How does *Salmonella* evade the adaptive immune system?

by

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Abstract

Salmonella enterica serotype Typhimurium is a Gram-negative facultative intracellular bacterium that causes a typhoid-like disease in mice. *Salmonella* invades the gut epithelium and establishes a systemic infection via invasion of phagocytes and replication within the *Salmonella*-containing vacuole (SCV). *Salmonella's* gene expression is a response to its environment and allows the bacterium to avoid macrophage killing and to establish persistence. Our understanding of the mechanisms and virulence factors necessary for *Salmonella* to invade and initiate infection are far better understood than those required for thwarting the adaptive immune response, preventing clearance and establishing a long-term infection. It was the aim of this study to identify novel genes required in the evasion of the adaptive immune response.

To identify *Salmonella* genes responsible for evading the adaptive immune response, we performed a microarray-based negative selection screen. Using a mutagenesis library, we infected RAG- mice that are missing B cells and T cells, as well as RAG+ mice, and compared the presence of mutants from spleens recovered at days five, six and seven. Following transposon detection, labeling, hybridization, quantitation, normalization and

analysis, we identified a group of candidates. Using an allelic exchange protocol we individually knocked out these genes and used the strains in a competitive index experiment testing for persistence. Using qRT-PCR, we quantified bacterial numbers throughout the course of infection for each mutant strain as compared to control.

We identified two *Salmonella* factors that are likely to be involved in evading the adaptive immune response, granting the bacterium the ability to prevent its own clearance. Listed as coding for a putative outer membrane or exported protein (STM4242) and putative cytoplasmic protein (STM1110), these genes are good candidates for further analysis of function and mechanism.

Chapter 1: Introduction

Salmonella enterica serotype Typhimurium is a Gram-negative facultative intracellular bacterium that causes a typhoid-like disease in mice, making the murine infection a widely accepted experimental model for the systemic infection and enteric fever causing human pathogen *Salmonella enterica* serotype Typhi. *S. typhimurium* serves as a model organism for genetic studies, allowing insight into microbial pathogenesis and conversely host immunity.

Although *Salmonella enterica* serotypes are some of the best studied bacterial pathogens, much is still unknown about the mechanisms of pathogenesis and evasion of host immune response. *Salmonella* has a broad range of hosts, and infections result in drastically different diseases in different hosts. *Salmonella* is able not only to evade the innate immune response, but also to utilize phagocytes to its advantage. It is also able to subvert the adaptive immune response and persist, as exhibited by the establishment of the asymptomatic carrier stage that serves as a reservoir of infection.[1,2] In recent years, there has been an increase in the number of multidrug resistant strains of *Salmonella* (MRS). This, combined with *Salmonella's* constant prevalence in developing areas such as Southeast Asia, Africa and South America, make further understanding of this organism and its interaction with the host of vital importance.[3-6]

In humans, *Salmonella enterica* serotype Typhi causes a severe systemic infection, whereas the *S. typhimurium* causes a localized infection manifesting as

gastroenteritis.[7,8] The important difference in pathogenesis of these organisms is their interaction with the human host.

Both *S. typhi* and *S. typhimurium* infections in humans are initiated by ingestion of the bacteria in contaminated food or water. *S. typhi* causes an intestinal influx of predominantly macrophages and dendritic cells, whereas *S. typhimurium* elicits a massive neutrophil response.[9,10] For typhoid fever infection in man, 10^3 to 10^6 organisms need to be ingested.[11] Following adherence to the intestinal epithelium, M cells of the lymphoid organs Peyer's patches are targeted and these provide a direct route to the engulfment by phagocytes, within which the bacteria survive and replicate in the lymphoid follicles, liver and spleen.[4,12] In typhoid fever, there may be minimal inflammation during the first seven to fourteen days of the disease and thus patients remain relatively asymptomatic.[4,12] Following this incubation period, bacteria are released from the intracellular phagocytic environment, enter systemic circulation and set up secondary infections in organs such as the spleen, liver, bone marrow, gall bladder and Peyer's patches.[4] This stage of typhoid is associated with fever, malaise, pain, and a variety of gastrointestinal symptoms and is usually diagnosed as fever of unknown origin pending blood culture. Antibiotics such as fluoroquinolones are used to resolve infection although relapses occur in five to ten percent of cases.[13,14] Additionally, S. *typhi* can persist in an asymptomatic individual in a carrier state where high numbers of bacteria are shed for months or years.[4,15]

Salmonella enterica serotype Typhimurium causes enteritis in humans eight hours to two days after ingestion of more than $5x10^5$ bacteria.[16,17] Following

bacterial colonization of the intestinal epithelium, a robust inflammatory response, characterized by massive neutrophil influx, is largely responsible for the symptoms of nausea, vomiting, abdominal pain and diarrhea.[16,18,19] *Salmonella typhimurium* infections are usually self-limiting within one week, with risk of sepsis existing mostly in the young, elderly, and immunocompromised. For this reason, and the chance of more serious infections with other bacteria such as *Clostridium difficile*, most patients are not treated with antibiotics in the United States.[20,21]

S. *typhimurium* infection in mice initiates with colonization of the small intestine following oral ingestion and penetration of the intestinal epithelium (M cells) via bacterial-mediated endocytosis.[22,23] Bacteria must survive the acidic pH of the stomach, antimicrobial peptides produced by certain intestinal cells, a thick mucus layer and overcome the barrier caused by the endogenous microbiota. Bacteria preferentially adhere to the M cells of the Peyer's patches of host epithelium, aided by *Salmonella*-expressed fimbriae.[24] Attachment is followed by drastic host cytoskeletal rearrangements via secreted proteins that directly interact with actin as well as stimulate host signal transduction.[25-28] The M cells of the lymphoid organs Peyer's patches are targeted by the invading bacteria. Because M cells are specialized endothelial cells that sample intestinal antigens via pinocytosis, they offer direct access to the lymphoid antigen presenting cells and host circulation.[29,30] *Salmonella* can also be taken up by migrating phagocytes that express CD18 and carry the bacteria to the circulation (Figure 1).[31,32]

Figure 1. (Bueno et al, 2007)

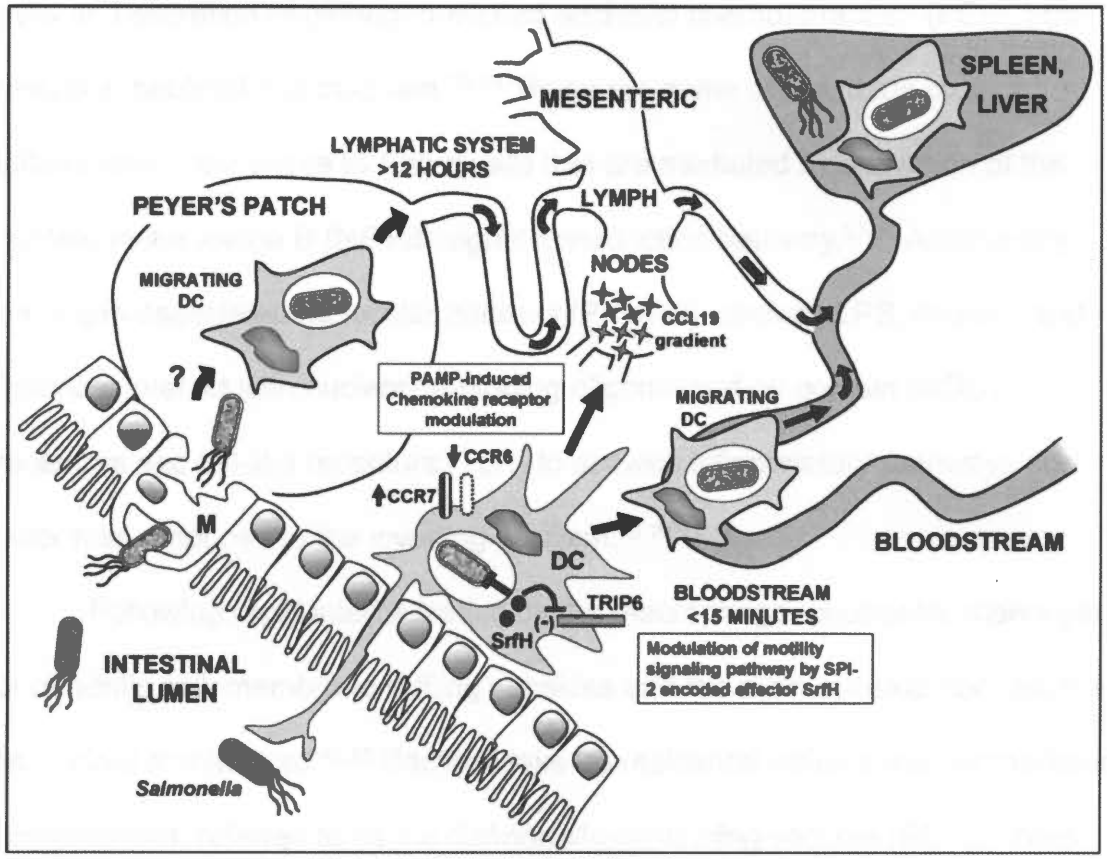

Figure 1. Model for systemic dissemination of virulent Salmonella. See text for details.

Concurrent with invasion is the stimulation of IL-8 secretion by epithelial cells and secretion of pathogen-elicited epithelial chemoattractant (PEEC) that results in neutrophil recruitment.[33-36] These are some of the many pro-inflammatory responses to *Salmonella* that are mediated by activation of the nuclear factor-kappa B (NF-kB) signal transduction pathway.[37-39] Additionally, pathogen-associated molecular patterns (PAMPS), such as LPS, flagellin and fimbriae, interact with nucleotide-binding oligomerization domain (NOD) receptors and toll-like receptors (TLR) to activate inflammatory pathways and tailor host response to the invading microbe.[40] [24,41]

Following rapid internalization by the macrophage, neutrophil, monocyte or dendritic cell, membrane ruffling subsides and the actin cytoskeleton resumes its original architecture.[42,43] Bacteria take up residence within a membrane-bound compartment, referred to as the *Salmonella*-containing vacuole (SCV), where they are protected from endosomal fusion with the lysosomal compartment by interfering with vesicular trafficking.[44-47]

In mice, replication of *Salmonella* within phagosomes is controlled by the expression of the innate resistance gene *Nramp1*. Nramp1 (natural resistance associated macrophage protein), also called Slc11a1, is a phospoglycoprotein that localizes to the membrane of the SCV and functions as a divalent metal ion pump.[48] The gene has two allelic forms, Nramp1^{resistant} and Nramp1^{susceptible}, the resistance allele being dominant.[49,50] Mutations in Nramp are also associated with increased sensitivity to several intracellular pathogens such as *Mycobacterium* and *Leishmania*.[48]

The proteins responsible for invasion during both the intestinal and the systemic phase of the disease are among the effector proteins that are part of the Type Three Secretion System (TTSS). The TTSS is encoded by two pathogenicity islands, SPI-1 and SPI-2, that are thought to have been part of pathogen evolution via horizontal gene transfer, suggested by remnants of bacteriophage or transposon insertion sequences.[51] Both pathogenicity islands code for the Type Three Secretion Associated Needle Complex, a needle-like structure spanning the inner and outer bacterial membranes (Figure 2).[52] The tip of the apparatus makes contact with the target host cell membrane where additional components of the secretion apparatus provide a pore to allow injection of effector proteins or virulence factors. SPI-1 encoded TTSS is expressed during the intestinal phase of infection by extracellular bacteria and along with associated effector proteins, is required for invasion as well as stimulation of an inflammatory response.[27] SPI-2 encoded TTSS is expressed during the systemic phase of infection and is required for survival and replication of bacteria in the intracellular environment.[53]

SipA, SipB and SipC are SPI-1 TTSS effectors key in direct manipulation of the cellular cytoskeleton. SipC and SipB comprise the translocon or pore but may encode additional virulence functions.[54] The C-terminal of SipC has been shown to nucleate the assembly of actin filaments with the same efficiency as the eukaryotic nucleating factor Arp2/3 complex, leading to rapid filament growth.[55] Additionally, the C-terminal of SipC has also been shown to mediate effector protein translocation via modulation of translocon assembly.[56] SipA has been

Figure 2. (Kuhle and Hensel, 2004)

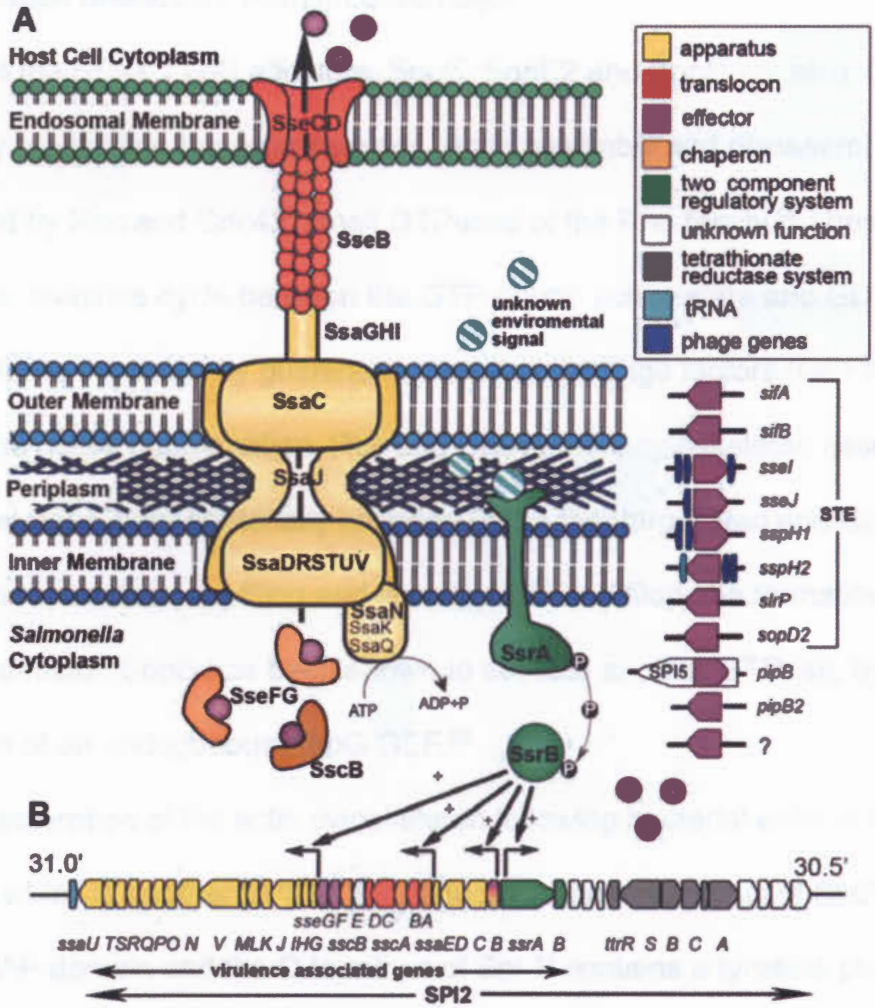

Figure 2. Salmonella pathogenicity island 2 (SPI-2) and model of the SPI-2 encoded Type Three Secretion System (TTSS). See text for details.

shown to promote actin filament polymerization by decreasing the monomer concentration needed for filament assembly.[57]

Of the SPI-1 TTSS effectors, SopE, SopE2 and SopB are also key in indirectly modulating the actin network. Actin assembly and disassembly is controlled by Rac and Cdc42, small GTPases of the Rho family.[58] These molecular switches cycle between the GTP-bound active state and GDP-bound inactive form, mediated by guanine nucleotide exchange factors (GEFs) (Figure 3).[59] In the active conformation, Rac and Cdc42 drive cytoskeleton assembly.[58] SopE and SopE2 are bacterially encoded GEFs that target Rac and Cdc42 and thus induce membrane ruffling and lamellopedia and filopodia formation.[60,61] Another effector, SopB has been shown to activate another GTPase, by activation of an endogenous RhoG GEF.[59]

Restoration of the actin cytoskeleton following bacterial entry is modulated by SptP, which is another SPI-1 TTSS effector. The N-terminus of SptP contains a Rho-GAP domain and the C-terminus of Spt-P contains a tyrosine phosphatase domain.[62] The GAP domain mimics that of native GAPs and is thus thought to catalyze the deactivation of Rac and Cdc42.[63] Although injected at the same time as the effectors whose activity it antagonizes, it's degradation rate is slower and thus its GAP activity predominates at later stages of bacterial entry.[64]

SPI-2 TTSS effectors are thought to mediate survival of the bacteria in the *Salmonella*-containing vacuole (SCV) by prevention of maturation and fusion with the lysosomal compartment.[65,66] This is thought to occur via SPI-2 TTSS effector induced filamentous, tubular structures called Sifs.[67,68] SifA has been shown to

Figure 3. (Patel and Galan, 2006)

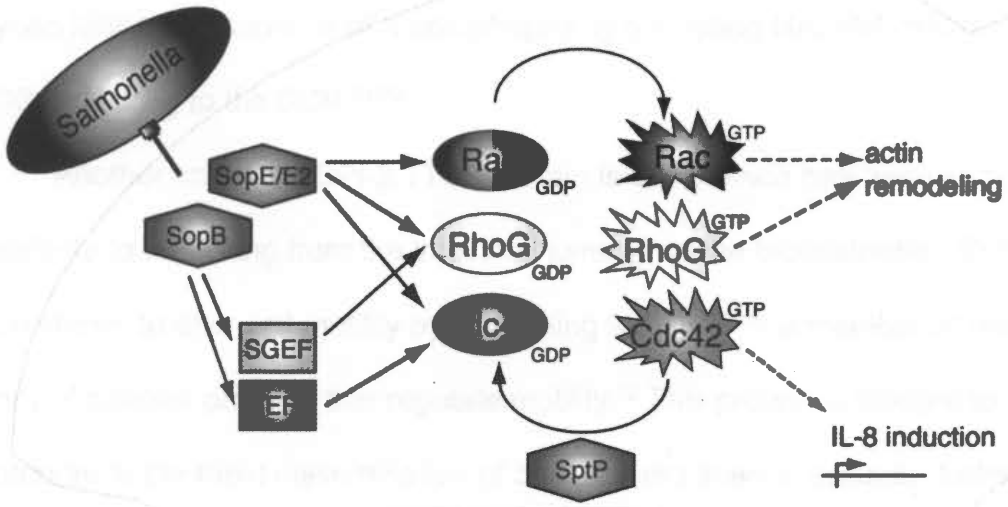

Figure 3. Model for *Salmonella* signaling to Rho family GTPases. See text for details.

induce these structures by displacing dynein and kinesin, microtubule motor proteins, from the SCV.[69] SseJ, SopD2, SseF and SseG are all thought to contribute to regulation of Sif dynamics.[65] Additionally, *Salmonella* evades the oxygen killing mechanisms of macrophages by disrupting NADPH oxidase and iNOS trafficking to the SCV.[70-72]

Another important SPI-2 TTSS protein is SrfH, which has been shown to contribute to trafficking from the intestinal lumen into the bloodstream. SrfH has been shown to alter cell motility by interacting with TRIP6, a member of the zyxin family of adaptor proteins that regulate motility.[73] This protein is thought to contribute to the rapid dissemination of bacteria into internal tissues. *Salmonella* has also been shown to alter chemokine receptor expression on dendritic cells, resulting in alteration of trafficking.[74,75]

Dendritic cells (DCs) are professional antigen presenting cells, necessary for activation of naïve T cells.[76,77] They are considered to be the link between the innate and adaptive immunity as they are phagocytes that capture invading pathogens, migrate to the lymph nodes, process the antigen and present it on MHC class II molecules to naïve T cells.[78,79] Infection with *Salmonella* induces DC activation but reduces antigen presentation on MHC class I and II to T cells.[80-86] One explanation for this is *Salmonella*'s ability to prevent endosomal trafficking and fusion of the SCV with the lysosome (Figure 4).[32] This not only allows the bacteria to survive but also prevents the processing and presentation of bacterial antigens on MHC molecules to T cells.[82,85,87,88]

Figure 4. (Bueno et al., 2004)

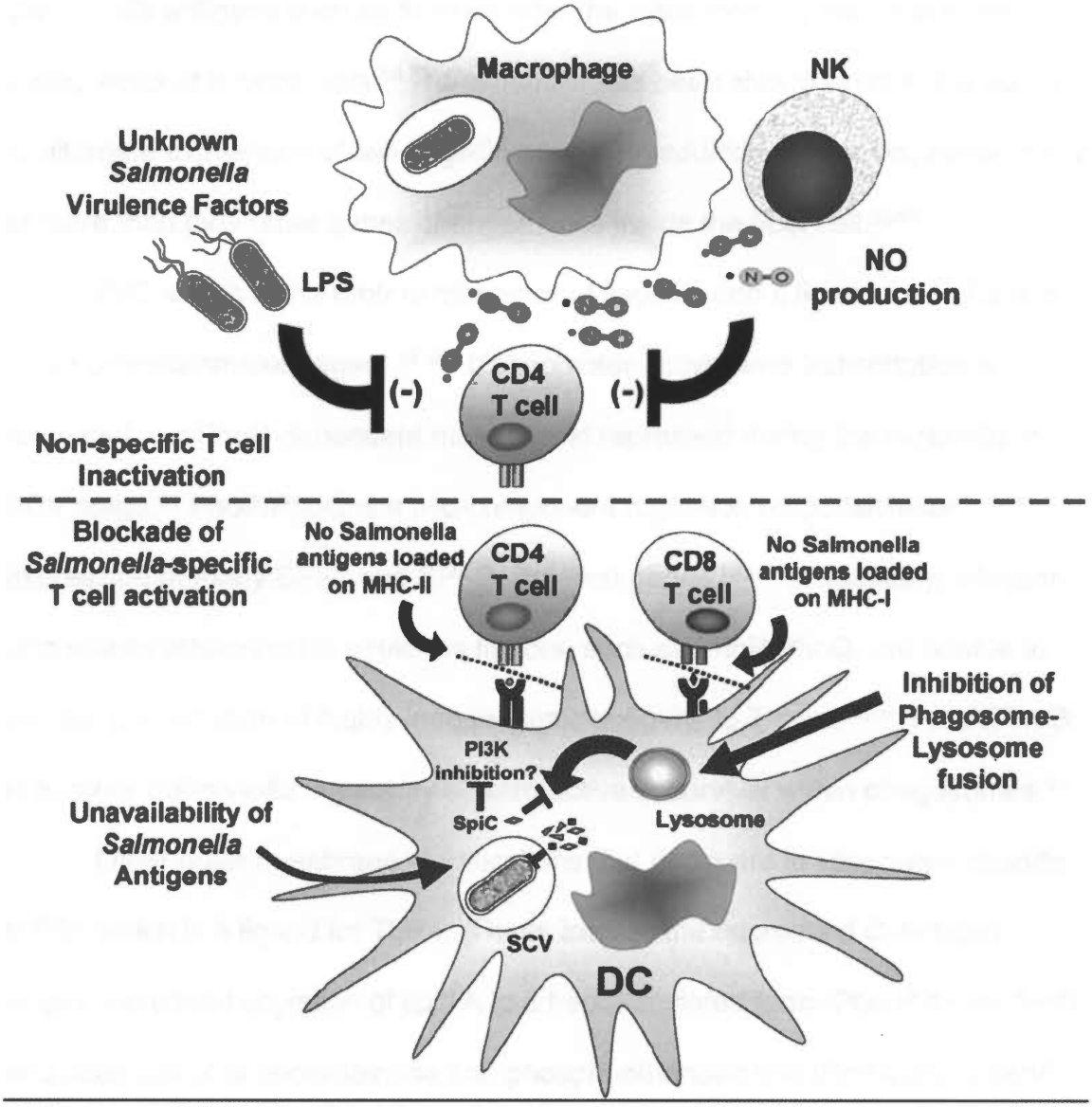

Figure 4. Molecular mechanisms used by Salmonella to impair T cell function. See text for details.

Another strategy used to prevent T cell activation is reduction of *Salmonella* antigens such as flagellin after the initial steps of bacterial entry, during which it is necessary.[86,89] *Salmonella* has been shown to have the ability to alternate expression of two flagellin genes. In addition to flagellin, transcription of more than forty other genes changes once inside the host cell.[90-93]

FliC, which is the protein monomer of flagellin and a ligand for TLR5 is a major proinflammatory agent.[94 95] It's promoter activity and transcription is regulated in a PhoP-dependent manner and repressed during the intracellular SCV stage.[41] PhoP/PhoQ is a two-component regulator, responsible for expression of many SPI-1 and SPI-2 virulence genes.[96,97] Incidentally, infection with strains attenuated in virulence factors, such as PhoP/PhoQ, are unable to escape presentation of highly immunogenic antigens to T cells.[92,98] PmrA/PmrB is another *Salmonella* regulatory system active in survival within phagosomes.[99]

Other outer membrane modifications that occur are in lipopolysaccharide (LPS), which is a ligand for TLR4. These include the decreased O-Antigen length, increased acylation of lipid A to a hepta-acylated form (Pho-P dependent) and additions of aminoarabinose and phosphoethanolamine (PmrA dependent).[91,99-101] These modifications result in reduced inflammatory properties of lipid A of LPS and confer resistance to intracellular bacteria from cationic microbial peptides (CAMPs) and bile salts.[100,102] Mig-14 (PhoP regulated) is another surface protein that is upregulated, along with VirK and PgtE, and contributes to *Salmonella* resistance to antimicrobial peptides produced by activated

macrophages, such as cathelin-related antimicrobial peptide (CRAMP), by an as yet unknown mechanism.[103-105106]

Salmonella typhimurium induces a lethal infection in susceptible mice that results in death by day seven. Mice that are resistant to infection (Nramp1^{resistant}) are utilized as models for chronic typhoid as the infection is not cleared until six weeks later.[107,108] Chronic infection in mice has been shown to persist for up to one year.[1] Immune responses of the host are necessary to control bacterial replication and to eventually clear the infection.

Innate immunity is in place prior to infection, while adaptive immunity has to develop the antigen specific response. As *Salmonella* makes its way past endogenous microorganisms of the gut and antimicrobial peptides at epithelial surfaces, it is phagocytosed by macrophages. The killing mechanism of macrophages consists of the deployment of reactive oxygen and nitrogen intermediates (ROIs, NOIs) that chemically modify and inactivate the lipid, protein and nucleic acid components of the internalized bacterium.[109,110] The production of reactive oxygen species is under the control of the phagosite oxidase protein (phox) and the production of nitric oxide is catalyzed by the cytosolic enzyme nitric oxide synthase (NOS). A form of NOS, NOS2, is induced in phagocytes upon stimulation with bacterial products such as LPS and inflammatory cytokines such as IL-12, IL-18, IL-1, IFN-gamma and TNF-alpha.[111,112] Once NOS2, referred to as iNOS, expression is induced, there is a high level of output of NO. Although toxic to internalized bacteria, NO also functions to non-specifically inactivate CD4 T cells (Figure 4).[113]

IL-18 is produced by macrophages and monocytes upon initial infection with *Salmonella* and is in this case considered a part of the innate response. IL-18 production is dependent on the activation of caspase-1 by the *Salmonella* effector SipB.[114,115] IL-18 contributes to non-specific activation of CD4 T cells.[116] During the early phase of infection, CD4 T cells, once activated, produce the macrophage-activating factor IFN-gamma and thus stimulate macrophages to control bacterial replication via the previously described iNOS response.[117] This innate activation of T cells is thought to be key in amplifying the effector function of cytokine production at sites of infection, especially when the pathogen is capable of inhibiting antigen presentation.[118]

IFN-gamma, in combination with IL-12, is crucial in eliminating *Salmonella* infection. Individuals harboring mutations in the IFN-gamma receptor, the p40 component of IL-12 or the IL-12 receptor show profound susceptibility to infection.[119] Persistent chronic infection in mice has been shown to be reactivated by IFN-gamma neutralization.[1] TNF-alpha may be important in controlling infection since it contributes to macrophage activation, and additionally, patients who were given anti-TNF-alpha antibodies developed *Salmonella* septicemia.[120] TNF-alpha has been shown to be key in granuloma formation, suggesting it is important during the stage of infection where replication of the bacterium is controlled.[121] IL-10, an anti-inflammatory cytokine, is also produced during *Salmonella* infection. This benefits the bacterium in preventing macrophage killing by deactivating macrophages.[122] It also aids the host pathology by counteracting the inflammatory cytokines and reducing

damage from excessive inflammatory response by macrophages and natural killer cells.[123,124]

Although CD4 T cells are crucial in the eventual clearing of infection, CD8 T cells play a considerable role. It has been observed that the CD8 T cell expansion is delayed, as is the subsequent contraction.[125] The general paradigm for differentiation, expansion and contraction of CD8 T cells has been derived from several mouse infection models.[126,127] CD8 T cells are stimulated when peptides from intracellular pathogens are presented on MHC class I molecules.[128] Presentation occurs within the first few days of infection and the subsequent CD8 T cell expansion follows and the specifically primed response peaks around day seven after infection.[129-131] Contraction of 90% of these CD8 T cells is completed within two to three weeks.[126] In Salmonella infection, the CD8 T cell response peaks at about day 21 of infection, and is followed by a protracted contraction.[125] Despite an initial rapid increase in bacterial load, Salmonella fail to mount a prompt CD8 T cell response. The reasons for this delay seem to be related to the replication and survival in the *Salmonella*-containing vacuole within the phagocyte. Additionally, there is emerging evidence that *Salmonella* hinders T cell activity and proliferation in a contact-dependent manner.[108,116] However, evidence remains that mice missing CD8 T cells are capable of clearing a *Salmonella* infection.[132]

Much like *Salmonella, Mycobacterium tuberculosis* (Mtb) resides in a phagosome that does not fully acidify or undergo phago-lysosomal fusion. Multiple mechanisms by which Mtb prevents phagosome maturation have been

shown.[133,134] IFN-gamma has been shown to reverse this block, as in *Salmonella* infection.[1,135] The interaction of CD4 lymphocytes and macrophages has been shown to be key in eliminating this bacterium. Specifically, Mtb antigens are processed and presented on the macrophage MHC class II and subsequent antigen recognition by CD4 T cells then leads to the release of pro-inflammatory cytokines, IFN-gamma and TNF-alpha. The resulting macrophage activation consists of upregulation of MHC class I and II molecules as well as the production of reactive nitrogen and oxygen species.[136,137] In the process of autophagy, which occurs in IFN-gamma activated macrophages, the autophagosome fuses to lysosomes resulting in degradation of the bacterial components.[138,139] Although CD4 T cells are largely responsible, there is evidence that CD8 T cells also play a role in controlling infection.[140,141]

Salmonella's finely tuned and regulated gene expression response to the environment allows it to evade the host's innate and adaptive branches of the immune system, evidence of millions of years of co-evolution. Our understanding of the mechanisms and virulence factors necessary for *Salmonella* to invade and initiate infection are far better understood than those required for thwarting the adaptive immune response and establishing a long-term infection. It is the aim of this study to identify novel genes required in the evasion of the adaptive immune response.

Chapter 2: Materials and Methods

To identify *Salmonella* genes responsible for evading the adaptive immune response, we performed a microarray-based negative selection screen. Using a mutagenesis library, we infected RAG- mice that are missing B cells and T cells, as well as a WT, and compared the presence of mutants from spleens recovered at days five, six and seven. The *Salmonella* mutagenesis library consisting of 39,000 mutants was made in Brian Ahmer's laboratory at The Ohio State University using the SaEZ::TN™ <T7/KAN-2> from Epicentre which contains a T7 promoter allowing for easy mutation detection (Figure 5). The transposon is only 1248 base pairs (bp) long facilitating some laboratory manipulations. It does not encode a transposase thus stabilizing insertions and avoiding rearrangements and deletions that often accompany transposon insertions. The selection marker is kanamycin and there are 19bp mosaic ends that are recognizable by the Tn5 transposase allowing for random insertions. The T7 promoter is pointing outward of the left mosaic end. The transposase is available commercially from Epicentre and can be combined with the transposon to make complexes that integrate the transposon into the chromosome following electroporation.

The transposon bank, which contains mutations in every non-essential gene, was used to infect two groups of thirty mice: RAG- and RAG+. The mice were ordered from Jackson Laboratories. The Rag1<tm1Mom> targeted

Figure 5. SaEZ::TN™ <T7/KAN-2> from EPICENTRE

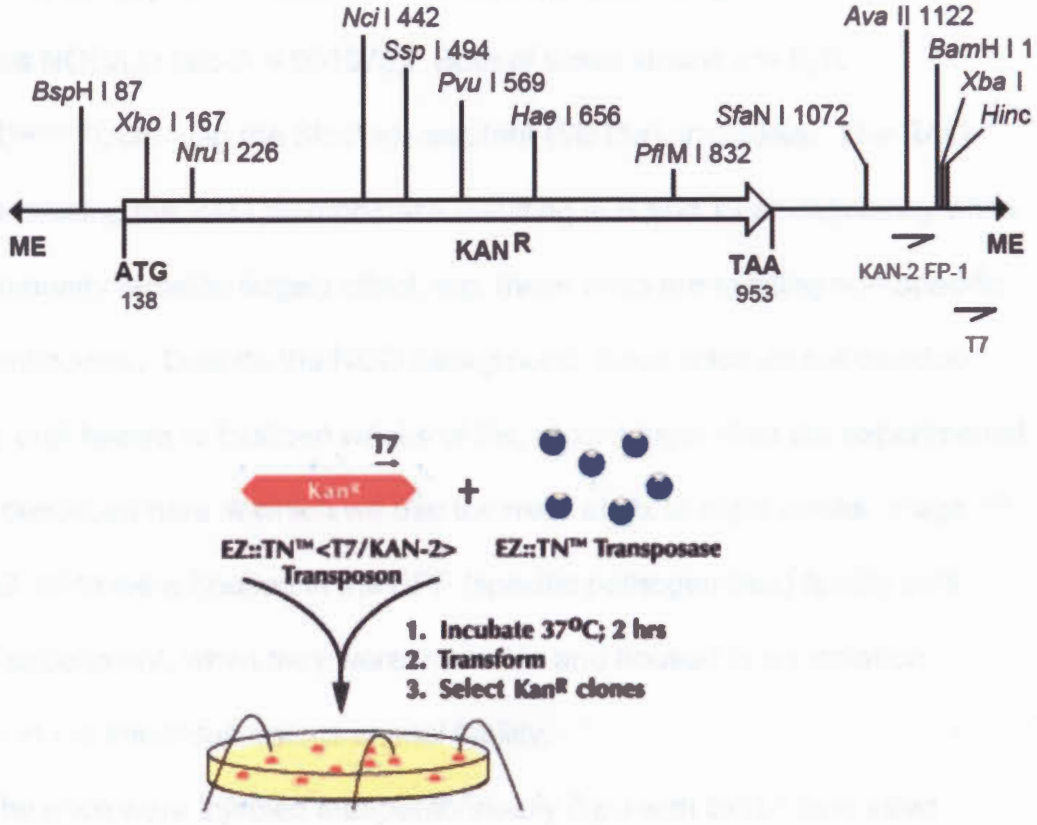

Figure 5: See text.

mutation was only available on the NOD background (NOD.129S7(B6)-Rag1<tm1Mom>/J, stock # 003729) and thus the most congenic RAG+ mice used were NOD/LtJ (stock # 001976). Both of these strains are ItyR (Nramp1^{resistant}), carrying the Slc11a1-resistant (Slc11a1<r>) allele. The RAG- mice are missing the RAG recombinase resulting in B and T cell deficiency while innate immunity remains largely intact, e.g. these mice are missing non-specific natural antibodies. Despite the NOD background, these mice do not develop diabetes until twelve to fourteen weeks of life, much longer than the experimental method described here in which we use the mice at six to eight weeks of age.[142] The RAG- mice were housed in the SPF (specific pathogen free) facility until onset of experiment, when they were moved to and housed in an isolation chamber in the infectious agents animal facility.

The mice were injected intraperitonneally (i.p.) with $5x10^5$ heat killed *Salmonella* mutagenesis library seven days prior to injection. This priming may have been required to allow RAG- mice to survive until day seven of infection. We are conducting further experiments to confirm this. Seven days later, mice were infected intraperitoneally (i.p.) with $5x10^5$ bacteria of a live library, grown overnight in LB (Luria Bertani) broth, dilutions were plated for next day counting to confirm correct dosage and mouse spleens were collected on days five, six and seven. I.p. infection is commonly used in a mouse systemic disease model and was chosen for our experiment to avoid the significant bottleneck that exists when the oral route of infection is used.[22,143,144]

The spleens were processed with frosted slides, lysed in 1% Triton-X and a tenth (150uL) of the solution was plated as was the rest (1.35mLs) on separate 150mm LB agar plates. The next day, colonies were counted and the mixture of colonies was resuspended in phosphate buffered saline and frozen in aliquots for later analysis. Plates with lawns or with low numbers of colonies were excluded from array analysis. Plates from spleens of dead mice always resulted in lawns and were also excluded. The bacterial colony numbers harvested per sample are shown in Table 1. To harvest colonies from plates, 1mL of PBS was added and colonies were scraped off gently with a tissue culture scraper. After thorough vortexing, approximately 3mLs of each sample were divided into six aliquots, some of which were pelleted and others frozen back in 50% glycerol. DNA extractions were made from the pellets using the Sigma GenElute Bacterial DNA kit according to manufacturer's instructions. DNA concentrations were determined using a spectrophotometer.

The samples from each plate were pooled proportionately such that the DNA in the experimental sample reflected the number of colonies from which the DNA was prepared. Thus, if there were 50 colonies on one plate and 5000 on the next the final mixture would contain 99% from the plate with 5000 and 1% from the plate with 50 colonies. In most cases, pooling from multiple mice was necessary to give an adequate representation of the original mutagenesis library. An adequate sample should reflect at least 10x times the library size (approximately 400,000) or many genes would be absent by chance alone as the presence of insertions in a given pool follows the Poisson distribution.

Table 1.

Day 5 RAG -	colony count	Day 5 RAG +	colony count	Day 6 RAG -	colony count
mouse 1	lawn (dead)	mouse 1	20000	mouse 11	lawn (dead)
mouse 2	80000	mouse 2	500	mouse 12	lawn (dead)
mouse 3	30000	mouse 3	30000	mouse 13	lawn (dead)
mouse 4	80000	mouse 4	20000	mouse 14	80000
mouse 5	80000	mouse 5	20000	mouse 15	90
mouse 6	80000	mouse 6	50000	mouse 16	20000
mouse 7	40000	mouse 7	20000	mouse 17	30000
mouse 8	100000	mouse 8	2000	mouse 18	80000
mouse 9	80000	mouse 9	8000	mouse 19	80000
mouse 10	10000	mouse 10	died post inj.	mouse 20	50000

Day 6 RAG +	colony count	Day 7 RAG -	colony count	Day 7 RAG +	colony count
mouse 11	10000	mouse 21	lawn (dead)	mouse 21	2000
mouse 12	10000	mouse 22	lawn (dead)	mouse 22	7000
mouse 13	20000	mouse 23	lawn (dead)	mouse 23	2000
mouse 14	30000	mouse 24	80000	mouse 24	1000
mouse 15	3000	mouse 25	80000	mouse 25	30000
mouse 16	10000	mouse 26	90000	mouse 26	9000
mouse 17	4000	mouse 27	80000	mouse 27	4000
mouse 18	20000	mouse 28	90000	mouse 28	4000
mouse 19	20000	mouse 29	80000	mouse 29	3000
mouse 20	4000	mouse 30	died at inj.	mouse 30	2000

Table 1. Output colony counts. Pink represents samples included in pools. See text for details.

The transposon detection, labeling, hybridization, quantitation and normalization were carried out in Michael McClelland's laboratory at The Sidney Kimmel Cancer Center with the guidance of Carlos Santiviago. For overview of procedures, see Figure 6, courtesy of Carlos Santiviago. Detailed protocols and array information can be found on the Sidney Kimmel Cancer Center website: (http://www.skcc.org/mcclelland_protocols_arrays.html). The initial step of random primer extention was performed using the Klenow Fragment of DNA Polymerase I (New England Biolabs) with a degenerate primer (DOPR1). Arbitrary primed PCR amplification was carried out at a low number of cycles to ensure equal representation of each insertion mutation and flanking DNA. In the next step, a transposon specific primer (KAN2FP1-B) and a primer corresponding to the 5' end of the degenerate primer (DOPR2) were used to amplify transposon plus flanking chromosomal DNA. Following this step, three microliters of PCR products were electrophoresed on an agarose gel and samples were quantified using a spectrophotometer. Gel electrophoresis confirmed fragments of different sizes suggesting that the procedure worked (Figure 7).

In vitro T7 transcription was carried out using the AmpliScribe T7 Transcription Kit from Epicentre. Three microliters of each sample were run out on an agarose, samples were quantified by spectrophotometer and purified using a RNeasy Mini Protocol for RNA Cleanup (Qiagen) (Figure 8). Samples were subsequently labeled using SuperscriptII reverse transcriptase (Invitrogen) in a reaction that included Rnasin (an RNase inhibitor from Roche). Labeled probes

Figure 6.

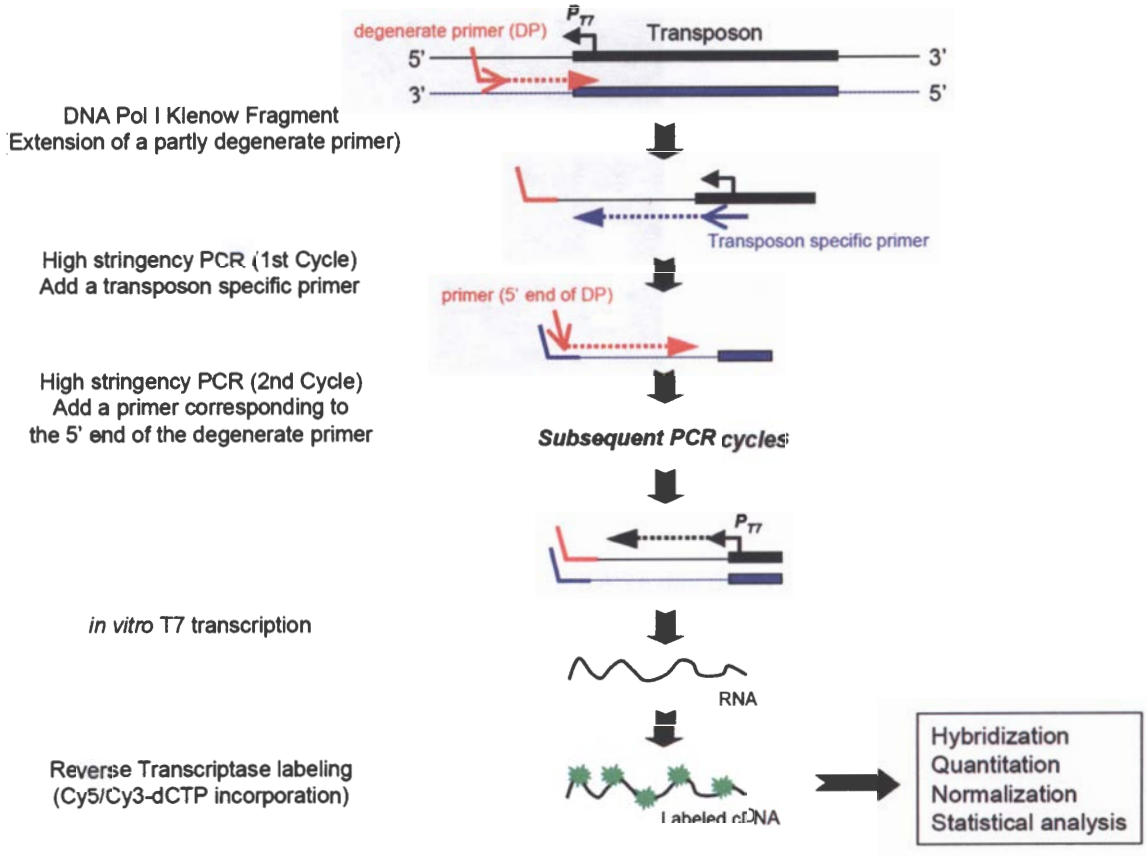

Figure 6. Overview of transposon detection protocol. Slide courtesy of Carlos Santiviago. See text for detail.

Figure 7.

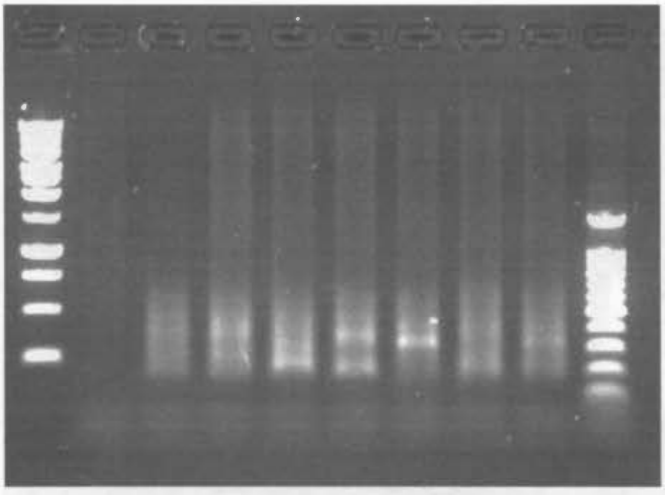

Figure 7. PCR amplification, DNA.

1) unlabeled *Salmonella* 14028
2) input mutagenesis library
3) output RAG- day 5
4) output RAG- day 6
5) output RAG- day 7
6) input RAG+ day 5
7) input RAG+ day 6
8) input RAG+ day 5+6

Figure 8.

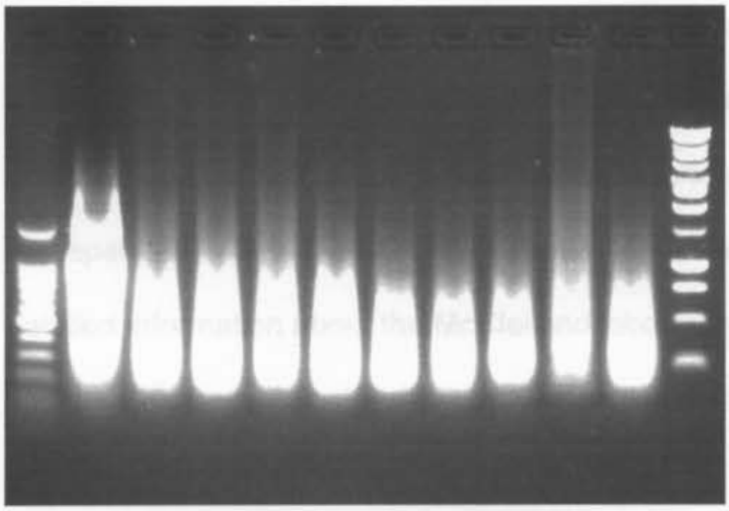

Figure 8. T7 *in vitro* transcription.

1) control DNA
2) input library
3) input library
4) input library
5) output RAG- day 5
6) output RAG- day 6
7) output RAG- day 7
8) output RAG+ day 5
9) output RAG+ day 6
10) output RAG+ day 5+6

were purified using the Qiagen PCR Purification Kit. The input library was labeled with Cy5-dCTP and each of the pooled output libraries were labeled with Cy3-dCTP.[145] The labeled samples were then hybridized to chips containing amplified and purified genomic *Salmonella*-specific probes that were resuspended in 50% DMSO before spotted onto the slides in triplicate. For detailed information about the McClelland laboratory *Salmonella* ORF microarray, see the SKCC website: (http://www.skcc.org/mcclelland_protocols_arrays.html). The chip design version used for this study was the STv7S, which covers 98% of all ORFs and annotated pseudogenes in the following *Salmonella enterica* genomes: *Typhimurium* LT2 (STM), *Typhi* CT18(STY), Typhi Ty2 (STT), *Paratyphi* A SARB42 (SPA) and the *Typhimurium* SL1344 (SSL) plasmid.[146] Pre-hybridization, hybridization and post-hybridization washing were also performed in Michael McClelland's laboratory according to standard protocols and hybridization of samples to array slides was carried out in the Corning Hybridization Chamber. Data acquisition and quantifications were also carried out at The Sidney Kimmel Cancer Center. Microarray data was analyzed at OHSU and by Jason McDermott at PNNL.

The candidates for *Salmonella* genes responsible for evading the adaptive immune response, chosen based on survival of mutants in RAG- mice vs. RAG+ mice, were individually mutated using a modified Datsenko and Wanner method for allelic replacement called "Red swap" (Figure 9).[147] In this procedure, a DNA fragment was created by PCR using a template containing a kanamycin antibiotic resistance cassette flanked by FRT sites for the flip recombinase and primers

Figure 9. (Datsenko and Wanner, 2000)

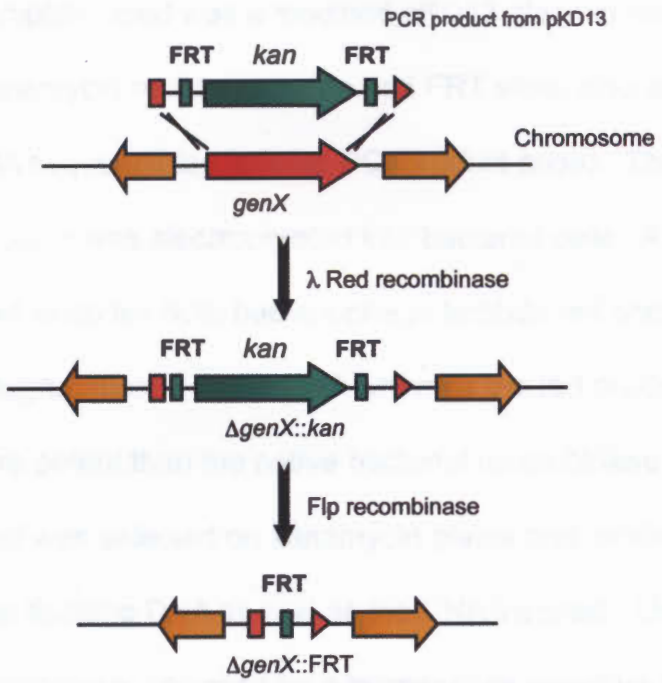

Figure 9. "Red swap," Lambda red allelic replacement. See text for details.

containing 40 bp sequences at either end that correspond to the gene being replaced. The template used was a modified pKD13 plasmid that contained, in addition to the kanamycin resistance gene and FRT sites, also a T7 promoter and a unique DNA sequence tag labeled PC (product code). The linear double stranded PCR product was electroporated into bacterial cells. A helper plasmid in the recipient cell encodes both bacteriophage lambda red and gam. The gam product inhibits degradation of linear DNA whereas the red product is a recombinase more potent than the native bacterial recombinase recA. Each allelic replacement was selected on kanamycin plates and verified using primers that correspond to flanking DNA as well as the DNA inserted. Using the FRT sites and flip recombinase, provided by a temperature sensitive pCP20 plasmid in another electroporation, we removed all but 135 bp from the kanamycin resistant recombinant gene replacing all but 8 codons of the original gene. The inserted sequence contained an open reading frame without stop codons that was fused between the first codon and the last seven. The constructs all included a unique 24 bp sequence product code for each mutant analyzed (Figure 10).

From our list of candidates, we wished to distinguish mutants that were more sensitive to the adaptive immune system from those that were either false positives or sensitive to innate immune mechanisms. To do this we compared persistence within normal mice (SvJ129, Nramp^{resistant}, RAG+). Since the innate immune system components are in place before infection, they result in an immediate antimicrobial response whereas the adaptive response only appears at days five and beyond. To distinguish between these categories of mutants, we

Figure 10.

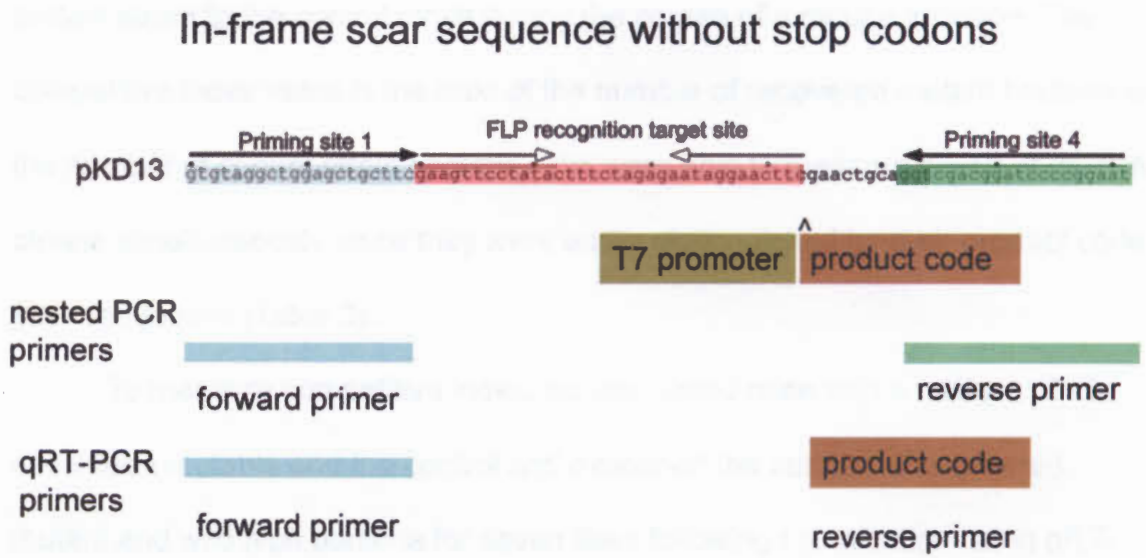

Figure 10. In-frame scar sequence without stop codons containing the unique 24 bp "product code" (PC). See text for details.

employed a competitive index method, which is a sensitive way of comparing a mutant strain to the control strain during the course of a mouse infection. The competitive index value is the ratio of the number of recovered mutant bacteria to the number of control strain bacteria. We were able to measure the CI of multiple strains simultaneously since they were easily distinguished by their product code scar sequences (Table 2).

To measure competitive index, we inoculated mice with a mixture of all seventeen mutants and the control and examined the number of recovered mutant and wild type bacteria for seven days following i.p. infection using qRT-PCR. As a control, a product code was introduced into a gene that appears to be non-essential for *Salmonella* virulence (STM 0314; Hyunjin Yoon and Fred Heffron, unpublished observations). Thirty mice (SvJ129, $Nramp^{resistant}$, RAG+) were infected i.p. with a mixture of strains containing equal numbers of each mutant (a total of 10^4 bacteria, containing 5.5×10^2 of each strain). The loss of a specific mutant strain was used as a measure of fitness. Individual strains were grown overnight in LB, washed, Optical Density was determined (OD1= $1x10^9$ bacteria/mL) and cultures were diluted and mixed accordingly. Dilutions were plated to confirm titer used to infect mice and equal distribution of strains.

Spleens were collected from groups of four mice on days one through seven. Spleens were processed with frosted slides, lysed with 1% Triton-X, filtered and several dilutions were plated. After counting the following day, colonies were harvested as previously described such that each sample from each mouse on each day contained approximately 10,000 colonies. For day

Table 2.

ID		PC "product code"	function
PSLT071		PC47	pseudogene; two frameshifts
STM0028	bcfH		putative thiol-disulfide isomerase
STM0289			putative cytoplasmic protein
STM0600	cstA		carbon starvation protein
STM0828	glnQ		ABC superfamily (atp-bind), glutamine high-affinity transporter
STM0839			putative inner membrane protein
STM1024			Gifsy-2 prophage
STM1110		PC1	putative cytoplasmic protein
STM1244	pagD	PC4	PhoP regulated
STM1397	sseA		Secretion system effector
STM1482	ydgF	PC7	putative membrane transporter of cations and cationic drugs
STM1504	ynfA	PC8	putative inner membrane lipoprotein
STM1615		PC72	putative nucleoside triphosphatase
STM2087	rfbX, wzxB	PC17	LPS side chain defect: abequosyltransferase
STM2098	galF, wcaN, yefG	PC18	putative UDP-glucose pyrophosphorylase, non-catalytic subunit
STM2209			putative inner membrane protein
STM2450	amiA	PC24	N-acetylmuramoyl-l-alanine amidase I
STM2471	tnpA_3		transposase for IS200
STM2679	yfjD	PC26	putative membrane protein
STM3096	yqgE		putative transcriptional regulator
STM3192		PC31	putative arylsulfate sulfotransferase
STM3248	garR	PC33	tartronate semialdehyde reductase (TSAR)
STM3765	yicL	PC34	putative permease, integral membrane protein
STM3785		PC35	putative regulatory protein, gntR family
STM3998	yihG	PC36	putative endonuclease
STM4156			putative cytoplasmic protein
STM4169	yjaG		putative cytoplasmic protein
STM4242		PC43	putative outer membrane or exported
STM4291	pmrB		sensory kinase in two-component regulatory system with BasR
STM4333		PC45	putative aminomutase
STM0314		PC3	control: appears to be non-essential in virulence

Table 2. List of candidates. See text for details.

one, samples from all four mice were pooled proportionately. Additionally, the input library mixture of eighteen strains used to inject the mice was used as a control. A 1:1000 dilution of the vortexed mixtures was used as a template for the nested PCR using priming sites 1 and 4 and Taq Polymerase (Invitrogen) using the manufacturer's protocol, and a portion of the mixture was stored at -70C in 50% glycerol. Nested PCR was also performed on the input library (Figure 10).

Following confirmation of nested PCR product and PCR purification using the Qiagen PCR Product Purification Kit, the concentration of DNA was determined and was used as template for the second PCR reaction used to distinguish individual strains. The concentration of the product of the nested PCR reaction was determined and diluted to the concentration of $7x10^5$ PCR products per reaction as a template for the specific PCR. Primers corresponding to the individual product codes (PCs) were used in combination with "prime site 1" or forward primer in a qRT-PCR reaction using the Qiagen QuantiTect SYBR Green PCR Kit according to the manufacturer's instructions.

The CI value of the results of qRT-PCR data means the ratio between mutant colony and wild type. To interpret our results from the qRT-PCR data, a calculation was developed (Yoon and Heffron, unpublished observations). The CI value in our case is defined by the difference between delta output and delta input: $CI=\Delta Ct_{output} - \Delta Ct_{input} = (Ct_{control} - Ct_{mutation})_{output} - (Ct_{control} - Ct_{mutation})_{input}$. To verify the methodology, *in vitro* qRT-PCR was carried out using several

combinations of three strains to determine that a CI value of 1 is a two-fold difference (Yoon and Heffron, unpublished observations).

Chapter 3: Results and Discussion

The numbers of colonies recovered from spleens of RAG- mice were drastically greater than those of RAG+ mice by the fourth day of infection. In the case of the RAG+, the entirety of what was plated (the entire processed spleen) was harvested to yield sufficient colonies. Additionally, the samples from each day were pooled to yield numbers of colonies sufficient to be representative of the mutagenesis library. As the original library consisted of about 39,000 independent insertions, the ideal number of colonies in a pool should be at least 10x this many. Samples containing less than 10,000 colonies and samples from dead mice that resulted in bacterial lawns were excluded from the pools. Day seven of control RAG+ mice yielded colony counts that were far too low to be used with the exception of one mouse and thus a sample of days five and six combined was used in its place.

The red Cy-5 labeled input library was hybridized to the chips and compared with each of the green Cy-3 labeled experimental group output libraries. A red spot on the microarray corresponds to loss of a mutation in the corresponding gene. A green or yellow dot corresponds to recovery of mutations in a specific gene. As one can see in Figure 11, far more mutations are lost in the output library of the RAG+ mice versus the RAG- mice as expected based on the drastic differences in surviving mutants, and as exhibited by the colony numbers from recovered spleens. In fact the difference is so great that it suggests a high rate of false discovery. The figure showing day five is representative of

Figure 11.

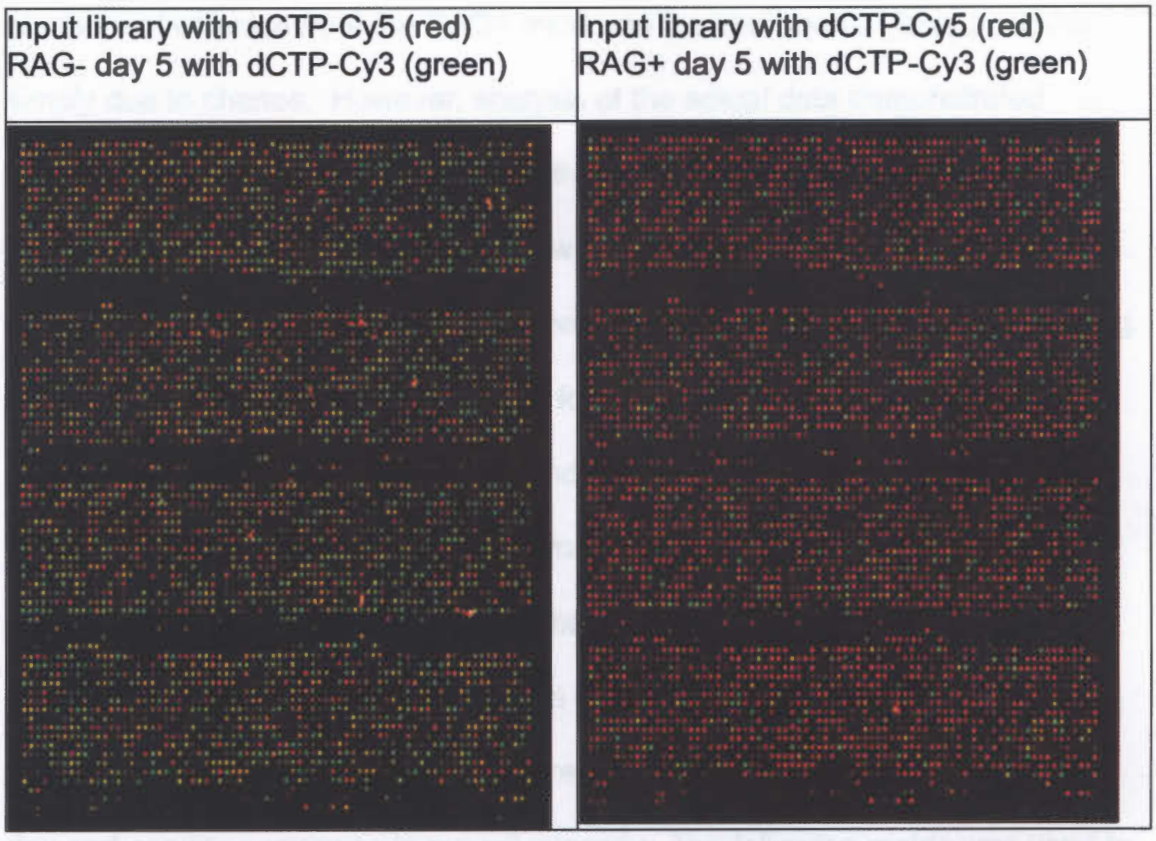

Figure 11. Comparison of Day 5 Output (red) to Input (green) in RAG+ and RAG-. See text for details.

differences seen in other day comparisons. The concern with recovering lower numbers of colonies from the RAG+ mice was the likelihood of false positives simply due to chance. However, analysis of the actual data demonstrated differences in recovery for RAG+ mice that were not evident in this figure.

To eliminate the false positives, we were able to employ the data from consecutive days and eliminate mutations that were not consistently present e.g. if mutations in a gene were lost on day four but recovered on day five and six. For each of the days and treatment conditions, the standard deviation was calculated from the expression values for all genes (not normalized). The z-score was calculated for each gene. The z-score is the number of standard deviations from the mean for each gene on each day. The z-score is an appropriate value for a normalized expression value since it takes into account day and condition variance in overall intensity. The following metric was used to identify the best differentially expressed genes on each day: ((RAG-)-(RAG+)*(RAG-))/((RAG+)+margin). This metric is skewed so that it represents data points that are differentially expressed but have a low RAG+ value and thus are placed higher on the ranked list. While insertions in many genes appeared to be lost in the RAG+ mice as shown in the microarray, the statistical analysis allowed us to identify differences that were not obvious to the naked eye. This method of comparing z-score between RAG+ and RAG- was used to rank all *Salmonella* genes according to the probability that the gene was selected against in RAG+ mice but not in RAG- mice.

To eliminate false positives, we compared our results to a complementary study of persistence in RAG+ SvJ129 mice without comparison to RAG-.[107]. The genes isolated during our study should be a subset of those identified by Lawley et al (2006) because mutants in genes required for resistance to the adaptive immune response should not persist in this mouse strain. We compared the top 130 genes on our list to the top 130 genes from their study and found that there was an overlap of 30 genes. The probability of this occurring by chance alone is statistically very low, on the order of 10^{-18}. The following factorial equation is used to determine this: (130!/4400!-100!/4370!). This calculation determines the chances of the overlap of thirty genes from the top 130 from each list, when the total number of non-essential genes is 4400. In order to maximize our chance of success we focused on only these 30 genes and were successful in constructing non-polar in-frame insertions in seventeen and control as indicated.

To determine if the mutants identified effect the adaptive or the innate/ adaptive immune response we compared survival of each of the mutants in a normal RAG+ mouse strain (SvJ129). In general we would expect defects that do not effect the initiation of an adaptive response but rather the effector phase in which individual mutants could be eliminated via T cell production of cytokines that activate infected macrophages. Because we are infecting with a pool of mutations, many mutations would never be recovered such as those that effect T cell replication as only a dominant mutation could be recovered. We anticipate that there would be different kinetics of loss for the various mutants as some may be effected in innate response as well as adaptive and that false positive mutants

could be eliminated by this simple experiment. Figure 12 shows the averages of each of the seventeen mutants from multiple mice on each day post-infection as compared to the control. Although the error bars are high for some of the data points, we can see a general downward trend for most of the mutations as compared to control by day seven of infection. For reasons unknown to us, most of the mutants actually have a better rate of survival on day one as compared to the control. Because some of the days only contained as few as three mice, and the other mutations were consistent between days, the data is shown in this format, as well as each mutation is shown individually throughout the course of infection as represented in each mouse.

As expected, we can categorize the mutations based on their survival during the seven-day infection in RAG+ SvJ129 mice into three categories: those responsible for evading the innate response, the adaptive response and false positives. As hypothesized, we identified two mutants of genes likely to be responsible for evading the adaptive immune response, as exhibited by the decline in growth after day five (Figure 13). Listed as coding for a putative outer membrane or exported protein (STM4242) and putative cytoplasmic protein (STM1110), these genes are good candidates for further analysis of function and mechanism. Since there are many factors responsible for clearing a *Salmonella* infection, these genes are likely to be coding for factors that block not the onset but the progression of the immune response with clearance of the organism. IFN-gamma is produced by CD4 T cells when in contact with APCs that express the cognate epitope or when activated non-specifically by IL-18.[116] Expression

Figure 12.

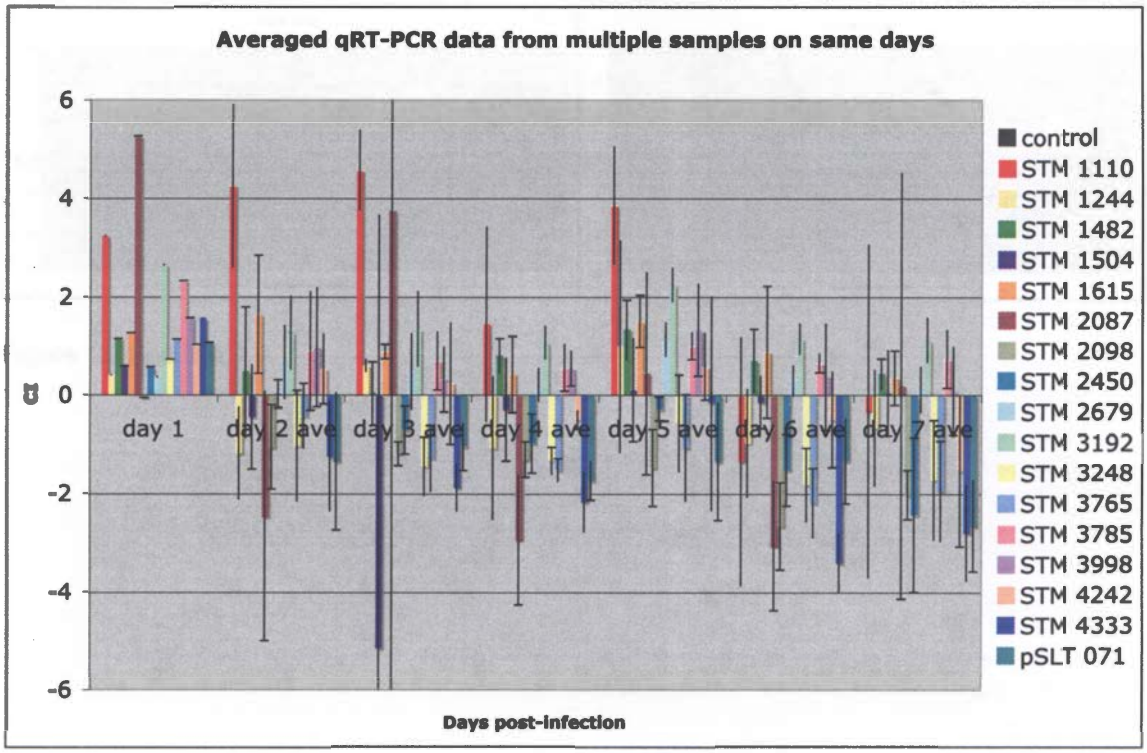

Figure 12: See text.

Figure 13.

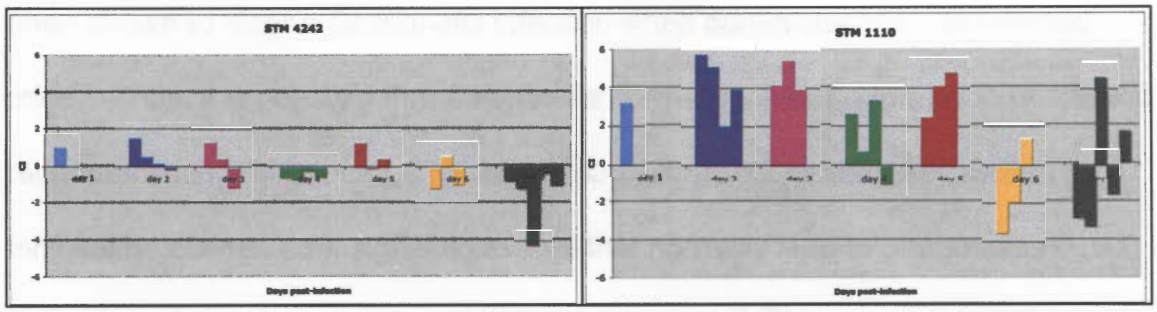

Figure 13: See text.

of IFN-gamma activates macrophages, increases the iNOS response and has been shown to clear a *Salmonella* infection when administered i.v. to infected mice.[1] Thus, it is possible that *Salmonella* normally inhibits expression of specific receptors such IFN-gamma, TNF-alpha, IL-18, or IL-12 or alternatively that it inhibits the downstream signaling events that normally lead to phagosome activation. According to this model, administration of IFN-gamma by i.v. injection simply overcomes the inhibition presumably because of the large amount that must be administered compared to the amount normally observed. Another possibility is that interaction between the T-cell and infected APC results in transfer of proteins via the synaptic junction leading to direct inhibition of T cell response. Our study may have identified *Salmonella* factors that are key to dissecting this state of the immune response and identifying factors that *Salmonella* makes to prevent its own clearance.

In Figure 14, we see a pattern of survival of strains of bacteria mutated in genes that are likely to be active in the evasion of the innate immune response as well as adaptive as exhibited by a decrease in persistence after day one. The mutants we would expect to recover include those more sensitive to the innate immune response as the criteria we used to select them was a decrease in RAG + on days five, six and seven. Since there are numerous genes that allow *Salmonella* to invade and set up systemic infection in phagocytes and clearance of *Salmonella* depends on factors of the innate immune response such as IL-12, IL-18, IL-1, TNF-alpha and as of yet unknown chemokines or cytokines, we

Figure 14.

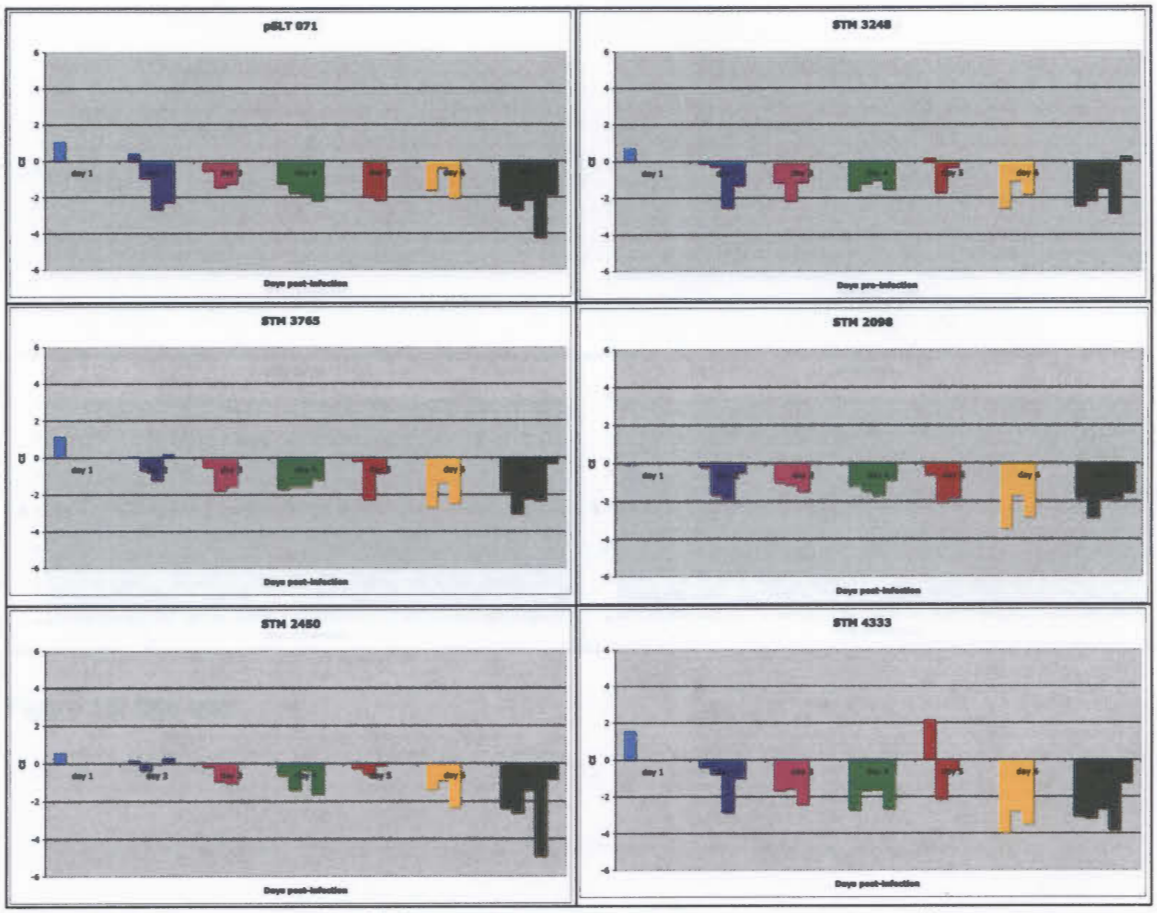

Figure 14: See text.

Figure 15.

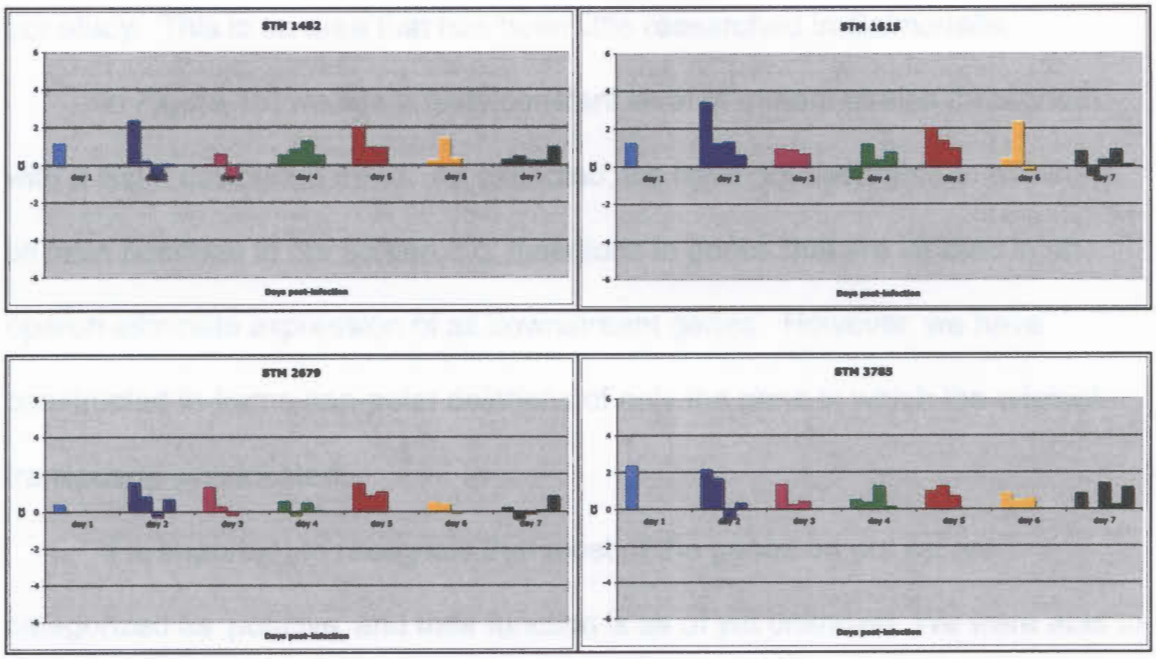

Figure 15: See text.

would expect to find mutations in genes responsible for blocking these factors in our study. This is an area that has been little researched in *Salmonella*.

In Figure 15, we see a fairly constant level of mutant strains throughout, with a slight downward trend. As expected, we have not been able to eliminate all false positives in our screen e.g. insertions in genes that are located in an operon eliminate expression of all downstream genes. However, we have constructed in-frame non-polar deletions of only the gene in which the original transposon was located.

It is important to recognize that most of the genes on our list are categorized as 'putative' and their function is as of yet unknown. We were able to confirm that many have a reduced virulence and reduced ability to persist in a seven-day infection of wild type mice. The variety of virulence factors, known and putative, shows that *Salmonella* has numerous mechanisms to subvert the immune system. Using the mutants we identified, especially STM1110 and STM4242, in further studies to elucidate their role in evasion of the adaptive immune responds is of great interest to us. Focusing on the role the proteins coded by these genes have in interfering with phagosome activation whether via the IFN-gamma response or via other cytokines is the most likely avenue for future research.

Conclusions

In this study, we have identified *Salmonella* factors that are likely to be involved in evading the adaptive immune response, granting the bacterium the ability to prevent its own clearance. Listed as coding for a putative outer membrane or exported protein (STM4242) and putative cytoplasmic protein (STM1110), these genes are good candidates for further analysis of function and mechanism.

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