically in the spleen following infection (Table 3-4, Figure 3-4A). These are infiltrating phagocytic cells that play a crucial role in innate immunity. We wished to determine whether secretion in these cells correlated with the presence of viable intracellular bacteria. GR-1+/CD11b+ cells were FACS sorted, lysed, and plated on LB agar to determine the number of CFU present. GR-1+/CD11b+ cells consistently contained most of the total intracellular CFU recovered from the spleen of infected mice (Figure 3-4D, ~90% on average). Determination of CFU counts in FACS sorted GR-1 Int/CD11c Hi and GR-1 Hi/CD11c Lo populations revealed that ~70% of the intracellular CFUs are associated with neutrophil enriched cells while only ~20% were associated with monocyte enriched populations.

Determining whether targeted cells contain intracellular Salmonella

We wanted to determine whether secretion in cells was associated with intracellular *Salmonella*. However, attempts to FACS sort blue were unsuccessful due to

the rarity of these cells, the weakness of the signal generated and because the machines used for FACS sorting are less sensitive than the machine we used for FACS analysis. Therefore, we used microscopy to visually inspect blue cells for the presence of bacteria. *S. typhimurium* simultaneously expressing SteA-Bla and harboring pWKS-Tomato (a red fluorescent protein expression vector) was used to infect C57BL/6 mice via i.p. inoculation. After two days, the spleen was homogenized and loaded with CCF2-AM and stained with the nuclear stain DRAQ5. Visually scanning slides through microscopic examination revealed dozens of blue cells among tens of thousands of green cells (Figure 3-5). It is likely that microscopic examination of blue cells is less sensitive than FACS analysis, since only the brightest blue cells could be detected with confidence. We estimate that the number of blue cells detected by eye, represent less than 0.1% of the cells on the slides. Although we could not conclusively identify the cell types, every blue cell, out of dozens examined, also contained red-fluorescent bacteria. 3D reconstruction of z-sections that were taken of the blue cells confirmed that the bacteria associated with these cells are intracellular (Data not shown). Conversely, some cells that contained bacteria had not turned blue (Figure 3-5, left panel). Taken together, our data strongly suggests that the secretion in GR-1+/CD11b+ neutrophils is a result of viable intracellular *Salmonella*.

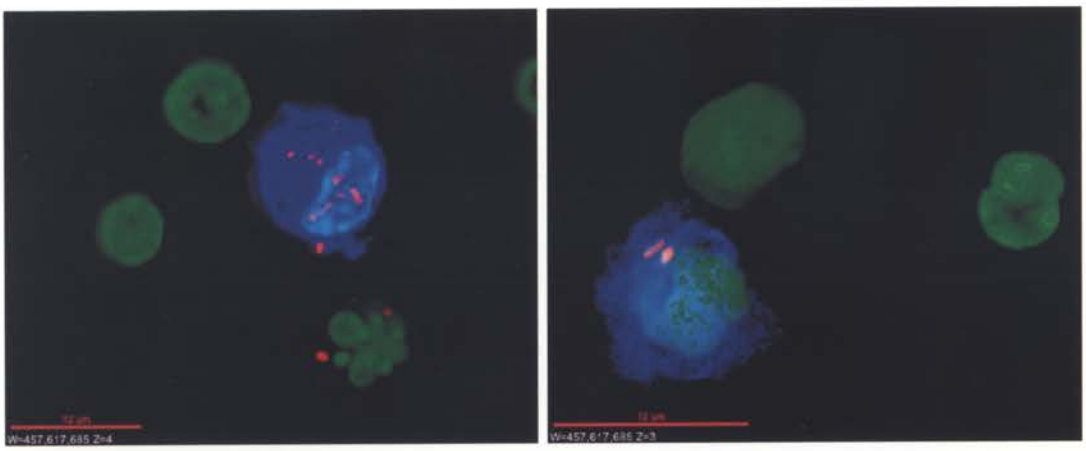

Figure 3-5. Microscopic analysis reveals that blue spleen cells contain intracellular *Salmonella*. Mice were infected i.p. for 48 hours with 14028 expressing a chromosomal SteA-Bla construct and harboring pWKS30-Tomato, a red fluorescent protein expression vector (shown in red). Spleen cell suspensions were prepared then loaded with CCF2-AM (blue) to detect secretion and a DNA stain, DRAQ5 (green), to visualize nuclei. The images in the left and right panels were taken at 40X and 60X magnification, respectively. The red reference bars represent 12 μm in both panels.

Discussion

This is the first report detailing the cell types targeted by *Salmonella*'s T3S during a course of infection. We investigated *in vivo* secretion patterns of effectors with different secretion patterns in cell culture, and found that only effectors capable of passing through T3S-2 were secreted into mouse spleen cells. This correlated well with the observation that only T3S-2 was required for lethality and spleen colonization in our infection model. We detected secretion in all cell types examined, except mature macrophages. Since the role of *Salmonella*'s T3S in subverting host cell function is well documented, our results imply that *Salmonella* targets and manipulates the function of both innate and adaptive immune cells in order to promote survival within its host. The importance of the role of the innate immune response during this early stage of infection was highlighted by the fact that most of the secretion was found in neutrophils and monocytes. Neutrophils were the most frequently targeted cell type and the major reservoir for intracellular bacteria. In addition, the presence of fluorescently labeled bacteria in secretion positive cells indicates that secretion is likely to be a result of viable intracellular *Salmonella*. These results demonstrate the importance for *Salmonella* to control the function of innate immune cells, especially neutrophils, at an early stage of infection in order to establish itself within the host.

Salmonella interactions with mature macrophages and monocytes

We found that *Salmonella* targets a very specific subset of newly recruited splenic monocytes. Although tissue monocytes can be referred to as macrophages, we make this distinction because these cells are morphologically distinct from mature macrophages based on Wright-Giemsa staining. These monocytes express CD11b, intermediate levels

of GR-1, and high levels of CD11c. Another study has suggested that *Salmonella* infects a specific subset of highly phagocytic macrophages (42). We concur with this hypothesis and believe that the presence of bacteria and secretion in these monocytes is most likely because they are highly phagocytic. These monocytes are probably precursors for the apoptotic mature macrophages that have been observed by others at later times of infection (44). It was surprising to see a complete absence of secretion in mature macrophages. However, it is likely that *Salmonella* killed the mature macrophages *in vivo* and dead or dying cells were excluded from our FACS analysis. This might explain the decrease in the percentage of mature macrophages in the spleen by day 2 of infection (Table 3-4).

Salcedo et al. found in a previous study that most of the intracellular *Salmonella* were in macrophages by the second day following i.p. infection (42). We found that using similar infection conditions as Salcedo et al., by two days post inoculation, most of the bacteria were inside neutrophils. The cell surface markers used to identify macrophages by Salcedo et al., including F4/80, may have confounded their findings. Although F4/80 is considered a relatively specific marker for macrophages, we find that during *Salmonella* infection, this marker is present on most CD11b+/GR-1+ cells and cannot distinguish splenic monocytes from neutrophils.

Salmonella survival in neutrophils

We were surprised to find that most of the targeted cells and cells containing viable *Salmonella* were neutrophils because these cells are generally considered too bactericidal to allow for intracellular bacterial survival. Neutrophils produce defensins and other antimicrobial proteins that are thought to inhibit bacterial growth and prevent

host colonization (303). For example, neutrophil elastase (NE) has been shown to degrade components of *Salmonella*'s T3S-1 *in vitro* (304). Our results indicate that despite the presence of NE, *Salmonella* is still capable of secreting virulence factors into neutrophils *in vivo*. In addition, neutrophils generate a robust NADPH oxidase dependent respiratory burst that contributes to bacterial killing (305, 306). However, *Salmonella*'s T3S-2 is known to prevent normal NADPH oxidase function in macrophages (208, 209), and presumably disrupting NADPH oxidase activity could also promote bacterial survival in neutrophils.

Dunlap et al. found that most of the *Salmonella* was present within neutrophil 24 hours after i.v. inoculation (43). However, the significance of the presence of *Salmonella* in neutrophils is disputed since most researchers believe that neutrophils kill *Salmonella*. This is primarily due to several reports indicating that neutrophils kill *Salmonella* efficiently *in vitro*, at least at early time points after infection (two hours or less) (307-311). However, in other studies the number of *Salmonella* in neutrophils began increasing after the initial killing phase (312-314). This pattern of an initial killing phase followed by replication is identical to the pattern of growth seen in primary macrophages. In fact, in the past, most people believed that macrophages kill *Salmonella*. However, it is now widely accepted that overall *Salmonella* survives and grows in macrophages, but not in neutrophils.

Based on our results and those of Dunlap et al., it seems that *Salmonella* can in fact survive within neutrophils just as they survive within macrophages. Therefore the presence of T3S-2 is likely to play a critical role in intracellular survival in neutrophils. Cheminay et al. tested this possibility by analyzing the growth of *Salmonella* lacking a

functional T3S-2 in neutrophils (315). However, in their study the infection was of short duration and any impact of T3S-2 was likely too subtle to be detected. Therefore, to fully assess the role of T3S-2 on survival within neutrophils, we are comparing various deletion mutations *in vivo* and in neutrophil infection models.

Depletion studies of neutrophils to determine their role in *Salmonella* infections

Cheminay et al found that a T3S-2 deficient strain was still attenuated in mice following GR-1 antibody-dependent cell depletion, and this was interpreted as meaning that T3S-2 is not involved in neutrophil survival *in vivo* (315). However, we feel that neutrophil depletion studies using antibodies to GR-1 have been inconclusive. We found that GR-1 is expressed on splenic monocytes as well as neutrophils. Others have observed that peritoneal macrophages and Kupffer cells (liver macrophages) are also depleted following GR-1 antibody injection during *Listeria monocytogenes* infection of mice (316, 317). Therefore, the interpretation of GR-1 depletion studies becomes more complicated. It is unlikely that many people would argue that T3S-2 does not play a role in survival within the macrophages depleted by GR-1 antibody. Instead this suggests that a T3S-2 dependent niche for *Salmonella* replication still exists in the absence of GR-1 expressing cells, but the role of T3S-2 in GR-1 expressing cells is not determined.

Is there any direct evidence that neutrophils kill *Salmonella in vivo*? Since neutrophil depletion results in increased bacterial load (315, 318-320), it is generally interpreted as meaning that bacteria are not efficiently killed in the absence of neutrophils. However, our data indicate that from one to two days post infection, when most of the *Salmonella* in the spleen are within neutrophils, the number of *Salmonella* continues to increase (Figure 2A). One possibility is that a high death rate caused by

neutrophil killing is compensated by an even higher rate of replication in another cell type. However, two studies using different methods to measure both death and growth rates of *Salmonella* in mice found that the death rate is actually very low (321, 322). In fact in one study, the rate of bacterial killing from 4-44 hours post i.v. infection was negligible (321). An explanation for all these observations would be that *Salmonella* grows poorly within neutrophils, but is not killed.

Our work shows that *Salmonella* injects virulence factors into neutrophils and that viable bacteria can be recovered from these cells, indicating that they are capable of surviving within these cells. It appears that evolution has honed the ability of this remarkable bacterial pathogen to combat even the harshest of intracellular environments. It is clear that neutrophils play a key role in controlling *Salmonella* infection. However, these findings challenge the current paradigm that *Salmonella* evades neutrophil killing *in vivo* by hiding within macrophages. Rather *Salmonella* appear to use its T3S to moderate neutrophil function. It is likely that a balance between *Salmonella* killing by neutrophils and *Salmonella* survival within neutrophils is established *in vivo*. In some cases neutrophils can prevent *Salmonella* penetration into systemic sites, whereas in other cases, *Salmonella* survives within neutrophils and thrives at systemic sites such as the spleen. This concept has significant implications for understanding the pathology of human disease and may provide an important clue as to what factors determine whether *Salmonella* causes gastroenteritis or septicemia.

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Chapter 4: Discussion

I. Summary

Type III secretion systems are essential for *Salmonella* pathogenesis. A role for *Salmonella*'s T3Ss has been identified for practically every aspect of infection. However, the precise mechanisms by which T3S-1 and T3S-2 contribute to certain aspects of infection remain poorly defined. To fully comprehend the role of T3Ss in Salmonellosis there are three essential requirements: I, identifying all secreted effectors, II, identifying all phenotypes associated with T3S, and III, identifying the targets of all the effectors. In the experiments presented Chapters 2 and 3, we made contribution to all three of these areas. First, we identified three new secreted effectors: SteA, SteB and SteC. Second, we identified a virulence phenotype for SteA and determined its subcellular localization. Third, we identified neutrophils as the primary spleen cell type targeted by *Salmonella*'s T3S-2 during mouse infection.

II. Identifying all secreted effectors

The work described in this dissertation has brought us one step closer to the goal of identifying all of *Salmonella*'s effectors. Through the screening strategy described in Chapter 2, we identified three new effectors as well as several previously identified effectors. Still, we believe that many more effectors yet remain to be identified.

A. Potential for using mini-Tn5-cyler to identify more *Salmonella* effectors

It is probable that continued screening using the mini-Tn5-cycler transposon would yield additional secreted proteins. Screening *Salmonella* mini-Tn5-cycler insertion libraries using different cell lines, and different infection conditions will likely yield

additional effectors. For example, extending the infection time to 22 hours may identify other effectors, such as SseK2, that have a late secretion phenotype (204). Additional screening in various cell lines could identify effectors preferentially secreted into specific cell types. We screened approximately 30,000 additional mini-Tn5-cyler mutants using a dendritic cell line, JAWSII, however, this effort did not yield any secreted proteins that were not identified in J774s. Based on our finding that neutrophils are the preferred target for T3S-2 *in vivo*, a neutrophil cell line would be an excellent candidate for screening for additional effectors.

B. Identification of effectors in other pathogenic bacteria

The most promising application for mini-Tn5-cylcer is for other pathogenic organisms that are known to have T3S, but that have few or no known effectors. Several other groups are attempting to use our system in other pathogens, such as *Burkholderia pseudomallei* and other pathogenic bacteria known to have T3Ss (323), but with few known effectors (only one effector has been dentified so far in *B. pseudomallei* (324)). In addition, since the CyaA' secretion is promiscuous and it is known to be secreted via several secretion systems (274, 292, 325), our system can potentially be used to identify secreted proteins in pathogenic bacteria that do not necessarily possess known secretion systems. Efforts are currently under way in our lab to modify the mini-Tn5-cycler transposon for use in *Francisella tularensis*, an important human pathogen and potential bio-warfare agent with no known virulence associated secretion systems.

C. Limitations on using mini-Tn5-cycler for finding more effectors in *Salmonella*

Due to transposition hot spots, such as the one we found in SlrP, insertions in some genes may be extremely rare. Therefore, it may take a herculean effort in order to screen a library large enough to completely saturate the genome. An alternate approach would be to systematically delete genes encoding all the known effectors and then screen a library in a *Salmonella* background that is lacking all the known effectors (thereby avoiding identification of effectors that are already known). Unfortunately, a mutant lacking all known effectors will be severely attenuated, as it will not invade epithelial cells, will not survive well in macrophages, and may not have normal T3S activity. Therefore, it is doubtful whether this strategy would work. Another limitation to the mini-Tn5-cycler transposon is that insertions can only occur in permissive sites. Therefore the transposon insertion cannot occur in a region that disrupts secretion or virulence either by disruption of the gene that it inserted into or due to polar effects on downstream genes.

D. Alternative strategies for identifying new *Salmonella* effectors

Alternative strategies for identifying *Salmonella* secreted effectors are currently being developed by our laboratory. The availability of the genome sequence for several *S. enterica* serovars makes computational approaches feasible. Efforts are underway to generate computer algorithms that will reliably identify secreted effector proteins. Using data compiled from a combination of computer algorithms, *in vitro* and *in vivo* gene expression and proteome studies, as well as results from virulence analysis, we have

generated a list of candidate genes that are currently being tested to determine whether they are secreted effectors.

III. Identifying all phenotypes associated with T3S

Identifying all the effectors is just the first step in fully understanding the functions of *Salmonella*'s T3Ss. Once all the effectors have been identified, the roles of these effectors during infection must be determined. The role of an effector in *Salmonella* virulence only becomes evident when a phenotype is associated with that effector.

A. Virulence phenotypes of SteA, SteB and SteC

The simplest phenotype to assay for is overall virulence in a mouse model or in cell cultures. We found that of the three new effectors we identified, only SteA is involved in mouse virulence. Interestingly, SteA was also targeted to the Golgi network in infected cells. This localization may be of significance since interactions with the Golgi appear to be involved in mediating Sif formation and intracellular survival. SteB and SteC are not involved in mouse infection. However, SteC was required for infection in a chick model, another model for systemic disease (294). It is possible that SteC is a host adapted virulence factor that is not required for virulence in mice. However, its conservation in *S. typhi* implies that it is likely to play a role in systemic disease in humans as well, since *S. typhi* is a host adapted pathogen that only causes typhoid in humans. No phenotype was identified for SteB, however, its presence in another human pathogen encoding a T3S, *Chromobacterium violacium* (295), would lead one to speculate that SteB is likely involved in the pathogenesis of these oganisms.

B. Differential secretion phenotype

We found that effectors have different secretion characteristics. Another group found that effectors whose expression is regulated by *ssrB* were secreted only via T3S-2 and other effectors with an unknown regulatory mechanism are secreted via both T3S-1 and T3S-2 (215). We also found that effectors regulated by *ssrB* are only secreted by T3S-2 and that another group of effectors is secreted via both T3S-1 and T3S-2. In addition, we identified one effector, SipA, strictly dependent on T3S-1 for secretion into host cells. Interestingly, two effectors secreted via T3S-1 and T3S-2, SptP and AvrA, are actually encoded in SPI-1. The dual secretion characteristic had not been previously reported for either of these proteins and their secretion was thought to be restricted to T3S-1. Interestingly, AvrA is involved in inhibiting Nf-κB dependent inflammatory responses, a phenotype associated with T3S-2 (260, 262). In cell cultures, SptP is secreted at very low levels through T3S-2, however, we did not detect any secretion of SptP in the mouse spleen in our *in vivo* analysis. Although T3S-2 mediated secretion of SptP does not appear to contribute to systemic virulence, we think that it is possible that T3S-2 mediated translocation of SptP is important for the reversal of actin polymerization during epithelial cell invasion.

We attempted to identify the regulator(s) for SteA expression, an effector secreted by both T3S-1 and T3S-2. *S. typhimurium* strains containing translational and transcriptional β-galactosidase fusions to SteA were generated then mutagenized with a transposon. Transcriptional β-galactosidase fusions to SteA were light blue when plated on LB, or N-salts media (a media for inducing T3S-2 expression) containing X-gal. However, after screening >50, 000 transposon mutants for changes in β-galactosidase

activity, we isolated seven unique insertions in β-galactosidase itself. We could not find an *in vitro* condition in which the translational fusion of β-galactosidase to SteA was expressed and none of the transposon insertions resulted in increased expression. Therefore, the identity of the regulator responsible for regulating SteA expression, or any of the proteins secreted by both T3S-1 and T3S-2 remains unknown. It is possible that the regulator of SteA transcription and/or translation is required for bacterial metabolism and transposon insertions in the gene encoding the regulator are non-viable. Another possibility is that the regulator is an extremely small target, such as an anti-sense RNA molecule, and the probability of transposition into the target is extremely low.

C. Identification of a new 'fast trafficking' phenotype

One reason why the role of some effectors has eluded researchers is because the processes that they mediate have not been identified. Recently, our laboratory discovered that a previously described phenotype in *Salmonella* infected mice requires T3S-2 and is mediated in part by the secreted effector, SrfH (326). *Salmonella* can be found in the bloodstream within CD18 expressing monocytes just 30 minutes after oral inoculation (41). This phenotype was originally thought to be part of a host antigen sampling mechanism and an entirely passive event on the part of the bacteria. However, we found that bacteria required the presence of T3S-2 in order to reach the bloodstream and that this phenotype was largely dependent on SrfH (Figure 4-1). Prior to this discovery, the role of SrfH was unknown, as it did not have any overt virulence defect. Now we have discovered a 'fast trafficking' phenotype where T3S-2 is directly involved in accelerating the spread of *Salmonella* to systemic sites of infection.

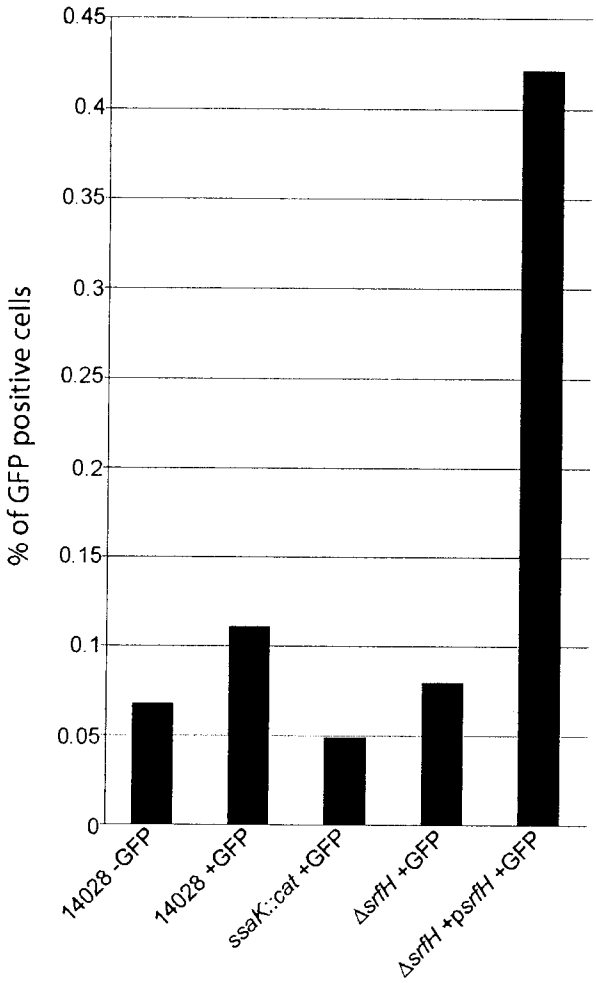

Figure 4-1. The requirement for T3S-2 and SrfH in order for GFP expressing *Salmonella* to reach the bloodstream in infected mice. Mice were orally inoculated with *S. typhimurium* 14028 derivatives expressing GFP. The percentage of GFP positive cells, containing *Salmonella*, was determined by FACS analysis of peripheral white blood cells that were isolated after 30 minutes of oral infection. (Cells from three mice were pooled and at least 500, 000 cells were analyzed for each sample.) The percentage of GFP positive cells following infection using a *ssaK*::*cat* background (*ssak*::*cat* +GFP), lacking T3S-2, is similar to background auto-fluorescence (wild type infected, but no GFP present (14028 -GFP)). The percentage of GFP positive cells is reduced in Δ*srfH* background (Δ*srfH* +GFP) when compared to wild type GFP (14028 +GFP) infected mice. When Δ*srfH* is complemented with a plasmid expressing SrfH (Δ*srfH* +p*srfH* +GFP), there is a dramatic increase in the number of GFP positive cells present in the bloodstream.

The discovery of this 'fast trafficking' phenotype illustrates the importance in identifying new phenotypes mediated by *Salmonella*'s T3Ss. There are several effectors, such as SteB that do not have any known affect on *Salmonella* virulence, and it is likely that some of the processes that these effectors mediate have simply not yet been discovered. One potential obstacle to discovering new phenotypes is the wide host-range of some *S. enterica* serovars, such as serovar *typhimurium* that we use in our laboratory. Some effectors, such as SteC, have host specific effects and their role in virulence will only be evident in the appropriate host. Several laboratories are developing new animal model systems for studying *Salmonella* infections, and these are likely to be important tools for furthering our understanding of the role of T3Ss in Salmonellosis.

IV. Identifying the targets of all the effectors

Identification of the targets of secreted effectors is critical in understanding the contribution of effectors to *Salmonella* virulence. The targeted host cell must be identified, because some effectors may mediate cell-type specific processes. In addition, the molecular targets of the effectors must be identified, in order to fully comprehend the mechanism by which a specific effector manipulates a host cell.

A. Identification of cell types targeted by Salmonella's T3Ss

The manuscript presented in Chapter 3 is the first report of the cell types targeted by *Salmonella*'s T3Ss infection during a course of infection. We found that only T3S-2 dependent secretion was detected in the spleen during acute infection of a mouse. T3S-2 targeted all cell types we examined except for mature macrophages. Interestingly, the primary cell type targeted was neutrophils. In addition, most of the viable *Salmonella* was

associated with neutrophils, suggesting that T3S-2 mediates intracellular survival within these cells. This was a very surprising result since macrophages are generally believed to be the main reservoir of *Salmonella* during the systemic phase of infection. However, now that it is evident that *Salmonella* injects effectors into neutrophils, many new questions arise. Is T3S-2 mediated *Salmonella* survival in neutrophils similar to survival in macrophages? Some groups have shown *Salmonella* survive in neutrophils (312, 314). The fact that the oxygen dependent killing mechanisms of neutrophils and macrophages are similar suggests that T3S-2 mediated *Salmonella* evasion of these processes will be similar in both cell types (306). Do some T3S-2 effectors have specialized roles for survival in neutrophils? Since neutrophils possess oxygen independent killing mechanisms that are not present in macrophages (303, 304), it is possible that some effectors specifically mediate survival of these oxygen independent killing mechanisms. Does *Salmonella* mediate T3S dependent cell death in infected neutrophils? *Salmonella* infection causes a delayed cell death in primary human neutrophils (after ~24 hours) however the role of T3Ss in this process has not been examined (312). Do T3S-2 effectors alter cytokine production or other inflammatory responses in neutrophils? The answer to this and many other questions is unknown. It is clear that the finding that neutrophils are an important target for *Salmonella*'s T3S has opened many new avenues of research.

Our report was the first study of the *in vivo* targets of *Salmonella*'s T3Ss. Similar studies can be performed to identify T3S targets in various hosts and organs following different infection conditions. For example, it would be interesting to determine the cell types targeted during the intestinal phase of *S. typhimurium* infection of a calf. Perhaps

during the intestinal phase of infection only T3S-1 dependent secretion will be detected after 24 hours, however during later stages of intestinal infection T3S-2 dependent secretion will be detected, mirroring the requirement of these secretion systems for intestinal inflammation at different times of infection (258). It would also be interesting to see what cell types are targeted by T3S-1 and T3S-2 during intestinal inflammation, as it may provide a clue as to how these secretion systems interact to mediate the inflammatory responses seen in a cow.

B. Identification of the molecular targets of effectors

Identification of the molecular targets of effectors is essential for understanding the mechanisms by which an effector manipulates host cell processes. Unfortunately, this aspect of effector characterization has proven extremely difficult. Two common approaches for identifying binding partners for effector proteins are two-hybrid screens, and co-immunoprecipitation (co-IP) assays. These approaches have had a limited success rate for a variety of reasons. For example, we wished to identify a eukaryotic binding partner for SteA by screening a yeast two-hybrid library. However we were unable to perform the screen because SteA expression causes high transactivation of the yeast reporter strain. This high level of transactivation means that the signal to noise ratio is very low and the probability of isolating false-positive interactions is high. Breaking SteA down into smaller domains did not resolve this problem and therefore it was decided that SteA was simply not a good candidate for this yeast two-hybrid system. Co-IP assays are often limited by the sensitivity of the methods used to identify host proteins interacting with the effector protein. Hopefully, advancements in the technology for proteomic analysis will improve the sensitivity of protein identification and this should

improve the usefulness of this approach. Although the existing means of identifying binding partners for effectors have limitations, improvements in existing technology and development of new technology, such as protein arrays, will improve the success rate of the efforts to identify the molecular targets of effectors.

Even when a binding partner of an effector protein is identified, the function of the target host cell protein is often unknown. For example, SpiC binds both TassC and Hook3, however the function of these proteins is poorly defined and the way in which these interactions contribute to manipulating vesicle trafficking and fusion in infected cells is not known (241, 243). However as more research is performed in areas of cell biology relating to vesicle trafficking and fusion, the role of TassC and Hook3 in *Salmonella* pathogenesis will become clearer. Our laboratory performed a yeast two-hybrid screen and identified the eukaryotic binding partner of SrfH as Trip6 (326). When this discovery was first made, the function of Trip6 was very poorly defined. However, several reports emerged that implicated Trip6 in regulating cell motility and inflammatory pathways (327-332). It was this revelation that lead to the discovery of the fast trafficking phenotype and helped us understand the role of SrfH in *Salmonella* pathogenesis. Clearly some luck is involved and it was very fortunate that Trip6's role in mediating cell motility was discovered.

V. Conclusions

Many challenges remain in order to fully comprehend the roles of T3Ss in *Salmonella* infection. Discovery of more new effectors, such as SteA, SteB, and SteC, will eventually lead to identification of the complete repertoire of virulence factors secreted via T3S-1 and T3S-2. Characterization of the effectors' role in virulence will

provide more clues to the mechanisms by which effectors mediate virulence. Finally, identification of effector targets, such as the host cell types, the cellular organelles, or the host proteins, will help uncover the cellular mechanisms that are being manipulated.

As the knowledge base for effectors, phenotypes, and targets grows, this will hopefully have a synergistic effect on developing our understanding of the roles of T3Ss in Salmonellosis. Identification of new effectors will lead to identification of new targets; identification of new targets will lead to identification of new phenotypes; and the identification of new phenotypes will help identify more cellular processes that are targeted as well as more effectors involved in these processes; and so on. Advances in other fields of research, such as cell biology and immunology will also make major contributions to our understanding of *Salmonella* pathogenesis and will help shed light on the cellular processes and host responses that are being manipulated by T3Ss.

The experiments presented in this dissertation have made a substantial contribution to our undestanding of the role of type III secretion systems in *Salmonella* pathogenesis. However, we are still just beginning to understand the complexity and diversity of the host cell processes that effectors manipulate. Many more questions remain to be answered before we truly comprehend all the functions of *Salmonella*'s type III secretion systems.

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