IDENTIFICATION OF NOVEL VIRULENCE GENES OF *SALMONELLA ENTERICA* USING AN ARRAY BASED ANALYSIS OF CISTRONS UNDER SELECTION

A Dissertation

by

MOLLIE MEGAN REYNOLDS

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

May 2010

Major Subject: Genetics

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Approved by:

Chair of Committee,	Helene Andrews-Polymenis
Committee Members,	L. Garry Adams
	James Samuel
	Jonathon Skare
Disciplinary Faculty Chair,	James Wild

May 2010

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ABSTRACT

Identification of Novel Virulence Genes of *Salmonella enterica* Using an Array Based Analysis of Cistrons Under Selection. (May 2010)

Mollie Megan Reynolds, B.S., Texas A&M University

Chair of Advisory Committee: Dr. Helene Andrews-Polymenis

Pools of mutants of minimal complexity but maximal coverage of genes of interest facilitate screening for genes under selection in a particular environment. Prior to this work, mutants were generated by random transposon insertions, which yielded highly complex pools for *in vivo* studies. Recent advances in polymerase chain reaction (PCR)-based mutagenesis in bacteria using the lambda red recombinase, as well as whole genome sequencing, enable a more directed approach for the generation of mutants. The lambda red approach was used to construct individual mutants in 1,023 *Salmonella enterica* serovar Typhimurium genes, including almost all genes found in *Salmonella,* but not in related genera. All the mutations were confirmed simultaneously using a novel amplification strategy to produce labeled ribonucleic acid (RNA) from a T7 RNA polymerase promoter, introduced during the construction of each mutant, followed by hybridization of this labeled RNA to a Typhimurium genome tiling array. To demonstrate the ability to identify fitness phenotypes using our pool of mutants, the pool was subjected to selection by intraperitoneal injection into BALB/c (Bagg Albino) mice and was recovered from the spleen. Changes in the representation of each mutant

were monitored using T7 transcripts hybridized to a novel inexpensive minimal microarray. Among the top 120 statistically significant spleen colonization phenotypes, 51 were mutations in genes with no previously known role in this model. Fifteen phenotypes were tested using individual mutants in competitive assays and eleven were confirmed in individual mixed intraperitoneal infection in mice, including the first two examples of attenuation for sRNA mutants in *Salmonella*. We refer to our method as Array-Based Analysis of Cistrons Under Selection (ABACUS).

Among the confirmed mutants identified in the ABACUS screen was a component of the twin arginine transport (Tat) system, *tatC*, required for transport of folded proteins across the cellular membrane. TatC is the highly conserved component necessary for recognition of the twin arginine containing signal sequence S/T-R-R-x-F-L-K. We confirmed Δ*tatC* mutants are defective for colonization of the liver and spleen in competitive infections with wild type ATCC14028 after intraperitoneal infection in *Salmonella*- susceptible (BALB/c). We also found that Δ*tatC* mutants were defective for swimming motility, but not swarming motility, which was linked to the ability to elaborate flagellins on the bacterial surface under different conditions.

DEDICATION

First and foremost, the work contained within this dissertation would not be possible without strength and wisdom from God, who guides my path daily. He has leveled every seemingly insurmountable obstacle and has been a light in the darkness.

I would also like to dedicate this work to my parents, who taught me the value of hard work by the example of their lives. They instilled in me the strength of the individual human spirit, which cannot be broken and a will to keep pursuing my dreams. Although my father did not live to see me reach this goal, he always told me that his children were his greatest legacy and I know he would be proud. Thank you Mom for keeping his spirit alive and being my greatest cheerleader.

To my children Kyle, Abigail, and David: may my life and work be an example to you as my parents were for me. Follow your dreams, believe in yourself and know that you can achieve anything you set your mind to accomplish.

Last, but certainly not least, to my husband, my best friend, and my rock: I love you dearly, Shawn. You have supported me, put up with me, pushed me, and I know this would not have been possible without you. I thank God for bringing you into my life everyday.

"To strive, to seek, to find, and not to yield."

Ulysses

Alfred, Lord Tennyson

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I have had the privilege during my pursuit of graduate studies to work with two wonderful committees. I would like to thank Dr. Geoffrey Kapler, Dr. Mike Kladde, Dr. Gary Kunkel, and Dr. Dorothy Shippen for your mentorship, support and insights. I truly learned a great deal from each of you. I would also like to thank my current committee, Dr. Garry Adams, Dr. James Samuel, and Dr. Jon Skare, for welcoming me to a new department, being patient as I learned a bit about immunology, and for offering new approaches and unique points of view on my project.

I would especially like to thank my advisor, Dr. Helene Andrews-Polymenis, without whom I might not have reached this goal. You taught me that it is possible to have a career in science and a balanced family life. At a time in my life when I truly needed to know this truth, you were there and I thank you. I also thank you for guiding me through the scientific process and being so enthusiastic about my projects. I am so excited about the direction of the lab and I wish you all the best.

Collaboration has been the key to the success of this project. I would like to acknowledge and thank the lab of Dr. Michael McClelland, especially Dr. Carlos Santiviago and Dr. Steffen Porwollik. You are true innovators, may you continue to conquer new frontiers for years to come.

Finally, I would like to acknowledge those who have struggled alongside me, both in my lab and in graduate school in general. To Lydia Bogomolnaya, Chris Carvin, and Archana Dhasarathy: thank you for long discussions, scientific and otherwise. Your

mentorship has been invaluable to me. To my co-workers, Hee-Jeong Yang, Tiana Endicott, Christine Sivula, and Christine Shields: I have thoroughly enjoyed working with you and I wish you all the best in your endeavours. Finally, to my classmates, especially Bonny and Ryan Milimaki, Anastasia Saccharidou, and Charles Greenwald: thank you for staying up all night and studying with me at Denny's, sharing your lives (weddings and children), and being supportive of me in good times and bad. You have all inspired me in your own ways and I love you all.

To all of my family and friends and mentors, you have each made me what I am today and I pray that I may continue to learn from you and lean on you. Thank you and God Bless you all!

NOMENCLATURE

ABACUS	Array based analysis of cistrons under selection
ABC	ATP-binding cassette transporter
Amp^R	Ampicillin resistance
ATCC	American type culture collection
CFU	Colony forming units
Cm^R	Chloramphenicol resistance
DFI	Differential fluorescence induction
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DOC	Deoxycholate
FACS	Fluorescence activated cell sorting
FAE	Follicle associated epithelium
FBS	Fetal bovine serum
FLP	Flippase recombination enzyme
FRT	Flippase recognition target
FUN	Genes of unknown function
GALT	Gut associated lymphoid tissue
GFP	Green fluorescent protein
GSP	General secretory pathway

IVET	*In vivo* expression technology
IVI	*In vivo* induced
Kan^R	Kanamycin resistance
LB	Luria Bertani medium
LD_{50}	Lethal dose at which 50% death occurs
LPS	Lipopolysaccharide
Nal^R	Nalidixic acid resistance
NRAMP	Natural resistance associated macrophage protein
OD	Optical density
P22	Phage used for transduction of *Salmonella*
PAGE	Polyacrylamide gel electrophoresis
PAMP	Pathogen associated molecular pattern
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline with 0.5% TWEEN (polysorbate)
PCR	Polymerase chain reaction
PVDF	Polyvinylidene fluoride membrane
RIVET	Recombinase based in vivo expression technology
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SCOTS	Selective capture of transcribed sequences
SCV	*Salmonella* containing vacuole
SDS	Sodium dodecyl sulfate

SEC	Translocase of the general secretory pathway
SPI	*Salmonella* pathogenicity island
SSC	Saline sodium citrate buffer
STM	Signature tagged mutagenesis
STM#	*Salmonella* serovar Typhimurium gene number
Tat	Twin arginine translocation
TATFIND	Twin arginine signal prediction software
TatP	Twin arginine signal prediction software
TCA	Tri-chloroacetic acid
TdT	Terminal transferase enzyme
TMDH	Transposon mediated differential hyridization
TraSH	Transposon site hybridization
TTSS	Type three secretion system
XP	5-bromo-4-chloro-3-indolyl-b-D-phosphate

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CHAPTER I

INTRODUCTION TO *SALMONELLA*

Background

Salmonella are responsible for millions of cases of food-borne illness and millions of cases of Typhoid Fever worldwide, despite efforts to reduce transmission of these organisms [1]. The majority of cases of salmonellosis in the United States are the result of food-borne infection with broad host range serotypes Typhimurium and Enteriditis [2,3,4]. One factor that has impacted the ability to control infection and transmission of this organism is that *Salmonella* are able to survive in numerous niches, including in diverse animal hosts, on vegetables and fruits, in the environment for livestock or vegetable production (farm, water, soil, etc.), and in other locations during food production.

Salmonella was first isolated in 1885 by Salmon and Smith as the bacterium thought to be responsible for hog cholera (later shown to be caused by a virus). Currently there are two species recognized: *Salmonella enterica* and *Salmonella bongori*. *Salmonella enterica* is divided into 6 subspecies containing over 2000 serotypes (**Figure 1**). *Salmonella* serotypes are differentiated and classified based on outer membrane structures LPS (O antigen), flagella (H antigen) and capsule (Vi antigen).

This dissertation follows the style of *PLoS Pathogens*.

[5,6]. Additional methods, such as phage typing, allow more detailed differentiation of *Salmonella* within a particular serotype. Approximately 1454 serotypes belong to subspecies I, a group of isolates that is responsible for 99% of mammalian and avian infections. Other subspecies are naturally found in reptiles and are commensals in this niche, but can be transmitted to and occasionally cause disease in mammals.

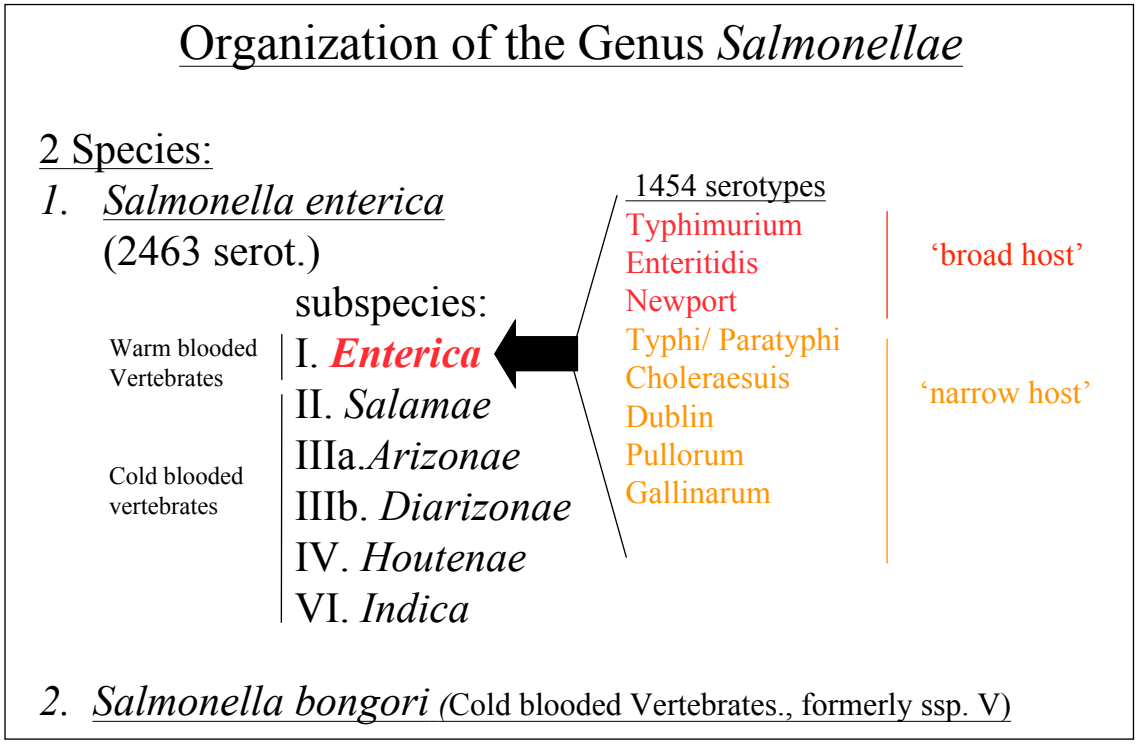


Figure 1 : Organization of the Genus *Salmonellae*. The Genus *Salmonellae* is organized into 2 species, *Salmonella enterica* and *Salmonella bongori*. Subspecies I of *S. enterica* is responsible for 99% of mammalian infections.

Salmonella are capable of causing two distinct diseases depending upon the infecting serotype and the nature of the host. Typhoidal serotypes, such as *Salmonella enterica* serotypes Typhi and Paratyphi, lead to a systemic enteric fever, but are limited to the infection of humans. Non-typhoidal serotypes, on the other hand, cause gastroenteritis or enterocolitis in a broad range of warm-blooded hosts. The most common non-typhoidal serotypes isolated from humans are Typhimurium and Enteriditis. The ability of certain serotypes to infect a broad range of hosts, while other serotypes are host limited may be attributed to the differences in genetic make-up of each strain, however, this is only a small percentage of the genome.

Animal Models of Infection

Salmonella enterica serotype Typhimurium can be used to study both typhoidal and non-typhoidal disease. Infection with serotype Typhimurium leads to murine typhoid in susceptible mice (BALB/c, C57B/6), that carry a non-resistant allele of the *NRAMP* (natural resistance- associated macrophage protein)[7,8,9] gene. These susceptible mice succumb to typhoid symptoms within 5 days of oral infection, thus they are a model for systemic disease in immunocompromised individuals, such as those with HIV, as well as Human Typhoid Fever. Mice that carry a wild type *NRAMP* gene (129sV, CBA, A/J) are not susceptible to systemic infection and are used to study long-term persistence of *Salmonella* because they colonize the intestine in high numbers [10,11].

Calves are a natural host of serotype Typhimurium and their use as a model system has real implications for food safety, as well as understanding human disease, due to the similarities in the pathogenesis and progression of the disease[12,13,14]. Orally infected calves develop similar clinical symptoms, including diarrhea [12,13,15,16], and intestinal pathology [17,18]. In a diarrheal model of infection, ligation of the ileal loops is used to observe changes in fluid accumulation, comparing the pro-inflammatory response of calves infected with wild type versus mutant strains [19,20,21,22]. Other relevant livestock models of infection include swine, chickens and rabbits.

Pathogenesis

Salmonella enterica serovar Typhimurium is a zoonotic bacterium that causes gastroenteritis in healthy individuals, but can lead to systemic disease in immunocompromised individuals. The onset of symptoms of gastroenteritis occur within 12-72 hours after ingestion of contaminated food or water (**Figure 2**); clinical symptoms include diarrhea, abdominal cramps, nausea and vomiting lasting 4-7 days in otherwise healthy individuals. Resolution of symptoms typically occurs without the use of antibiotics and it has been shown that use of antibiotics actually prolongs symptoms, as well as the period of asymptomatic shedding that follows. Complications such as septicemia and Reiter's syndrome (arthritis) are rare [23]. In individuals with a depressed immune system, infection with *Salmonella* can lead to a chronic infection and even death. The effects on the immunodeficient is of special importance in developing

countries with a high incidence of HIV, which is often correlated with a high incidence of non-typhoidal *Salmonella* infection.

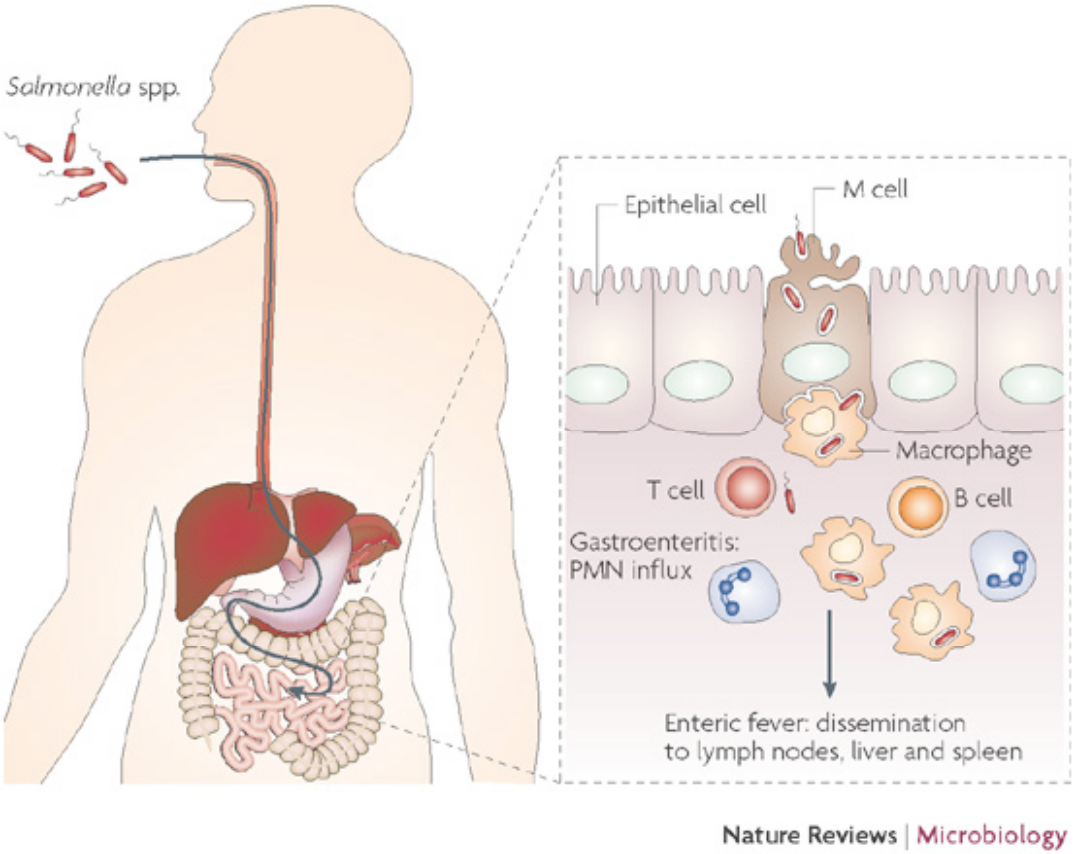


Figure 2: *Salmonella* Pathogenesis. Orally ingested *Salmonella* passes through the stomach and the upper intestine before invading the epithelium of the small intestine. Once the epithelial layer is breached, *Salmonella* may be disseminated though the reticuloendothelial system. Adapted from Haraga et al. [24].

Salmonella must survive in many niches of the host during infection. First, the Acid Tolerance Response is induced to protect against low pH in the stomach, but in addition, global regulation by *rpoS* induces cross-protection against many other environmental conditions [25]. The bacteria move down the intestinal tract, where they invade the intestinal epithelium, the Gut Associated Lymphoid Tissue (GALT) [26], using the SPI-1 (*Salmonella* Pathogenicity Island) type-three secretion system (TTSS). There are 13 annotated fimbrial operons [27]that potentially aid in attachment to epithelial cells; *fim* encodes a Type I fimbriae [28,29], *agf* encodes curli fimbriae [30,31], and there is little information about *lpf, pef*, *bcf*, *stb*, *stc*, *std*, *stf*, *sth*, *sti*, *saf*, and *stj*. Recently, *lpf, bcf, stb, stc, std*, and *sth* were shown to be involved in intestinal persistence in a mouse model of infection [32].

Salmonella are able to heavily colonize the Peyer's patches [33]. Invasion of M-cells, specialized lymphoid cells on Peyer's patches, and intestinal epithelial cells is regulated by *hilA* [34,35] and the SPI-1 TTSS [36]. SPI-1 effectors SopB, SopE and SopE2 trigger cytoskeletal rearrangements that are necessary for invasion [37,38]. SopB induces macropinosome formation and fission of the *Salmonella* Containing Vacuole (SCV), thus affecting trafficking within the cell [39,40].

SPI-1 invasion mediates inflammation and fluid accumulation in the infected intestine [13,41,42,43,44,45,46]. The release of chemokines, including IL8 and CXC chemokines from epithelial cells is promoted by the TTSS-1 effectors SipA, SopA, SopB, SopE1 and SopE2 [47]. Once inside the cell, *Salmonella* is contained inside the SCV. Inside this compartment, secretion of effectors from the TTSS-2 is induced. The

effectors of the TTSS-2 modulate the fate of the SCV, leading to inhibition of lysosomal fusion [48,49,50] and altered vacuolar trafficking [49,50,51], including inefficient trafficking of vesicles containing oxidative compounds [51,52]. *Salmonella* escape into the lamina propria, either passing through epithelial cells, slipping between cells via disrupted tight junctions or by sampling of dendritic cells. Once the *Salmonella* have breached the boundary of the epithelium, they are phagocytosed by macrophages. The SPI-1 effector *sipB* has been shown *in vitro* to bind Caspase-1 of the resident macrophages and induce apoptosis [53,54]. Caspase-1 also induces IL1B and IL18, leading to a pro-inflammatory response, benefiting the bacteria by affecting the normal intestinal flora [55]. Proinflammatory signaling results in a large influx of polymorphonuclear neutrophils (PMNs) leads to fluid secretion, likely by producing prostaglandins that affect Na+/Cl- channels of the host cells [56]. The result is damage to the intestinal epithelium and diarrhea.

SPI-2 TTSS is essential for intracellular growth [57,58,59] within macrophages, a process necessary for systemic infection [60]. Inside the macrophage, the bacteria reside within a specialized structure called the *Salmonella* Containing Vacuole (SCV). SPI-2 secretes effectors including SpiC that inhibit normal trafficking of endocytic vesicles, as well as inhibition of phagolyosomal fusion, seen in cultured J774 macrophages [49].

SPI-2 also inhibits localization of reactive nitrogen species (RNS) and vesicles containg NADPH-oxidase, which leads to the production of reactive oxygen species (ROS) [51,61]. ROS act to inhibit growth of *Salmonellae* early during infection [62],

and vesicles containing ROS are localized to the SCV through TNFα receptor p55 signaling [61]. RNS controls the growth of *Salmonellae* during both early and late phases of infection, in contrast to ROS [62,63,64]. Antimicrobial peptides have also been shown to be important for controlling growth of *Salmonella* within macrophages [65].

Growth rates of *Salmonellae* during infection are increased in susceptible models of infection [10,66]. A single amino acid substitution in Slc11a1/Nramp is associated with susceptibility to systemic infection in mice and leads to altered function of a divalent metal ion pump of the macrophage late endosome [8,9]. The macrophage is only able to slow growth of the organism, and eventually, the bacteria are able to escape the macrophage through the production of necrotic cytotoxins [67,68,69].

Toll-like receptors of the macrophages are able to recognize pathogen associated molecular patterns (PAMPs) such as LPS, flagella, and bacterial DNA. TLR4, which recognizes Lipid A of LPS, has been shown by *in vivo* and *in vitro* methods to be required by the host during innate immune response due to *Salmonella* infection [70,71,72,73]. TLR2 was shown to work with TLR4 to promote TNFα production [74] and LPS induced shock is mediated by *waaN* mutation in *Salmonella* [75]. Also, curli amyloid fibrils, produced by the *csg* operon, have been shown to up-regulate Nos2 expression in a TLR-2 dependent manner, likely contributing to sepsis [76].

T and B cells are necessary for clearance, antigen specific immunity, and prevention of relapse or chronic carrier state of salmonellosis. $CD4^+$ $TCR\alpha\beta^+$ T cells are necessary for late stages of infection for control of growth, as observed in

immunodeficient mice infected with a Δ*aroA* strain of *Salmonella* [77]. Systemic infection with *Salmonella* leads to activation of $CD4^+$ cells ($CD44^{high}$ and $CD62L^{low}$) and a Th1 skewed phenotype, increasing the efficiency of the cellular immune response [78]. B cells are necessary for Th1 development [79,80,81]. B cells generally produce antibodies, but are also capable of presenting antigen to T cells upon infection with pathogens, including *Salmonella* [82,83]. *Salmonella* can inhibit T cell proliferation by direct contact inhibition in a SPI-1 and SPI-2 independent manner [84], as well as inhibiting MHCII in a *sifA* dependent process [85]. Genes involved in these mechanisms may be of special interest since inhibition of T cells blocks clearance and leads to a chronic carrier state, also known as long-term persistence.

Once the clinical symptoms of *Salmonella* gastroenteritis subside, an asymptomatic carrier state begins. Typhimurium can be shed in the feces of the infected individual for an average of 8 weeks [86]. Infected livestock may shed from six months to the life of the animal. Long- term asymptomatic shedding is a serious problem in the food chain because detection of these passive carriers is difficult. Infected livestock can shed 10^5 CFU/gram in their feces and remain asymptomatic. According to the USDA Hazard Analysis and Critical Control Points (HACCP), meat products are randomly sampled during production, but there are no methods to prevent contamination at slaughter and during pre-production and there is no microbial testing of carcasses for infection. Identification of novel genes involved in systemic infections, intestinal persistence and long term shedding, and the development of new methods to reduce subclinical and chronic carriage is of paramount importance to food safety.

Identification of Novel Virulence Factors

Salmonella have acquired a vast array of mechanisms that allow them to adapt and thrive in a variety of conditions. In the current genomic era, forward genetics, genomic sequencing and many technologies developed in the wake of complete genome sequencing are revolutionizing the identification of genes required for *Salmonella* survival in many environments. Identification of *Salmonella* genes necessary for growth in different niches has made rapid progress in recent years, as the molecular biologic tools for their identification become more sophisticated and are applied to study this organism in an increasing number of environments. In the following chapter I detail the identification of *Salmonella* genes required in niches that have been studied to date, and describe advances in forward genetic and functional genomic approaches that are advancing this important area of study.

Genomic Sequencing

Understanding the *Salmonella* biology comprehensively begins with sequencing. Recent reductions in the cost, along with improvements in sequencing technology have made it possible to rapidly sequence many *Salmonella* genomes. The current status of genome sequencing of non-typhoidal *Salmonellae* has recently been reviewed [87], and since the beginning of 2009 two additional genomes have been completed, and a number of sequences have been updated (**Table 1**).

Table 1: Sequencing Progress of Salmonella Isolates.

Sequences completed	Sequencing Center	GenBank Accession #	Citation
Arizonae 62:z4,z23	WU	CP000880.1	
Agona SL483	JCVI	CP001138.1	
Cholerasuis SC-B67	Taiwan	AE017220.1	Chiu et al. (2005) NAR 33:1690-1698 [88]
Dublin CT_02021853	TIGR	CP001144.1	
Enteriditis P125109 (PT4)	Sanger	AM933172.1	Thomson et al. (2008) Genome Res. 18:1624-1637 [89]
Gallinarum 287/91	Sanger	AM933173.1	Thomson et al. (2008) Genome Res. 18:1624-1637 [89]
Hadar	Sanger		
Heidelberg SL476	JCVI	CP001120.1	
Infantis	Sanger		
Newport SL254	TIGR/JCVI	CP001113.1	
Paratyphi A ATCC 9150	WU	CP000026.1	McClelland et al. (2004) Nat Genet 36:1268-1274 [90]
Paratyphi A AKU_12601	Sanger	AM412236	Holt et al. (2009) BMC Genomics 10:36-47 [91]
Paratyphi B SPB7 SGSC4150	WU	CP000886.1	
Paratyphi C RKS4594, SARB49	Peking University	CP000857.1	
Salmonella bongori 12149	Sanger		
Schwarzengrund CVM19633	TIGR	CP001127.1	
Typhi AG3/Vietnam 1998	Sanger	NZ_CAAY00000000	Holt et al. (2008) Nat Genet 40:987-993 [91]
Typhi CT18/Vietnam 1993	Sanger	AL513382.1	Parkhill et al. (2001) Nature 413:848-852 [92]
Typhi Ty2 Russia 1916	U Wisconsin/Sanger	AE014613.1	Deng et al. (2003) J Bacteriology 185:2330-2337 [93]
Typhimurium DT104	Sanger		

Table 1 Continued

Sequences completed	Sequencing Center	GenBank Accession #	Citation
Typhimurium LT2	WU	AE006468.1	McClelland et al. (2001) Nature 413:852-856 [27]
Typhimurium SL1344	Sanger		

Sequences under assembly	Sequencing Center	GenBank Accession #	Citation
4,[5], 12:I: CVM23701	TIGR/JCVI	NZ_ABAO00000000	
Hadar RI_05P066	TIGR/JCVI	NZ_ABFG01000000	
Heidelberg SL486	TIGR/JCVI	NZ_ABEL01000000	
Javiana GA_MM04042433	JCVI	NZ_ABEH02000000	
Kentucky CDC 191	JCVI	NZ_ABEI01000000	
Kentucky CVM29188	TIGR	NZ_ABAK02000000	
Newport SL317	JCVI	NZ_ABEW01000000	
Saintpaul SARA23	TIGR	NZ_ABAM02000000	
Saintpaul SARA29	TIGR	NZ_ABAN01000000	
Schwarzengrund SL480	JCVI	NZ_ABEJ01000000	
Tennessee CDC07-0191	CDC	NZ_ACBF00000000	
Typhi E98-0664/Kenya 1998i	Sanger	NZ_CAAU00000000	Holt et al. (2008) Nat Genet 40:987-993 [91]
Typhi 150(98)S/Vietnam 2004	Sanger		Holt et al. (2008) Nat Genet 40:987-993 [91]
Typhi 404ty/Indonesia 1983	Sanger	NZ_CAAQ00000000	Holt et al. (2008) Nat Genet 40:987-993 [91]
Typhi 8(04)N/Vietnam 2004	Sanger		Holt et al. (2008) Nat Genet 40:987-993 [91]
Typhi E00-7866/Morocco 2000	Sanger	NZ_CAAR00000000	Holt et al. (2008) Nat Genet 40:987-993 [91]

Table 1 Continued

Sequences under assembly	Sequencing Center	GenBank Accession #	Citation
Typhi E01-6750/Senegal 2001	Sanger	NZ_CAAS00000000	Holt et al. (2008) Nat Genet 40:987-993 [91]
Typhi E02-1180/India 2002	Sanger	NZ_CAAT00000000	Holt et al. (2008) Nat Genet 40:987-993 [91]
Typhi E02-2759/India 2002	Sanger		Holt et al. (2008) Nat Genet 40:987-993 [91]
Typhi E03-4983/Indonesia 2003	Sanger		Holt et al. (2008) Nat Genet 40:987-993 [91]
Typhi E03-9804/Nepal 2003	Sanger		Holt et al. (2008) Nat Genet 40:987-993 [91]
Typhi E98-2068/Bangladesh 1998	Sanger	NZ_CAAV00000000	Holt et al. (2008) Nat Genet 40:987-993 [91]
Typhi E98-3139/Mexico 1998	Sanger	NZ_CAAZ00000000	Holt et al. (2008) Nat Genet 40:987-993 [91]
Typhi ISP-03-07467/Morocco 2003	Sanger		Holt et al. (2008) Nat Genet 40:987-993 [91]
Typhi ISP-04-06979/Africa 2004	Sanger		Holt et al. (2008) Nat Genet 40:987-993 [91]
Typhi J185SM/Indonesia 1985	Sanger	NZ_CAAW00000000	Holt et al. (2008) Nat Genet 40:987-993 [91]
Typhi M223/Unknown 1939	Sanger	AE014613.1	Holt et al. (2008) Nat Genet 40:987-993 [91]
Virchow SL491	TIGR/JCVI	NZ_ABFH02000000	
Weltevreden HI_N05-537	TIGR/JCVI	NZ_ABFF01000000	

Sequences in progress	Sequencing Center	GenBank Accession #	Citation
Arizonae 05-0715 ATCC BAA-1577	WU		
Diarizonae 61:1,v:1,5	WU		
Diarizonae ATCC BAA-1579 05-0625 48:i:z	WU		
Houtenae SARC13 ATCC BAA-1580 45:a:e,n,x	WU		
Houtenae SARC14 ATCC BAA-1581 11:b:e,n,x	WU		

Table 1 Continued

Sequences in progress	Sequencing Center	GenBank Accession #	Citation
Indica ATCC BAA-1576	WU		
Indica ATCC BAA-1578	WU		
Salamae ATCC BAA-1582 (05-0626) 47:b:1,5	WU		
Salamae SARC3 58:d:z6	WU		
Abortusovis SSM0041	WU		
Bovismorbificans 01-05481 PT13	WU		
Braenderup S-500	WU		
Brandenburg KMR12	Korea		
Dublin	U Illinois		
Enteriditis 48-0811	WU		
Enteriditis LK5	U Illinois		
Enteriditis P125109	Korea		
Enteriditis SARB17	WU		
Enteriditis SARB19	WU		
Indiana KMR53	Korea		
Infantis SARB27	WU		
Miami ATCC BAA-1586 (02-3341)	WU		
Montevideo SARB30	WU		
Muenchen SARB32	WU		
Muenchen SARB34	WU		
Muenster ATCC BAA-1575 (0065-00)	WU		

Table 1 Continued

Sequences in progress	Sequencing Center	GenBank Accession #	Citation
Newport CVM36720	UMIGS		
Panama KMR64	Korea		
Paratyphi B ATCC BAA-1585	WU		
Paratyphi B SARB47	WU		
Paratyphi B tartrate (+) [Java] ATCC BAA-1584 (S-1241)	WU		
Poona SGSC4934	WU		
Pullorum	U Illinois		
Schwarzengrund KMR78	Korea		
Sendai 55-2461	WU		
Senftenberg SARB59	WU		
Stanley SARB60	WU		
Thompson SARB62	WU		
Typhi SGSC2661	WU		
Typhi Ty21a	Naval Med Research Center		
Typhimurium 14028s	WU		
Typhimurium D23580	Sanger		
Typhimurium DT2	Sanger		

The data compiled from sequencing projects has allowed the development of microarrays, and comparative genomic, transcriptomic, and forward genetic studies using these tools [27,94,95,96,97,98]. Microarray technology has also recently been described as a novel method for the rapid molecular typing of *Salmonella* isolates [99].

Annotated genomes are the foundation for functional genomic studies, yet even annotation cannot completely describe a genome. The first complete *Salmonella* genome sequences were published in 2002, but gaps in our knowledge of these genomes persist. Annotation only assigns a putative function to genes based on sequence homology to genes of known function, and this is only a starting point for determination of the function of a gene. For example, the annotated genome of *Salmonella enterica* serotype Typhimurium LT2 contains three genes that are annotated as *citE* (*STM0060*, *STM0622*, and *STM3120*), and are assigned the function of a citrate lyase by homology (**Figure 3**). However, whether the proteins encoded by these genes really have an identical function or not, and the timing, conditions of the expression of these three genes and the importance of each of these genes in growth and survival, will only be determined by functional studies. Furthermore, in the Typhimurium LT2 sequence, there are approximately 1000 genes out of 4500 that have been assigned as putative, in terms of *location* in the cell, but have no assigned *function*. The case for other genomes is similar, for example, Typhi has approximately 200 hypothetical and 600 conserved hypothetical open reading frames [92]. In the future, it will be vital to our understanding of virulence mechanisms and pathogenesis, to assign well-characterized functions to all genes within the genomes of interest.

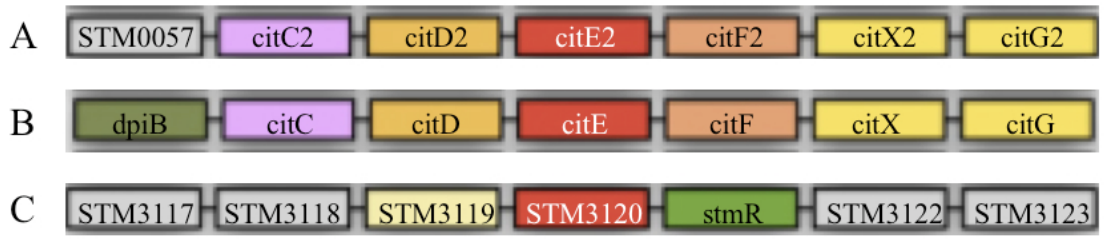


Figure 3: Context of Multiple Citrate Lyase Homologs within the *Salmonella* Genome. A) Region surrounding *STM0060* (*citE2*) B) Region surrounding *STM0622* (*citE*) and C) Region surrounding *STM3120* (Adapted from Goulding et al. [100]).

Genetic content, along with what is known about the niche preferences of different serotypes, may also provide information about the ability to survive within different hosts or environments. Comparison of the LT2 sequence with sequences of closely related serovars, such as Typhi, Enteriditis, Galinarum, Cholerasuis, and Dublin has revealed interesting differences between serovars that infect a broad range of hosts and those that are more specialized and infect only specific hosts. Host specialist genomes, such as Typhi and Cholerasuis, typically contain a large number of pseudogenes: Typhi has approximately 200 [92] and Cholerasuis has 151 [88], while the host generalist Typhimurium has only 39 [27]. Typhimurium also has a large array of fimbrial operons, encoding up to 13, presumed to be necessary for survival within different hosts [27]. More specialized strains, such as Dublin [97] and Typhi [92], encode a much smaller repertoire of fimbria. In contrast, *Salmonella* Pathogenicity Islands appear to be rather stable across serovars, leading some to believe that the

differences that do exist are necessary for host adaptation, while the machinery (i.e. type three secretion systems) remains intact.

In order to identify genes important in particular niches, two broad approaches have been employed. Promoter-trapping strategies and microarray-based approaches (**Table 2**) have been used to evaluate *gene expression* under particular sets of conditions. Such studies offer both a limited set of genes to explore for functional importance during a particular process, as well as a more comprehensive determination of gene expression changes that can be used to outline and analyze pathways necessary for a particular process. Forward genetic screens, and now functional genomics are being used to identify genes *required for survival and growth of Salmonella,* in particular niches. The generation of mutants using various methods, and the study of these mutants in various environments remains a definitive method for identifying necessary genes and beginning a molecular determination of their function in the environment under study.

Salmonella **Genetic Screens: A Review**

In Vivo *Expression Screens: IVET*

Promoter trap strategies were first developed in the 1980s [101] and they enabled the identification of promoters that are up-regulated in particular environments, including infected tissue. *In vivo* expression technology (IVET) was originally described in 1993 as a method for identifying genes specifically induced during *Salmonella* infection (*in vivo* induced genes, or *ivi*) [102]. In general this strategy works in the following way; a library of fragments of genomic DNA from the genome of interest is

cloned upstream of a gene necessary for growth under a particular condition and integrated into the *Salmonella* genome. This promoter trap library is then screened for reporter expression during infection, and constitutive promoters are excluded by comparison of *in vitro* expression with expression during infection. In the initial IVET screen, Typhimurium genomic DNA was cloned upstream of a promoterless *purA-lacZY* fusion, in a *purA* mutant background, and fusions were placed in single copy on the bacterial chromosome [102]. BALB/c mice were infected with a pool of these promoter trap chromosomal fusions and three days post-infection, bacteria were recovered from infected spleens [102]. Surviving bacteria had PurA expression during infection [102]. Constitutive promoters were excluded by testing the recovered strains for *in vitro* expression of lacZY on MacConkey lactose inhibitor plates. In this initial screen, genes necessary for *de novo* pyrimidine synthesis (*carAB*) and LPS synthesis (*rfb*) were shown to be expressed during infection of murine models, and requirement of the genes during infection was confirmed by determination of LD_{50} of *Salmonella* mutants in these genes [102].

The next generation of IVET was developed to broaden applicability to other host/pathogen systems and uses an antibiotic resistance reporter, *cat*, instead of *purA* [103]. After infection with a pool of chromosomal fusions, mice are supplemented with chloramphenicol to select for expression of the resistance marker during infection. Using this method, a gene required for fatty acid degradation, *fadB*, was identified and expression of the promoter fusion was confirmed in the presence of oleate [103].

This strategy was also used to identify *Salmonella* genes induced in RAW 264.7 macrophages [103]. Five *ivi* fusions were identified and individually tested for growth in macrophages in the presence of chloramphenicol. The strains, MT735-MT739, showed a 500-1000 fold increase in growth in macrophages supplemented with chloramphenicol compared with random "non-selected" fusions [103]. While these specific fusions were not characterized further, *ivi* fusions necessary for growth in macrophages found in later studies were shown to be PhoPQ regulated and/or iron responsive [104,105].

The two versions of IVET described above were also used to identify *Salmonella* promoters induced during infection of BALB/c mice by the oral and intraperitoneal routes, as well as during infection of RAW 264.7 macrophages [104]. Three different infection models were chosen to identify novel genes expressed during infection, and for internal validation. Genes required for global gene regulation (*phoP*, *pmrB*, *cadC*, *iviXIII*, and *vacBC*), during stationary phase (*spvB*, *cfa*, *otsA*), for metabolism (*recD*, *hemA*, *entF*, *fhuA*, *cirA*, *mgtAB*, *iviX* and *ndk*), and genes required for adhesion and invasion (*iviVI-A* and *iviVI-B*) were identified as induced during infection in this large-scale screen [104]. Three genes of unknown function were also identified and determined to be important for survival within macrophages, *iviXI*, *iviXII*, and *iviXV* [104]. Identification of fusions in 2 or more infection routes, such as the spleen after intraperitoneal infection and during macrophage infection, were used as internal controls and validation for the study. In a follow-up study, *ivi* fusions (*mgtAB*, *spvB*, *iviVI-A, iviXVI*, *phoP*, *pmrB*, *iviXVII*, *fhuA*, *cirA*, and *entF*) were tested for responsiveness to low pH and low Mg^{2+}, conditions that may exist within the *Salmonella* containing vacuole

[105]. Each of these genes may not individually be responsible for full virulence of *Salmonella* and the authors cite the *inv* locus of *Yersinia*, which shows no defect in LD_{50} studies, but is important for invasion of cultured epithelial cells and colonization of Peyer's patches [106]. Heithoff therefore argues that the summation of the "*in vivo* induction profile" is necessary for the ability of *Salmonella* to survive and replicate in a variety of niches, despite a lack of forward genetics.

IVET in animal models provides general information about promoters that are induced during infection, yet it does not provide any information regarding the specific location where these promoters are induced. In order to determine spatial induction of promoters, it is necessary to break the animal model down and examine specific organs that may be important during infection, such as the liver. IVET was used to identify promoters that are induced during infection of cultured murine hepatocytes [107]. Genes identified in this screen included known virulence factors *sodA*, *pagJ* and *ssaE*, as well as an iron transport operon, *sitABCD* [107]. The *sit* operon is located on *Salmonella* Pathogenicity Island 1 (SPI-1) and was shown to be required for full virulence in BALB/c and C3H/HeN mice infected by the oral an intraperitoneal routes of infection, but it is not required for initial invasion of Henle 407 epithelial cells or hepatocytes [107].

In Vivo *Expression Screens: RIVET*

IVET has been further modified using a highly sensitive recombinase method called Recombinase- based IVET (RIVET) [108]. A library of promoter fusions is

generated upstream of a promoterless resolvase, *tnpR*, that induces excision of an antibiotic resistance cassette when it is expressed. A loss of tetracycline resistance is a permanent marker of induction that occurred at some point during the infection, enabling the identification of transient events. RIVET was initially used the screen for promoters expressed in *Vibrio cholera* during infection of infant mice.

In *Salmonella enterica* serotype Typhimurium, RIVET was recently used to identify promoters expressed during infection in swine [109]. Genomic DNA was cloned upstream of a promoterless Cre-LacZ cassette and the resulting library of plasmids was transformed into a strain harboring a chromosomal cassette containing a gene for sucrose sensitivity (*sacB*) and kanamycin resistance flanked by *loxP* sites. When the upstream genetic element is activated, the Cre recombinase is expressed and both the *sacB* and the kanamycin resistance genes are excised, resulting in sucrose resistance and kanamycin sensitivity. In conjunction with a modified signature tagged mutagenesis strategy (described later in this chapter), this method was used to identify 32 unique promoters that are induced during infection in swine [109]. Huang et al. also found expression of regions that were cloned in a reverse orientation and suggest that they may have identified anti-sense regulators [109]. The recombinase plasmid used in this study also encodes a promoterless *lacZ* reporter downstream of Cre, which was used to measure the level of induction of certain promoters, using a β-galactosidase assay, under specific environmental conditions, such as high temperature and osmotic stress [109].

In Vivo *Expression Screens: DFI*

Differential Fluorescence Induction (DFI) is an alternative promoter trap strategy that utilizes GFP as a reporter along with fluorescence-activated cell sorting (FACS) analysis to find promoters that are active during infection, independent of antibiotic susceptibility or metabolic requirements [110,111,112]. A library of *Salmonella* genomic DNA fragments is cloned upstream of promoterless GFP and expression of GFP can be examined under a variety of conditions. Initially, DFI was used to identify promoters induced upon exposure to an acidic environment, such as *Salmonella* might experience in the stomach, or within macrophages and in intracellular vacuoles [110]. Promoters that were induced upon exposure to low pH, but repressed when exposed to high pH were termed acid inducible genes and included the promoter for cyclopropane fatty acyl phospholipid synthase (*cfa*) [110]. Induction of *cfa* is necessary for modification of the *Salmonella* membrane in response to stress [113]. DFI was subsequently used to examine promoters induced when *Salmonella* was associated with host cells, such as RAW 264.7 macrophages [111]. Among the genes induced within macrophages were PhoPQ regulated and OmpR/EnvZ regulated genes, as well as members of *Salmonella* Pathogenicity Island 2 (SPI-2), encoding the type three secretion system 2 (TTSS-2) [111].

Host cell auto-fluorescence, which can make it difficult to distinguish GFP expressing bacteria from host tissue, is a major drawback to using GFP as a reporter. This limitation has been reduced using two-color flow cytometry [112]. Three promoters expressed during infection have been identified during oral infection of BALB/c mice

using this two-color flow cytometry DFI method [112]. AroQ is one of many chorismate mutase genes encoded in the *Salmonella* genome, and is necessary for biosynthesis of aromatic amino acids. Pathogenicity Island encoded protein B (*pipB*), which lies upstream of *sopB* (SPI-1 effector protein) on SPI-5, has been shown to be necessary for virulence in BALB/c mice infected orally [114] and subsequently shown to be secreted by the SPI-2 TTSS [115]. Another SPI-2 effector, *sifA*, was also identified in this screen and is required for virulence in mice associated with formation of filamentous structures of the *Salmonella* containing vacuole [116].

This flow cytometry approach has also been used to identify *Salmonella* promoters that are up-regulated in tumors in mice, a tissue for which *Salmonella* has a specific tropism [117]. Nude mice were infected with a library of 180,000 promoter-trap fusions to otherwise promoterless GFP, and clones were recovered from tumors and normal spleen tissues and sorted by FACS for GFP expression. Clones expressing GFP *in vitro* were sorted out, and the remaining clones were subjected to additional rounds of enrichment for tumor specific promoters. Promoters of genes required under hypoxic conditions, including *pflE* (pyruvate-formate-lyase actvating enzyme), *flhB* (flagellar apparatus) and *ansB* (asparaginase) were identified in this study as specifically induced in tumor tissue [117]. The identification of tumor specific promoters will be useful for targeting chemotherapeutic agents to be expressed specifically in tumors.

In Vivo *Expression Screens: Transcriptional Profiling by Microarray*

Transcriptional profiling by microarray is another high-throughput method to determine genes that have altered expression under a specific set of conditions. This method can be used in all phases of the *Salmonella* life cycle and has been used to analyze global transcript changes in regulatory mutants, gene regulation during infection, and during *in vitro* growth under infection relevant conditions, such as bile (see review [118]). Identification of *Salmonella* genes expressed during infection presents a unique set of challenges, however. Most importantly, bacterial RNA must be separated from the host RNA in order to determine only those bacterial genes that are differentially regulated. In 2003, Eriksson et al. described a novel method of bacterial RNA isolation from infected macrophages that involved the use of detergent to specifically lyse the eukaryotic cells and a phenol-ethanol mixture to stabilize the RNA [119]. High quality bacterial RNA was used to obtain a global transcriptional profile of bacterial genes, by probing a whole genome microarray bacterial RNA collected from *Salmonella* infected J774 macrophages at 4, 8, and 12 hours post-infection. Such work has shown that one quarter of the Typhimurium genome (919 genes) displayed altered expression at 4 hours post-infection of macrophages [119]. *Salmonella* Pathogenicity islands, along with other genes necessary for survival within the *Salmonella* containing vacuole (SCV) were up regulated, while many gene products expressed on the bacterial surface including LPS, fimbria and flagellar genes were down regulated. Nearly half of the genes with altered expression when Typhimurium is inside macrophages are of unknown (FUN genes). FUN genes are also defined as genes with putative functions

based on homology to other organisms, but with no biological evidence for confirmation. In this study two regions, *STM0854-0859* and *STM3117-3120,* were identified as expressed in macrophages and also having structural features of pathogenicity islands, [119].

Transcriptional profiles of Typhimurium have also been determined in cultured epithelial cells at various time points after infection, and this data can be compared to earlier work in macrophages [120]. HeLa cells were infected with Typhimurium and RNA was isolated at 4, 8 and 12 hours. While a large proportion of genes show a similar pattern of expression in both cell-types, including 128 genes of unknown function, differences in the transcriptome signatures between the different cell types also have several significant differences.

TTSS and flagellar expression during infection of the two cell types are prominent features that differ during *Salmonella* infection of macrophages versus epithelial cells. In epithelial cells, SPI-1, encoding TTSS-1 necessary for invasion of normally non-phagocytic cells, was expressed throughout the infection. But in macrophages, a phagocytic cell type, the need for TTSS-1 appears to be less critical as SPI-1 transcription was down regulated in this cell type [120]. While SPI-2 genes (encoding the TTSS-2) were expressed in both cell types, SPI-2 encoded genes appear to be more highly transcribed in macrophages than in epithelial cells [120]. During infection of macrophages, SPI-2 transcripts were highly elevated at all time points, while in epithelial cells, transcript levels were reduced over the 12 hour course of infection [120]. In contrast, flagellar genes were up-regulated at later time points during infection,

which may signal the need for motility at this stage is a necessary bacterial adaptation to prepare for lysis from dying epithelial cells [120].

In Vivo *Expression Screens: SCOTS*

An alternative method for preferentially isolating bacterial RNA from infected eukaryotic cells, termed selective capture of transcribed sequences or SCOTS, is a subtractive hybridization method [121]. In this method, *Salmonella* genomic DNA is prepared and biotinylated for use as a probe. Eukaryotic cells are infected with *Salmonella*, and at a pre-determined time point post-infection, total RNA is isolated and cDNA is prepared. cDNA from infected cells that hybridizes to the biotinylated *Salmonella* genomic DNA is isolated using streptavidin, specifically enriching for the bacterial sequences transcribed during infection.

Transcriptional differences between broad host and host-adapted *Salmonellae* (Typhimurium versus Typhi), have been examined using SCOTS. These studies identified a fimbrial operon (*stfACDEFG*) and a LysR type regulator *stmR* (*STM3121*), which are present in Typhimurium, but are not found in Typhi and may be necessary for survival in a wider range of hosts [121]. The *stf* operon was also shown by a PCR based subtractive hybridization method to be absent in Typhi [122].

Recent studies using SCOTS have identified genes expressed in macrophages and have utilized microarray technology in order to bring this technology to higher-throughput. Typhi genes that were expressed during infection of human monocytes (THP-1 cells) at 0, 2, 8 and 24 hours post-infection were identified using this

combination of methods [123]. The expression of approximately 117 genes, including genes of unknown function, was elevated at all intracellular time points [123]. The TTSSs were up-regulated early in infection. SPI-1 expression fell off shortly after infection, while SPI-2 expression remained high throughout the infection. Genes encoding proteins involved in antimicrobial peptide resistance were expressed at high levels early and late, but many were repressed at 8 hours. Genes involved in motility and iron transport were repressed throughout Typhi infection of macrophages.

Differences were observed between this study of host specific serotype Typhi in THP-1 human monocytes and the results of earlier studies examining gene expression of broad-host serotype Typhimurium in murine are perhaps a result of the host specificity of the serovar that was studied. Specifically, *phoP*, *slyA* and antimicrobial resistance genes displayed differential regulation when comparing transcriptional profiling of Typhimurium and the SCOTS profile of Typhi [123], consistent with the different niches that each serotype is exposed to during infection. Such comparative analysis may yield further clues to the evolutionary basis of host preference.

In vivo expression and expression profiling have been heavily used in animal models and in cultured cells to provide lists of genes that may be critical during infection, or growth in a particular environment. Limitations do exist however, as these methods may not be able to detect transient events that occur during infection and may give the impression that genes are not heavily differentially regulated in a given environment are dispensable in that niche. Thus, gene expression studies are best used as a platform for determining groups of genes that are coordinately regulated, in an effort to

build an overview of the network of gene expression, and as a tool to determine and prioritize candidate genes for further genetic studies.

Comparative Genome Analysis

The genus *Salmonellae* is genetically diverse and contains over 2000 different serotypes. In the pre-genomic era, examinations of this genetic diversity among isolates were done using simple genomic DNA hybridization methods described previously. In the current genomic era, genome comparison includes the use of high-throughput technologies, such as sequencing and microarrays. Use of these methods has allowed genomic comparison between *Salmonellae* in greater depth. Genetic differences between closely related strains have led to a new designation, genovars, and widespread use of the genovar designation will hopefully reduce the inaccuracies of the current serotyping classification system of *Salmonellae* [124]. By comparing the genomes of broad host range serotypes with narrow host range serotypes, it has also been possible to identify genes that are potentially responsible for the ability of *Salmonella* to invade certain hosts [96,98].

Forward Genetic Screens

Large-scale genomic studies yield a broad overview of important aspects within the genomes of a variety of serovars, but they also create a platform for more focused studies using classical techniques, such as forward genetics. The power of forward genetics lies in the ability to examine the function of a gene by disrupting that gene and

looking for a change in phenotype. For decades, researchers had only tools that would enable mutagenesis in the absence of sequence data, such as chemical mutagens and radiation treatment. More recently, re-engineered, stable transposable elements have been used to create banks of mutants for phenotypic analysis [60,125,126,127,128,129,130,131,132,133,134,135,136]. Screening these mutants, however, continued to be tedious due to limitations on studying many mutants simultaneously, and because sequencing of candidate mutants one at a time was necessary to determine the location of transposons. The development of several negative selection techniques improved the throughput and allowed screening for mutants that were not viable during infection. The publication of the first genome sequences for *Salmonella* [27,92], and the development of microarrays, allowed high-throughput screening methods for both transposon mutants and targeted deletions.

Signature Tagged Mutagenesis: The Development

In 1995 Hensel et al. described the first negative selection strategy used during infection with *Salmonella*, called signature tagged mutagenesis (STM). STM combined transposon mutagenesis and tracking of each individual transposon mutant using short, exogenously introduced, unique sequence tags on the transposons themselves [137]. These unique tags, or barcodes, were used to identify each mutant when mutants were pooled for infection, using Southern blotting for these unique tags. Small pools of mutants were assembled and subjected to a genetic selection during intraperitoneal infection of BALB/c mice, an established model for Typhoid fever [137]. Mutants

differentially represented in the input pool versus the output pool recovered from the murine spleen after infection were identified using Southern analysis [137]. Approximately 40 mutants were identified as present in the output with reduced frequency in this study, and these mutants included those in a region that was later termed *Salmonella* Pathogenicity Island 2 (SPI-2), encoding a type three secretion system (TTSS-2) [138].

The development of STM was not only a technical advance in the methods available to study bacterial pathogens during infection, it was also a large advance in our understanding of the biology of complex organisms. Numerous STM studies in *Salmonella* have followed, building upon the important work of the initial study, by determining genetic requirements for infection in numerous niches and for different serotypes of *Salmonella*.

Signature Tagged Mutagenesis: Comparative Studies Across Species

STM has been used for comparative studies; specifically for comparing the genetic requirements for *Salmonella* survival and growth during infection of different host species. In order to compare the *Salmonella* genes that were required in mice versus calves, a small pool of 260 serotype Typhimurium mutants was used for comparison during oral infection of these two different host species. *Salmonella* genes required during infection of these two hosts were classified in three categories, those necessary in mice but not calves, those necessary in calves, and those necessary in both hosts. Surprisingly, the largest category of *Salmonella* mutants that were identified in this study

were necessary for survival in both mice and calves [139], providing a basis for future work that initially identifies *Salmonella* gene requirements in mice and later tests these in more cumbersome and expensive livestock models. Four mutants (*slrP*/*STN39*, *bcf*/*STN35*, *yaiO*/*STN08*, and *STN28*) were identified as important for virulence in only one host species; three genes were determined to be necessary in mice and one gene was required only in calves. One mutant, identified as defective for colonization in mice, but not calves, was further characterized. The defective gene in this mouse-colonization deficient mutant, in a *Salmonella* leucine-rich protein *slrP*, has significant homology to *ipaH* and *yopM*, secreted effectors of type three secretion systems in *Shigella* and *Yersinia*, respectively [139]. STM has also been employed to comparatively study genetic requirements for Typhimurium infection in the natural hosts calves and chicks, using a much larger number of transposon mutants than were previously studied [139,140]. In these studies, calves and chicks were infected with pools of signature tagged mutants to identify genes necessary for virulence in either or both host systems [140]. SPI-4 was identified as an important determinant of virulence with a specific role in cattle. SPI-4 is a 27 kb region encoding a type I secretion system and a very large non-fimbrial adhesin protein, *siiE* [140,141,142,143,144]. The *siiABCDEF* operon is regulated by *sirA*, *hilA* and *rfaH* [145,146,147], and mutants in *siiE* are attenuated for virulence in mice and cattle [140,143,144]. SPI-4 is also co-regulated with SPI-1 and required for apical invasion of polarized epithelial cells, destruction of tight junctions, cytoskeletal rearrangement and effacement of the brush border [146,148]. The large size

of *siiE* is a requirement for its function, as it must reach past the lipopolysaccharide of the cell to interact with the host cell [148].

Candidate genes necessary for virulence in swine have also been identified using STM [149]. Genes in pathogenicity islands SPI-1 through 6 were identified as candidate mutants that were under-represented after infection in swine. Mutants in *Salmonella* atypical fimbria (*saf*) genes after infection of swine suggest a role for this adhesin during infection, and a subtle role for *saf* was confirmed in a competitive oral infection of swine. Mutants in a structural component of SPI-1, *prgH*, were also defective in colonization of cattle and pigs [140,149], and the efficacy of the *prgH* mutant as a vaccine candidate has been explored [149]. Finally, over 40 genes encoding proteins of unknown or hypothetical function were identified after STM screening in swine, including *STM0557 (gtrC1)*, a gene that has since been characterized as necessary for O12 antigen form variation and persistence of Typhimurium in the murine intestine [150]. STM has also been used to identify serotype Typhimurium genes in an *ex vivo* assay, with swine stomach contents as the selective mechanism [151]. Several genes of putative function were identified from the 34 pools of 48 mutants tested, as defective in the ability to survive the harsh, acidic environment of the stomach.

STM has also been used to determine genes necessary for host-specific serotype Cholerasuis to survive in swine. A pool of 45 Cholerasuis mutants was used to infect pigs orally and intraperitoneally, in order to identify genes necessary for both routes of infection by this host specific serotype [152]. Mutants in the regulatory gene *hilA* (hyper-invasive locus A)were attenuated in oral, but not inteperitoneal infections in

swine [152], as observed for Typhimurium in cattle and mice [36,139,153]. STM mutants of serotype Cholerasuis have also been delivered to swine using an intranasal route of infection. Two genes required for virulence, *STM1459* (putative oxidoreductase protein) and *STM2626* (Gifsy-1 prophage replication protein) were identified in this study [154].

Gene requirements for survival of the host specific *Salmonella* serotype Dublin in calves have also been studied using STM methodology. 5000 *Salmonella* serotype Dublin STM mutants were studied during infection of calves and BALB/c mice to determine genes necessary for virulence in each host [155]. Mutants defective for virulence in one host, but not the other were rarely observed compared to mutants defective in both hosts, similar to results seen in previous studies of Typhimurium in two hosts [139,155]. In serot. Dublin, mutants in *ssaT*, encoding a component of the SPI-2 TTSS apparatus, were defective in both host models [155]. A second TTSS-2 mutant in *sseD*, encoding a SPI-2 secreted effector, was initially identified in this STM screen as specifically defective for virulence in mice. However, when examined in a *single* systemic infections, the *sseD* mutant was defective in both mice and calves [155]. These data indicate that *sseD* mutants may be transcomplemented by other Salmonella mutants during the calf infection, but not during the murine infection. The *sseD* mutant was further characterized in oral and ileal infections in the calf that are used to study enteric, rather than systemic disease. Consistent with previous conclusions that SPI-2 is not required for invasion of the intestinal epithelium, the *sseD* mutant and the wild type were recovered equally from ileal loop infection, while SPI-1 mutants was recovered at lower

levels [155]. During oral infection of calves however, *sseD* mutants were decreased significantly 2 days post-infection in feces compared to the wild type [155]. While the oral and ileal data initially seem disconnected, they are consistent with the hypothesis that *Salmonella* reseed the intestinal lumen from the mucosa, where SPI-2 is required for proliferation.

A defined, small-scale method was used by Pullinger et al. in 2007 [156]. Thirty-six mutations previously generated and characterized in Typhimurium were generated in serotype Dublin using the lambda red recombinase methods [140] [156,157]. These defined mutants were used to infect cattle orally and identify genes necessary for translocation from the gut to the mesenteric lymph node. This study showed that the SPI-4 T1SS is necessary for persistence and translocation from enteric to systemic sites while the TTSS-2 is not required for this translocation.

Improvements to Signature Tagged Mutagenesis: TraSH

As investigators have continued to use STM heavily, improvements have been made to the original technology. Alternate methods have been developed to use PCR to assay changes in representation of members of the pool [158], in studies using mutant pools with reduced complexity. Using this strategy, the requirement for serotype Gallinarum genes for growth and survival of this host specific serotype in chicks was determined after oral infection [159]. Mutants in genes encoded in SPI-10 (*sefD*) and SPI-13 (*STM3118-3120*) were defective during this host specific infection [159].

The use of unique sequence tags embedded within the transposon was revolutionary, but certain drawbacks limited the further applications of STM technology. Transposon-based mutagenesis and pool sizes limited to the number of available sequence tags, 96 or fewer, constrained the use of STM for comprehensive functional genomic studies. For large-scale genome-wide screening, two groups simultaneously developed methods to use microarray technology and the idea of signature tagging without exogenously added tags to study bacterial pathogens [160,161]. Each group designed transposons bearing outwardly facing T7 promoters that could promote *in vitro* transcription from genomic DNA flanking each insertion site, generating a unique transcript for each mutant. The representation of these transcripts in a pool was evaluated by using ORF microarrays [160,161]. This strategy was named Transposon Site Hybridization or TraSH [161], and made the use of additional exogenous sequence tags unnecessary. Although this method was developed for use in *Mycobacteria*, a similar approach was also used in *E. coli* [160] and was subsequently adapted for use in *Salmonella*.

TRASH screening was used to follow 50,000 pooled transposon mutants in serotype Typhimurium during intraperitoneal infection of BALB/c mice of short duration, as well as infection of cultured RAW 264.7 macrophages [162]. Many of the genes identified in this study, including those in genes of SPI-2, were previously identified as important in this murine model by Hensel et al. using STM. These data verified that this T7 based method worked in *Salmonella*. However surprisingly, in these studies genes encoded on SPI-1 were shown to play a role in replication and survival in

macrophages, and *sirA* was identified as a potential regulator of SPI-1 function within macrophages [162]. Finally, the vast majority of genes required for virulence in these experiments exist only in *Salmonellae* [162], suggesting that Salmonella-specific genes are an important category of genes for further study [94].

The genetic requirements for Typhimurium persistence in systemic sites has also been studied using this T7-microarray based method in Salmonella- resistant mouse models [163]. *Salmonella* resistant mice were infected for up to 28 days, in order to investigate genetic requirements systemic persistence [163]. Infected animals were sacrificed and the mutant pools were analyzed at 7, 14, 21 and 28 days post-infection, allowing specific identification of genes necessary for long-term intestinal persistence. Similar to short term systemic colonization, studies of long-term colonization of systemic sites also identified a large complement of *Salmonella* specific genes as necessary including genes of SPI-2. Genes required for restructuring the bacterial outer membrane, including the *rfa* and *rfb* clusters, also appear to be necessary for long term colonization of systemic sites by serotype Typhimurium [163]. Finally, mutants in SPI-1 effectors were underrepresented within 7 days post infection. The effectors SipCBD appear to be necessary not only for establishment of infection, but also for more long term role that is poorly understood [163].

Improvements to Signature Tagged Mutagenesis: TMDH

Until recently, pools of transposon mutants have been evaluated using primarily ORF microarrays. Because this type of microarray contains open reading frames,

transposon insertions in non-coding regions, or very small coding regions that can't be amplified by PCR, are not assayed. A new quantitative method termed Transposon Mediated Differential Hybridization (TMDH), that uses a novel high-density tiling array containing 60-mer oligonucleotides spaced every 100 base pairs along both strands of the genome alleviates these issues [164]. The transposons developed for generating mutants in this method, Tn5 and Mu, have an outward facing T7 promoter on one end and an outward facing SP6 promoter on the opposite end. Representation of transposon mutants in the pool was evaluated by hybridizing labeled transcripts to the tiling array. Use of this array allows determination of the location of each transposon insertion with much higher resolution than is possible with other types of arrays.

Approximately 10,000 serotype Typhimurium Tn mutants were studied, pooled in groups of 480 or 384 and used to infect mice intravenously. Comparison of the representation of individual mutants in pools used to infect mice (input pools) to the pools recovered from mice (output pools) were evaluated, however, the input and output are not directly compared to each other on the same slide. A second minor limitation is that multiple transposons located very near each other in the same region cannot be distinguished from each other, a factor that limits the pool size that can be tested. Using the TDMH method Chauduri et al. identified the position of 8,533 transposon mutations, with 6,108 transposons unambiguously mapped to 2,824 genes, roughly half of the *Salmonella* genome. A large data set of "unbiased relative fitness" was generated from this study, and 47 genes were targeted for deletion and studied further using single infections in BALB/c mice. Mutants in *trxA*, *atpA*, and *tolA* were further tested in

immunization and protection studies, and found to provide protection against i.v. challenge (*trxA* and *atpA*) or against i.v. and oral challenge (*tolA*) .

Problems with Transposon Mutagenesis

Transposon mutagenesis is a valuable tool for genetic studies, but its use is limited in particular environments where members are lost at random from pools of mutants, as sometimes occurs during infection. In order to generate a mutation in each gene in the *Salmonella* genome using random mutagenesis approaches, transposon libraries must be highly complex. Generally 5-10 times more transposon mutants are generated than there are genes in the genome of interest in order to have a high probability of generating a mutation in each gene in the genome. During genetic selections where many mutants are lost at random (also termed a "bottleneck"), it is difficult to accurately evaluate representation of individual mutants in such complex pools. This is one factor that necessitates the use of smaller pools of Tn mutants, such as in STM and in TMDH approaches used for *Salmonella*, during infection. Pool sizes that do not fluctuate at random must be determined empirically for each route of infection and niche to be studied. Use of small pool sizes also does not allow comprehensive screening of the genome in non-traditional animal models, such as livestock, where it is prohibitively expensive to use many animals for screening. Additional drawbacks of transposon-based mutagenesis include random location of the insertions themselves, and potential polar effects on downstream genetic elements.

Targeted Deletion Libraries

The rapid availability of complete genome sequence information, combined with straightforward techniques for generating targeted mutants in enterobacteriacae, allow the development libraries of targeted deletion mutants, thus generating one mutant in each gene in the genome. Reduction in the number of mutants, thus reducing the complexity when mutants are screened as a pool, is a major advantage of the generation of targeted mutants. Targeted deletion libraries that contain mutants in each non-essential gene have been developed in *S. cerevisae, Cryptococcus neoformans* [165] and *E. coli* (the Keio collection [166]). The following chapters detail work on the development of a similar library in *Salmonella enterica* serotype Typhimurium and a novel array based screen used to identify mutants defective during systemic infection.

CHAPTER II

ARRAY BASED ANALYSIS OF CISTRONS UNDER SELECTION

Introduction

Genetic screening remains one of the most efficient methods to identify genes associated with a phenotype of interest in bacteria. Array-based methods for these screens originated with the transposon-based "signature tagged mutagenesis" (STM) strategy that used unique signature sequences in each transposon to evaluate the relative abundance of individual mutants in pools after selection [167]. STM was later improved by modifying the mutagenizing transposon to include a T7 RNA polymerase promoter (P_{T7}) that is used to generate a unique transcript for each mutant from the genomic sequence adjacent the mutation. This modification makes exogenous unique sequence tags unnecessary. Relative abundance of the input and output P_{T7} transcripts is monitored using an ORF microarray [160,161,163,168].

Transposon mutagenesis suffers from several drawbacks. First, tens of thousands of random transposon insertion mutants are necessary to ensure that mutations occur in most small genes; mathematical simulations indicate that 40,000 random mutants would still fail to disrupt over 200 of the 1,100 annotated open reading frames in *Salmonella* that are less than 500 bases in length. Second, this need for a high complexity is a critical limitation for genetic screens in environments, including live animals, where the bacterial population may fall to low levels during infection. These 'bottlenecks' may

occur at various points during infection – for example, survival of the acidic environment in the stomach, invasion of Peyer's patches and survival in the bloodstream represent some of the processes where the founder population may be very small. Such 'bottlenecks' cause undesirable random loss of mutants and complicate forward genetic screening in such environments. In addition, the polar nature of transposon insertions makes mapping of a phenotype more difficult.

We used the lambda-red recombination method that includes features to minimize polarity [157] to construct targeted deletion mutants in *Salmonella*. We added a P_{T7} to the cassette inserted during mutagenesis, positioned to produce a gene-specific transcript from the genomic sequence adjacent to the insertion. Thus, our targeted deletion mutants can be pooled for genetic screens. Far fewer specific mutants are needed to ensure representation of every gene of interest than is needed when using random transposon mutagenesis. We introduce a novel and inexpensive array designed for monitoring changes in specific deletion mutant representation in the pool after selection. Finally, we demonstrate the utility of these techniques by identifying novel candidate mutations in genes encoding proteins and sRNAs that appear to affect the fitness of mutants in competitive infection with wild-type *Salmonella*, and we validate eleven of these fitness phenotypes.

Results

Genome Sequencing

The virulent *Salmonella enterica* serovar Typhimurium ATCC14028 is extensively studied both *in vitro* and *in vivo*. To design primers for generation of our deletion collection, we produced a near complete draft sequence of this genome using the 454 shotgun approach (GenBank accession in process). The ATCC14028 sequence was compared to the completed genome of the 1,000-fold less pathogenic laboratory strain Typhimurium LT2 [169]. As expected, over 95% of the two genomes were orthologous, and the orthologous regions had less than 1% divergence. The ATCC14028 and LT2 genomes differ only by a few hundred single base mutations (including a mutation in *rpoS* that is partly responsible for the attenuation of LT2 [170]), the absence of the two Fels phage in ATCC14028, and other insertions and deletions encompassing less than 40 kb (McClelland et al., unpublished data).

Generation of Specific Gene Deletions in ATCC14028

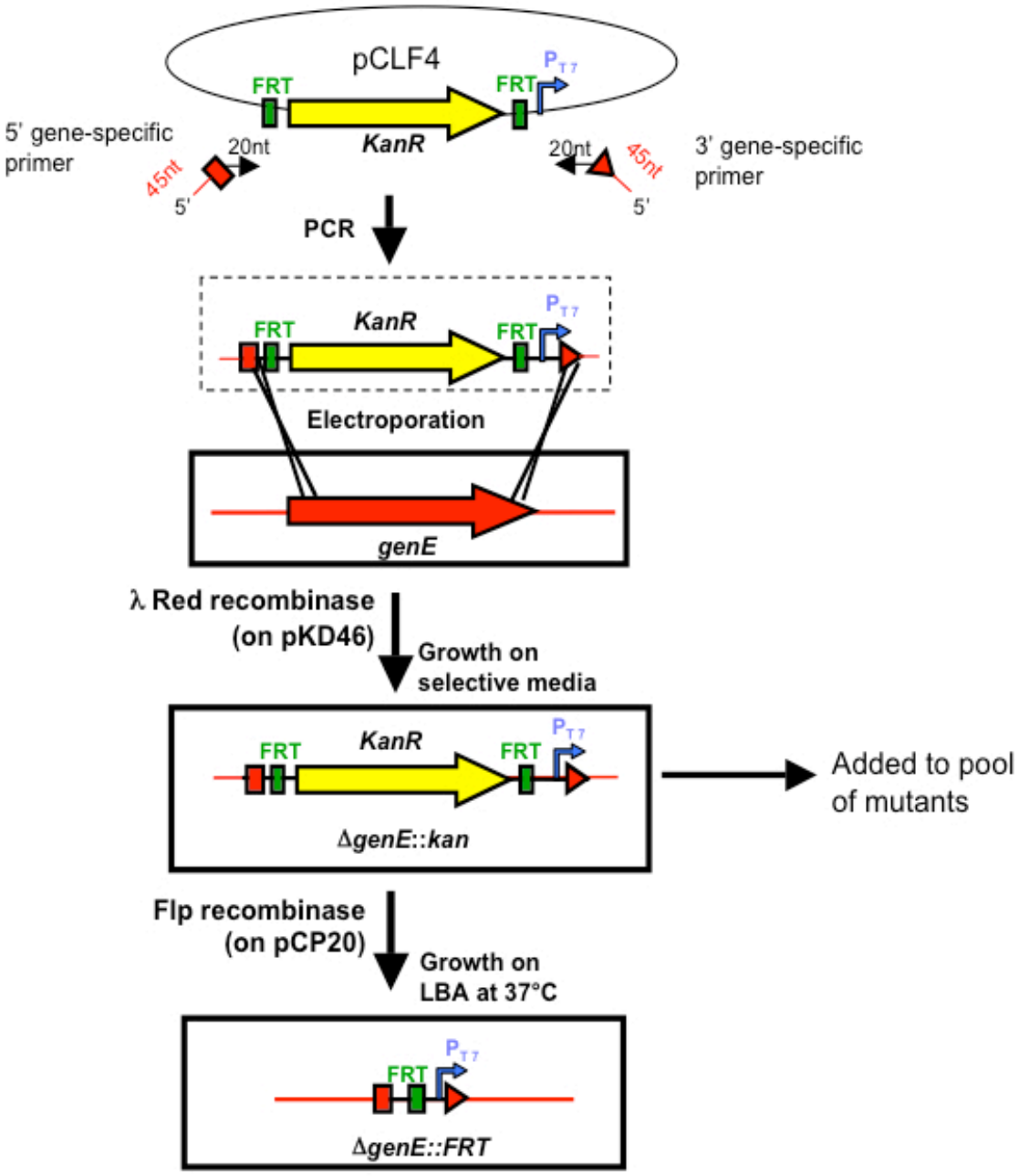
We targeted 1,052 genes for deletion [171], primarily genes in *Salmonella* that are not found in *E. coli* [169]. Such genes are usually in very A+T rich regions [172], and include nearly all of the ~200 genes previously associated with *Salmonella* virulence, including the Type III secretion systems (TTSS) and their known effectors. Targeted deletions were also generated in nearly all of the 100 genes in fimbrial and surface antigen regulons. Finally, we deleted a subset of genes shared by *Salmonella* and

E. coli, including 44 known and candidate sRNAs, and genes that have known motility, regulatory and pathogenesis functions.

The original vectors for the lambda-red swap strategy, pKD3 (Cm^R) and pKD4 (Kan^R) [157], were redesigned to include a T7 RNA polymerase promoter positioned to generate a unique transcript from the *Salmonella* genome directly downstream of each mutant. The construct includes an ATG and ribosome binding site (RBS) to preserve any translation coupling. An outline of our variation on the red-swap strategy is in **Figure 4**. The sequences of the redesigned vectors, pCLF3 and pCLF4, are available in GenBank (Accession numbers EU629213 and EU629214, respectively).

We designed 65mers that contain the same 3' sequence of 20 bases for vector PCR that were used previously [157], but with a 45-base rather than the conventional 35-base gene-specific portion, to allow a secondary function for the oligonucleotides as probes in a microarray (described later). Homology of the oligonucleotides with the genome was positioned so that each swap eliminated the entire gene except for the coding regions of 30 base pairs at the 5' and 3' ends of each gene. These two ends of each gene were preserved to minimize unintended effects on adjacent genes caused by removal of the gene sequence and insertion of the mutagenic cassette.

Figure 4:Generation of Specific Deletions in *S. enterica* serovar Typhimurium ATCC14028. Our procedure is identical to the Red-swap described in Datsenko and Wanner [157] with the exception that we re-engineered the original insert to include an in-frame T7 RNA polymerase promoter (P_{T7}), and the sequences used for recombination are longer. A gene, identified in the schematic as *genE*, is targeted for deletion. Two 65mer primers (red) are used to amplify the region containing the antibiotic resistance cassette, the P_{T7}, and the FRT (Flippase recognition target) sites (among other elements) from the plasmid pCLF4. The resulting PCR product has 45 base sequences at each end that are homologous to sequences near the 5' and 3' ends of the targeted ORF. Transformation of these PCR products into ATCC14028 expressing lambda Red recombinase *in trans* leads to a recombination event, resulting in the swap-in of the PCR product, and the swap-out of the targeted gene. A ribosomal binding site (RBS) and a downstream ATG start codon near the 3' end of the inserted sequence ensures that a 12 amino acid peptide is made from any RNA that is transcribed in this strand to reduce polar effects. Targeted mutants in many genes were pooled and used for Array-based analysis of cistrons under selection (ABACUS).


pCLF4
FRT
FRT
P_{T7}
KanR
5' gene-specific primer
20nt
45nt
5'
20nt
45nt
5'
3' gene-specific primer
PCR
FRT
KanR
FRT
P_{T7}
Electroporation
genE
λ Red recombinase (on pKD46)
Growth on selective media
FRT
KanR
FRT
P_{T7}
ΔgenE::kan
Added to pool of mutants
Flp recombinase (on pCP20)
Growth on LBA at 37°C
FRT
P_{T7}
ΔgenE::FRT

If FLP recombination is used to remove the antibiotic cassette after mutant construction then an open reading frame is generated including ten codons from the 5' end of the original gene, 39 amino acids from the lambda-red mutagenesis referred to as a "scar" **Figure 5** , and nine amino acids and the stop codon from the original gene. In our constructs, the T7 promoter remains in place in the scar after FLP recombination.

We also increased the level of throughput in the generation of our mutant collections. Lambda-red swap recombination in ATCC14028 was performed with a mixture of two PCR products, one originating from pCLF4 containing a kanamycin resistance cassette (Kan^R, sense orientation) and one originating from pCLF3 containing a chloramphenicol resistance cassette (Cm^R, antisense orientation). Each transformation was plated on LB-Kan and LB-Cm, and two transformants from each plate were colony purified and stored (a total of four transformants). These two collections marked with different antibiotic resistance cassettes facilitate the construction of double mutants by transduction.

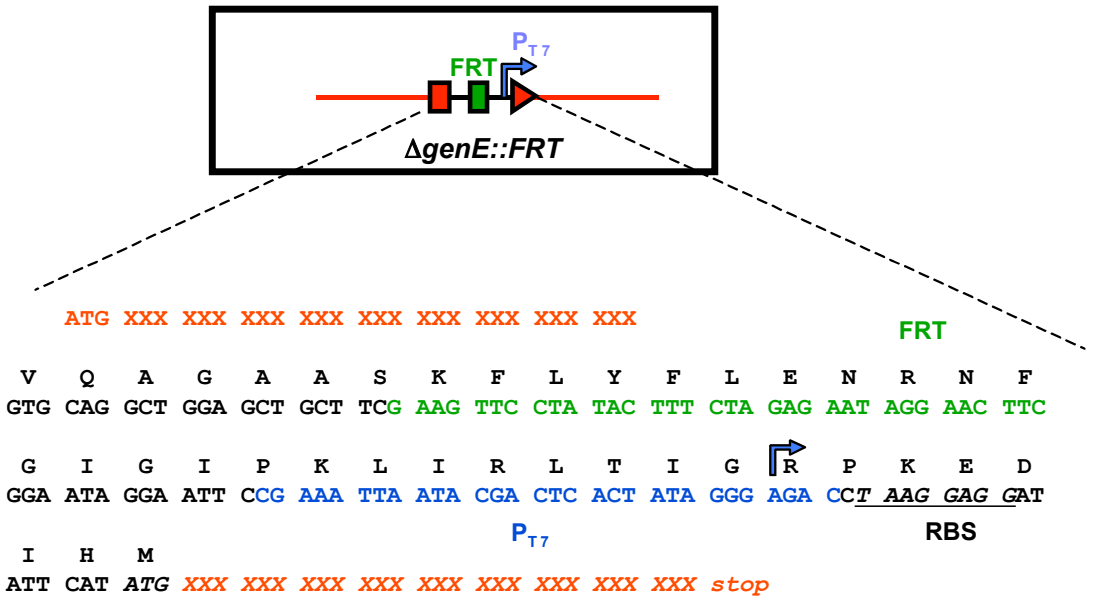


Figure 5: Anatomy of the "Scar" Left by the Antibiotic Resistance Cassette. Antibiotic resistance markers in targeted genes can be removed using FLP recombinase, resulting in a gene encoding a mini-protein of the first ten amino acids of GenE, 39 amino acids from the inserted DNA (called a "scar"), and the last nine amino acids of GenE, and retaining the P_{T7}.

The *Salmonella* genes targeted for deletion were spot-checked by PCR. Of 1,052 mutants attempted, 1,040 produced Kan^R or Cm^R clones, or both [171]. To confirm that mutations occurred at the targeted location, two clones obtained for the first 304 Kan^R mutants and for 231 Cm^R mutants were checked by PCR using primers to the flanking genomic regions. Only one mutation was incorrect.

The accuracy of FLP recombination to remove the antibiotic resistance marker was verified for twelve transformants (as detailed in the Methods and Materials section).

The mutant including flanking regions was amplified by PCR, and both strands of the amplification product were sequenced. All twelve swaps examined were precise, and each recombination event resulted in the intended truncated open reading frame.

Array-based Verification of P_{T7} *Location and Activity from a Pool of Mutants*

We used a NimbleGen tiling array to verify the correct insertion and activity of the P_{T7} in all of our mutants simultaneously. 1,031 Kan^R mutants were pooled by growing each mutant separately to stationary phase in LB, mixing in equal volume, and storing as glycerol stocks at -80ºC. The region 3' to each mutation in the pool of 1,031 Kan^R mutants was labeled by a novel method designed to generate uniform signals from every P_{T7} (Materials & Methods and **Figure 6**). For the necessary DNA fragmentation prior to amplification and labeling, we replaced the restriction digestion step used in previous protocols with genome shearing and polyA tailing. To identify active P_{T7} in the mutants, labeled RNA obtained by T7 *in vitro* transcription from the pool was hybridized to a custom NimbleGen tiling array of 50mer oligonucleotides covering the ATCC14028 genome in overlapping 24 base steps on both strands. Oligos directly adjacent to each functional insert and in the opposite strand from the transcript hybridized intensely, with a rapid decrease in signal over a 200 base region [171]Using this method we simultaneously identified 933 inserts from Kan^R mutants with functional P_{T7} sites in the correct location. 48 additional Kan^R mutants are likely correct but could not be formally confirmed, due to an overlapping transcript from a nearby mutation in another mutant in the pool. 50 Kan^R mutants (4.8% of all pooled mutants) did not

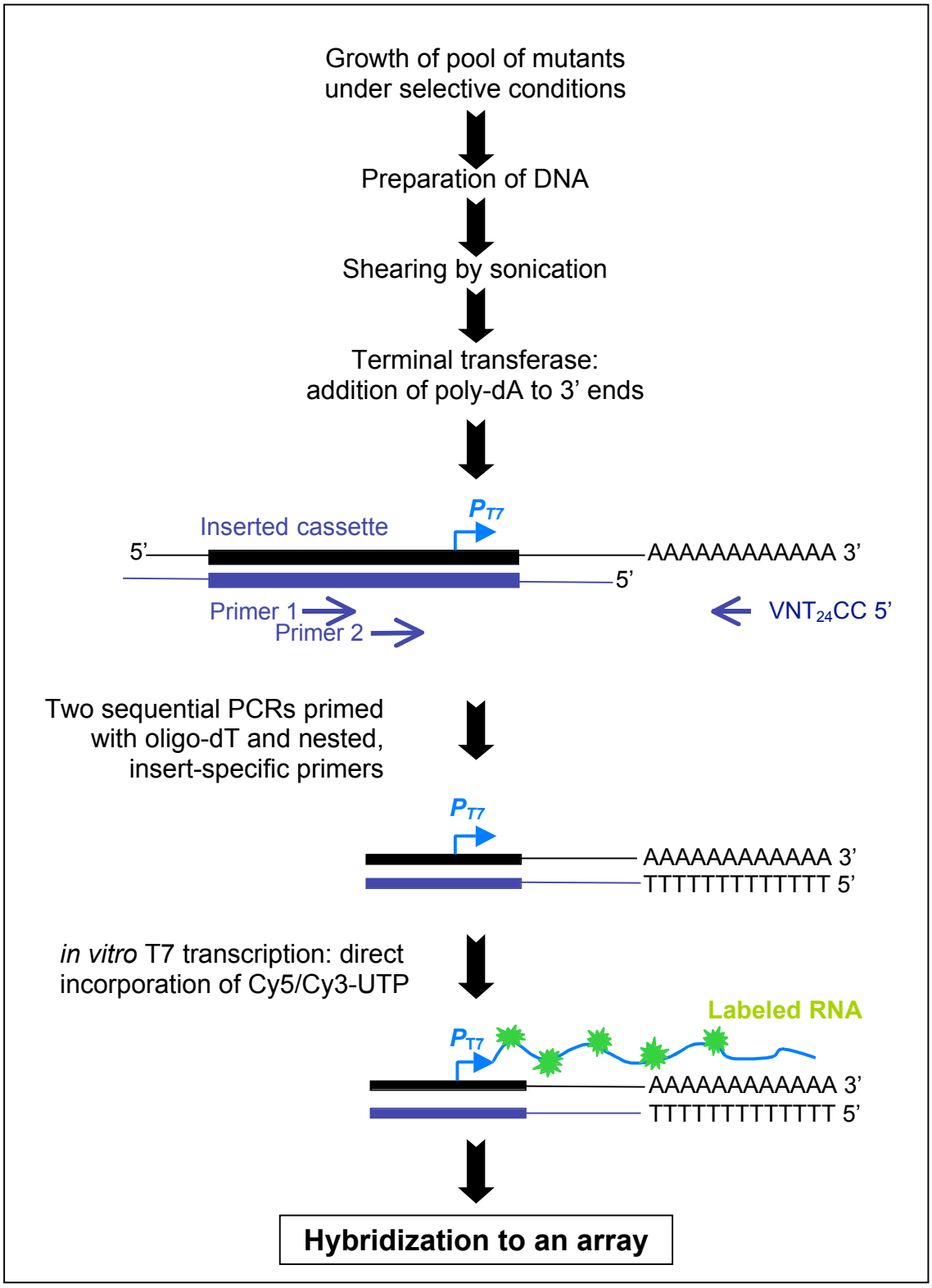


Figure 6: Labeling Technique for Detection of Mutants in a Pool by Array Hybridization.

display active transcription at the correct location perhaps due to a mutation in the P_{T7}. Four inserts were at an incorrect location.

We repeated these experiments with a pool of 972 Cm^R mutants and at least 892 of these have a functional P_{T7}. 78 Cm^R mutants were in genes where a Kan^R clone was not confirmed. Overall, Kan^R or Cm^R mutants, or both, were confirmed in 1,023 genes out of 1052 targeted for deletion. We have not studied the Cm^R clones further, but these mutants should be useful as a pool and also for the construction of double mutants in combination with Kan^R clones.

Failure to generate the correct targeted mutation could occur if the mutation was lethal. We did not attempt to delete any genes that are orthologous to genes previously reported to be essential in *E. coli* [166]. However, we did target 38 genes that were previously reported as essential in *Salmonella* [173] and succeeded in obtaining mutants in all but two; *STM1008*, a phage gene that has a close paralogue in the genome, and seems unlikely to be essential in LB, and *STM2087*, encoding an enzyme required for the synthesis of LPS side chains. It is likely that very few of the 29 failures to construct a mutant were due to lethality of the mutant.

Generation and Testing of an 'In house' Microarray for Screening

Pools of our mutants can be studied using competitive assays and the representation of each mutant can be determined using microarrays. For such detection, we manufactured an economical "in house" array based on 1,241 65mers derived from the 3' ends of genes, including all those previously utilized for mutant construction.

Oligonucleotides of 45 bases in length produce a more robust RNA hybridization signal [174] than the 35mers that are normally used for lambda-red recombination [157]. As negative controls we included in the array 308 65mer oligos with homology to the 3' end of mutants that had not yet been generated and were therefore absent from the pool, and 955 65mers designed on the 5' end of genes (previously used for mutant construction).

One aliquot of the pool of 1,031 Kan^R mutants was used to test the "in house" array. Labeled RNA probe was prepared from the pool, and hybridized to the array as described (Materials and Methods). A specific 27 base oligonucleotide, complementary to the conserved 27 bases of RNA produced from the inserted region common to every mutant, was included in the hybridization. This 27mer blocks cross-hybridization of this portion of the T7 transcripts to a complementary 20 base sequence present in each of the 3' gene probes (**Figure 7**). Specific signal at least three-fold over the mean signal of the negative controls was detected for 97% (905) of the 933 mutants known to be in the pool. Only seven (0.7%) of the 5' oligos included as negative controls in the array hybridized, possibly due to a transcript from a nearby mutant in the complementary strand.

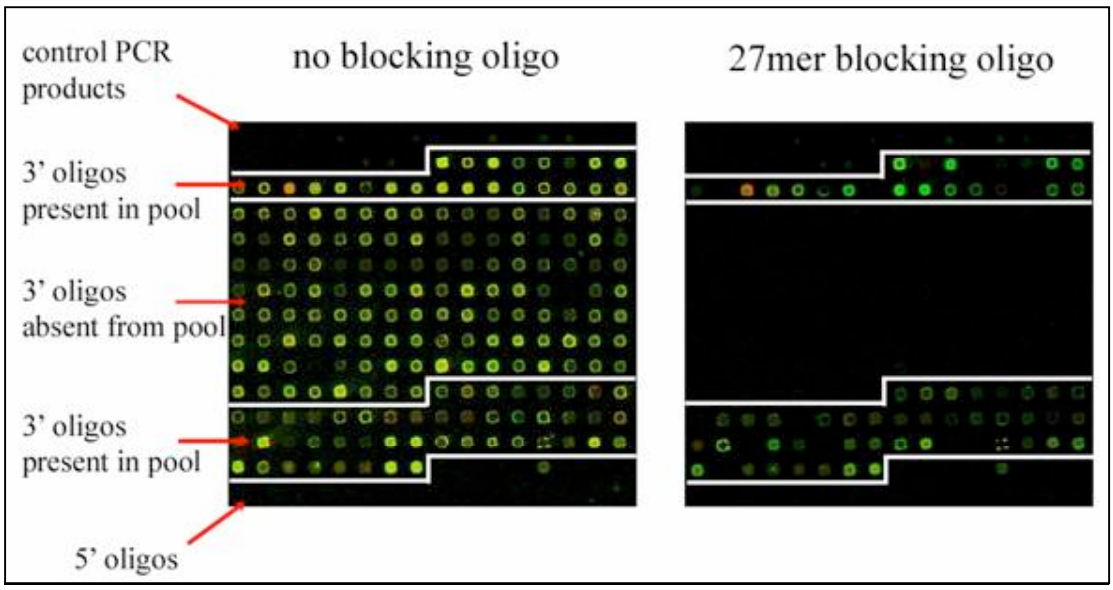


Figure 7: Addition of the 27mer Competitor Oligo Results in High Specificity of Array Detection.

Genes That Affect Fitness of Salmonella During Passage Through BALB/c Mice

BALB/c mice develop fatal systemic infection from *S. enterica* serotype Typhimurium, as can also occur in humans infected with *S. enterica* serotype Typhi. We used intraperitoneal infection in BALB/c mice, a well-studied model [126,129,136,137,162], to demonstrate that our mutant screening method could correctly identify both previously observed and novel gene requirements during infection. A group of six BALB/c mice were infected intraperitoneally (IP) and bacteria were recovered from the spleen after euthanasia at 48 hours post-infection. Labeled RNA

probe was prepared using aliquots of DNA from the input pool and output pools from each animal as outlined in **Figure 6**, and the labeling protocol was repeated with dyes reversed. Hybridizations were performed using twelve slides, each slide containing the array printed in triplicate. The data were processed using WebArray [175], as described in Materials & Methods, and are summarized in **Figure 8**.

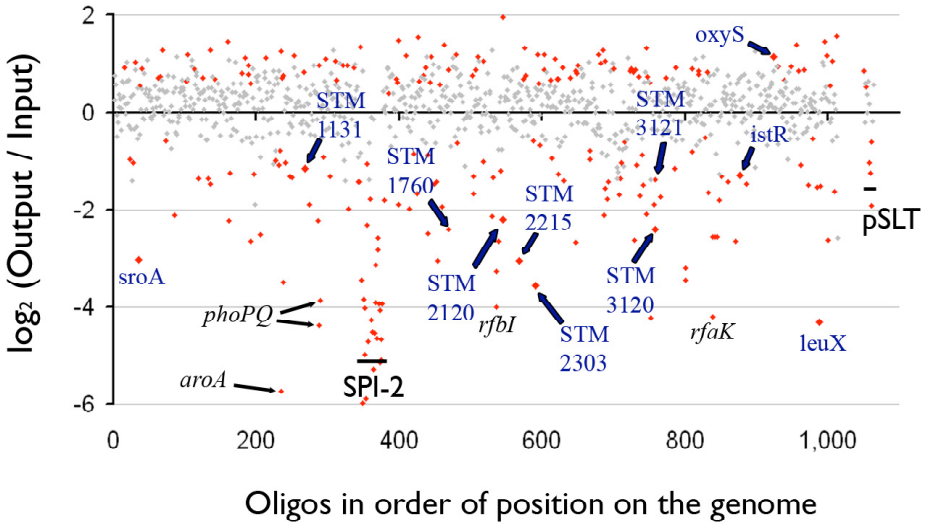


Figure 8: Loss of Defined Mutants From the Input Pool After Intraperitoneal Delivery to Six BALB/c Mice and Recovery From Spleen at 48h Post-Infection. Twelve array hybridizations were performed using two each for six mice. WebArray analysis results using quantile normalization for all oligos representing candidate deletions in the pool are shown. Data with P<0.0005 are depicted in red, those with P>0.0005 in grey. The X-axis plots the data for each of the mutants in the order in which those mutants occur in the *Salmonella* genome. Mutants confirmed individually in a competitive assay with wild-type are labeled in blue. Examples of important genetic elements are marked in black.

At a threshold of a two-fold change and P<0.0005, 120 mutants showed a change in fitness in IP infection. Among the most unfit were mutants in the TTSS encoded by SPI-2, and associated effector genes, and genes for cell wall biosynthetic enzymes, all known to be important during systemic infection [137,162]. We identified 51 mutants that have previously been identified during intraperitoneal screens as defective (**Table 2**).

Table 2: Mutants without Previously Known Phenotypes. We identified 51 mutants not previously identified during infection and grouped them into functional categories.

Unknown function	**Regulator**	**Enzymatic function**
STM0081 STM0731 STM1600 STM1760 (TPR repeat) STM1948 STM2303 (pmrM, pbgE3) STM2329 STM3047 (ygfY) STM3089 (yqgD) STM3155 STM3783 STM4504 STM4529 STM4599 (yjjY)	STM1547 (putatitive marR family) STM2639 (rseA, anti sE) STM3121 (stmR) STM3687 (mtlR) STM4417 STM0693 (Fur, transcript repressor)	STM0719 STM0857 STM1151 (mdoH) STM3120 (citE) STM3119 (maoC, ripB homolog) STM3339 (nanA) STM3846 (putative RT) STM4475 (valS, valine tRNA synthase) STM4489 (putative helicase)
	Small RNAs	
	leuX sroA sraA istR rybB oxyS (FOR)	
c-di-GMP	**Porin/transport**	**PhoP/Q related**
STM0551 STM1344 (cdgR/ydiV) STM1697 STM2215 (rtn) STM2672 (yfiN)	STM1131 STM3975 (tatC) STM1258 (ABC trans) STM3763 (mgtB), Mg Transport	STM1544 (pqaA, phoQ reg) STM1601 (ugtL)
		LPS
		STM2120 (asmA)
Flagellar biosynthesis	**Scavenger**	**Kinase**
STM1971 (fliH)	STM2777 (iroN)	STM1670 STM2116 (wzc)

Fifteen mutants without previously known fitness phenotypes during systemic infection in this model were selected from among the 120 statistically top-ranked candidate mutants. These included mutants in all five candidate sRNA, a supernumerary tRNA (LeuX), and nine randomly picked from among the protein coding genes. These 15 mutants were studied one at a time in competitive assays with wild-type ATCC14028, after P22 transduction of each mutation to a clean genetic background. The phenotypes observed in the pool of mutants were confirmed for eleven mutants, as listed in **Figure 9** and **Table 3.** To reduce the chance of polar effects on nearby genes, the Kan^R cassette was removed by FLP recombination from all eleven mutants with confirmed competitive index phenotypes and eight of these (*STM1131*, *STM2303*, *STM3120*, *STM3121*, *sroA*, *istR*, *leuX*, and *oxyS*) were retested in competitive infections in BALB/c mice (**Figure 9**). The phenotype was confirmed in all eight of these unmarked mutants that should have minimal or no polar effects. Four mutants failed to reiterate the phenotype observed in the mutant pool (*sraA*, *rybB*, *STM4529*, and *STM0857*) but this result does not rule out confirmation of a phenotype in a study involving more animals.

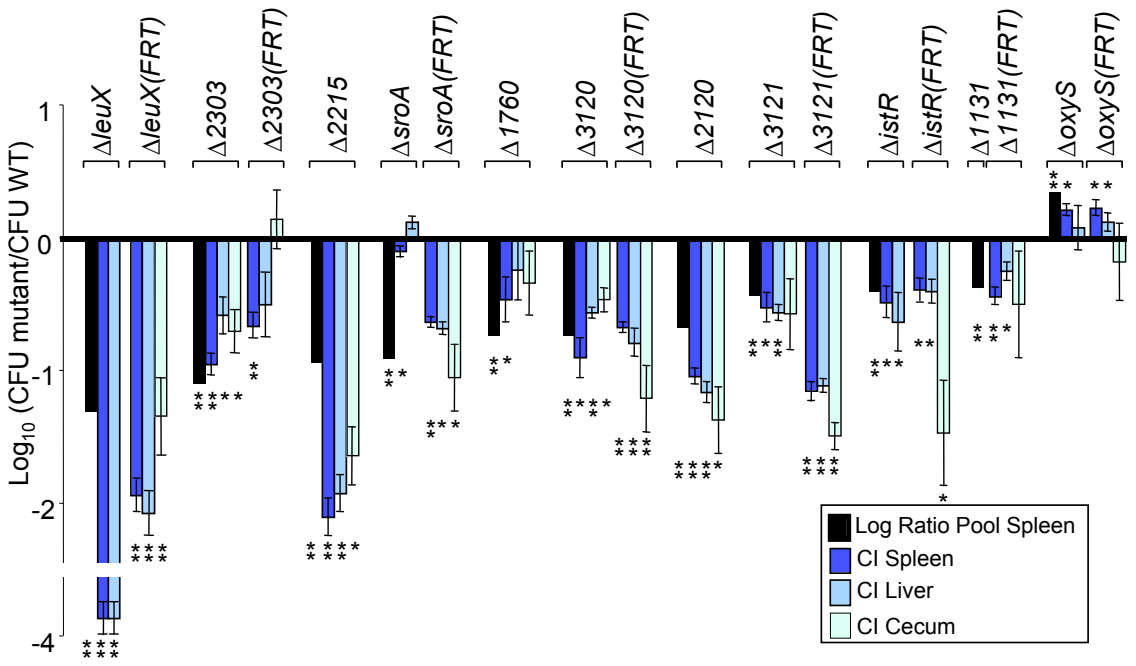


Figure 9: Competitive Index Experiments with Individual Mutants Versus Wild Type After Intraperitoneal Delivery and Recovery from the Spleen, Liver, and Cecum. 106 cfu of 933 mutants (pool) or a 1:1 mixture of individual mutants versus wild type ATCC14028 Δ*phoN*::Kan^R were injected into 5-6 mice and recovered from the spleen one or two days post infection. Statistical significance was determined using a Student's 2-tailed t-test, and asterisks indicate that normalized output ratios were significantly statistically different from the equivalent ratio in the inoculum. **P-value of <0.001, *P-value of <0.05. Strains marked with a (FRT) have undergone FLP-mediated recombination to reduce or eliminate polar effects. None of the mutants have a change in fitness during growth of the pool in LB for four serial passages to stationary phase with 100-fold dilutions (data not presented). Confirmation of mutants, identified by ABACUS, in competitive infections in BALB/c mice. ABACUS prediction for selected mutants (black bars) and confirmation data from competitive infections in BALB/c mice (dark blue bars are from spleen, lighter blue bars are from liver and cecum) of infected animals.

Table 3: Competitive Infection Data. Bold denotes statistical significance, *death after 24 hours, but prior to 48 hours post-infection.

Gene	Mutant	Locus	ABACUS (pool of 933 mutants)		Competition with wt (single mutant)		
			ratio spleen / input	p-value	CI	standard error	p-value
STM0731	Δ*STM0731*::*kan*	Spleen	**0.18**	2.E-04			
STM0732	[1]Δ*STM0732*::*kan*	Spleen*			0.68	0.06	**5.E-04**
		Liver*			0.55	0.07	7.E-02
		Cecum*			0.74	0.1	**7.E-03**
STM0857	Δ*STM0857*::*kan*	Spleen	**0.47**	8.E-03	1.28	0.03	**2.E-03**
		Liver			1.37	0.04	**1.E-02**
		Cecum			1.08	0.02	**7.E-03**
STM1131	[2]Δ*STM1131*::*kan*	Spleen*	**0.44**	1.E-04	0.0008	0.78	**2.E-02**
		Liver*			0.002	0.25	**5.E-04**
		Cecum*			0.007	0.78	6.E-02
	Δ*STM1131*::FRT	Spleen			**0.37**	0.06	**2.E-04**
		LIver			0.58	0.07	**3.E-03**
		Cecum			**0.32**	0.4	2.E-01
STM1760	Δ*STM1760*::*kan*	Spleen*	**0.19**	1.E-06	**0.35**	0.17	**4.E-02**
		Liver*			0.59	0.22	3.E-01
		Cecum*			**0.46**	0.24	2.E-01
STM2120	Δ*STM2120*::*kan*	Spleen	**0.22**	9.E-12	**0.09**	0.06	**1.E-04**
		Liver			**0.07**	0.08	**2.E-04**
		Cecum			**0.04**	0.25	**6.E-03**
STM2215	Δ*STM2215*::*kan*	Spleen*	**0.12**	2.E-08	**0.01**	0.14	**1.E-04**
		Liver*			**0.01**	0.14	**1.E-04**
		Cecum*			**0.02**	0.22	**1.E-03**
STM2303	Δ*STM2303*::*kan*	Spleen	**0.08**	6.E-12	**0.12**	0.08	**3.E-04**
		Liver			**0.27**	0.14	**2.E-02**
		Cecum			**0.2**	0.16	**2.E-02**
	Δ*STM2303*::FRT	Spleen			**0.22**	0.09	**1.E-03**
		Liver			**0.32**	0.24	9.E-02
		Cecum			1.42	0.22	5.E-01
	Δ*STM2303*::FRT pWSK29	Spleen			**0.23**	0.04	**1.E-04**
		Liver			**0.4**	0.09	**1.E-02**
		Cecum			0.52	0.08	**2.E-02**
	Δ*STM2303*::FRT pWSK29::*STM2303*	Spleen			1.51	0.04	**3.E-02**
		Liver			2.18	0.04	**2.E-03**
		Cecum			4.76	0.14	**8.E-03**

Table 3 Continued

Gene	Mutant	Locus	ABACUS (pool of 933 mutants)		Competition with wt (single mutant)		
			ratio spleen / input	p-value	CI	standard error	p-value
STM2639	Δ*STM2639*::*kan*	Spleen*	**0.15**	5.E-08	1.22	0.04	5.E-01
		Liver*			1.31	0.05	3.E-01
		Cecum*			1.2	0.25	9.E-01
STM3120	Δ*STM3120*::*kan*	Spleen*	**0.19**	1.E-06	**0.13**	0.15	**1.E-02**
		Liver*			**0.28**	0.04	**9.E-04**
		Cecum*			**0.35**	0.09	**2.E-02**
	Δ*STM3120*::FRT	Spleen			**0.22**	0.04	**<1.E-7**
		Liver			**0.17**	0.1	**7.E-04**
		Cecum			**0.06**	0.25	**6.E-03**
STM3121	Δ*STM3121*::*kan*	Spleen	**0.38**	1.E-05	**0.31**	0.11	**4.E-03**
		Liver			**0.28**	0.06	**2.E-04**
		Cecum			**0.27**	0.26	9.E-02
	Δ*STM3121*::FRT	Spleen			**0.07**	0.07	**8.E-06**
		Liver			**0.08**	0.05	**2.E-06**
		Cecum			**0.03**	0.1	**2.E-05**
	Δ*STM3121*::FRT pWSK29	Spleen			**0.14**	0.08	**1.E-04**
		Liver			**0.22**	0.19	**1.E-02**
		Cecum			**0.25**	0.32	9.E-02
	Δ*STM3121*::FRT pWSK29::*STM3121*	Spleen			3.75	0.09	**1.E-02**
		Liver			4.83	0.15	**3.E-02**
		Cecum			5.32	0.33	2.E-01
leuX	Δ*leuX*::*kan*	Spleen	**0.05**	6.E-12	**0.0001**	0.14	**<1.E-7**
		Liver			**0.0002**	0.14	**<1.E-7**
		Cecum					
	Δ*leuX*::FRT	Spleen			**0.01**	0.12	**<1.E-7**
		Liver			**0.01**	0.16	**<1.E-7**
		Cecum			**0.05**	0.29	**6.E-03**
istR	Δ*istR*::*kan*	Spleen	**0.41**	5.E-06	**0.34**	0.12	**3.E-02**
		Liver			**0.24**	0.22	7.E-02
		Cecum					
	Δ*istR*::FRT	Spleen			**0.42**	0.09	**1.E-02**
		Liver			**0.41**	0.09	**1.E-02**
		Cecum			**0.03**	0.4	**2.E-02**

Table 3 Continued

Gene	Mutant	Locus	ABACUS (pool of 933 mutants)		Competition with wt (single mutant)		
			ratio spleen / input	p-value	CI	standard error	p-value
sroA	Δ*sroA*::*kan*	Spleen	**0.13**	2.E-10	**0.81**	0.04	**5.E-03**
		Liver			1.34	0.05	6.E-01
		Cecum					
	Δ*sroA*::FRT	Spleen			**0.24**	0.04	**<1.E-7**
		Liver			**0.21**	0.05	**4.E-03**
		Cecum			**0.09**	0.25	**4.E-03**
oxyS	Δ*oxyS*::*kan*	Spleen	**2.3**	6.E-06	1.67	0.04	**3.E-03**
		Liver			1.23	0.17	7.E-01
		Cecum					
	Δ*oxyS*::FRT	Spleen			1.74	0.06	**3.E-03**
		Liver			1.34	0.07	**2.E-02**
		Cecum			0.68	0.29	8.E-01
rybB	Δ*rybB*::*kan*	Spleen	**0.5**	6.E-04	1.79	0.09	2.E-02
		Liver			1.24	0.14	4.E-01
		Cecum					
sraA	Δ*sraA*::*kan*	Spleen	**0.35**	1.E-07	1.03	0.02	**1.E-02**
		Liver			0.63	0.06	**4.E-03**
		Cecum					
tatC	Δ*tatC*::*kan*	Spleen	**0.47**	1.E-02	0.18	0.05	**8.E-05**
		Liver			0.05	0.37	**3.E-02**
		Cecum			0.03	0.14	**1.E-04**

In order to definitively link the deleted genes to the reduced fitness phenotype, two of our unmarked mutants (Δ*STM2303*::FRT and Δ*STM3121*::FRT) were re-tested after complementation *in trans*. Intact copies of these genes were cloned into a stable low copy plasmid vector, pWSK29 [176] , and the complementing plasmids were transformed into the corresponding unmarked deletion strains. The fitness defect of both

mutants was reversed when these strains were complemented with an intact copy of the corresponding gene *in trans* and retested in competitive infections with wild-type ATCC14028 in BALB/c mice. (**Figure 10**). The presence of the vector alone did not improve the fitness of these two mutants during infection.

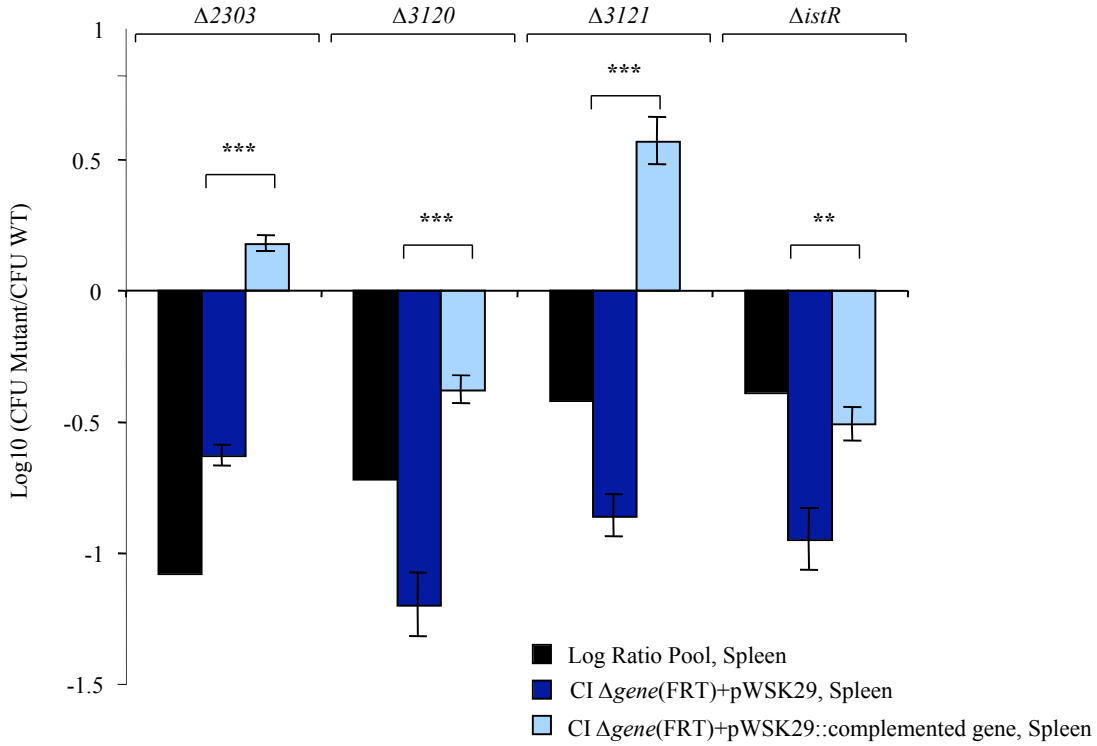


Figure 10: Complementation of Δ*STM2303*::FRT and Δ*STM3121*::FRT Mutants *in trans*. For complementation experiments animals were infected with either a 1:1 mixture of the mutant bearing pWSK29 vs. wild-type ATCC14028 Δ*phoN*::Kan^R (negative control, blue bars) or the mutant bearing the corresponding complementing gene on pWSK29 vs. the wild type ATCC14028 Δ*phoN*::Kan^R (complemented, grey bars). ABACUS prediction for selected mutants (black bars).

Materials and Methods

Strains and Standard Culture Conditions

All strains used in this study are derivatives of *Salmonella enterica* serovar Typhimurium ATCC14028 (Manassas, VA). Strains were routinely cultured in Luria-Bertani (LB) broth and plates, supplemented with 50 mg/l Kanamycin (Kan), 20 mg/l Chloramphenicol (Cm) or 100 mg/l Ampicillin (Amp) where appropriate.

Construction of Plasmids with T7 RNA Polymerase Promoters.

We used a PCR-based strategy to include a T7 promoter in the original pKD3 (Cm^R) and pKD4 (Kan^R) vectors for the lambda-red recombination method. Briefly, pKD3 and pKD4 were used as template for independent PCR reactions using primers PT72_EcoRI (ACTCGAA**TTCCGAAATTAATACGACTCACTATAGGGAGAC**CTAAGGAGGATATTCATATG) and FRT3-EcoRI (CATCGAATTCCTATTCCGAAGTTCCTATTCTCTAGAAAGTATAGGAACTTCGGCGCGCCT) or primers PT72-EcoRI and FRT4-EcoRI (CATCGAATTCCTATTCCGAAGTTCCTATTCTCTAGAAAGTATAGGAACTTCAGAGCGCT), respectively. The *Eco*RI sites are underlined and the P_{T7} is in bold. Each PCR product was digested with *Eco*RI, gel purified and self ligated. Electrocompetent *E. coli* EC100D *pir*-116 (Epicentre), was transformed with aliquots of the ligation, and transformants were selected at 37ºC on LB Cm or LB Kan plates. The sequence (both strands) of the resulting plasmids pCLF3 and pCLF4 was determined by the

conventional dye-terminating Sanger method, and deposited under GenBank accession numbers EU629213 and EU629214, respectively.

Generation of Specific Deletion Mutants

Deletion strains were generated using the lambda-red recombinase method [157], with the following modifications, as illustrated in **Figure 4**. Plasmids pCLF3 (Cm^R) and pCLF4 (Kan^R) were used as templates to generate unique PCR products for deletion of each gene of interest. PCR amplifications were carried out in 96 well plates using ExTaq polymerase (Takara) in a total reaction volume of 60 μl, and a PCR reaction of 30 cycles at an annealing temperature of 55°C. For each gene to be deleted, 30 μl of both PCR products bearing different antibiotic markers were combined and purified using the Qiaquick PCR purification kit in 96 well format (Qiagen). Mixed, purified PCR products (10 μl) were dialyzed against sterile water for 10 minutes using filters with 0.025 μM pore size (Millipore). 2-5 μl of mixed, purified PCR products were used for transformation by electroporation (Bio-Rad Gene Pulser) of electrocompetent ATCC14028 expressing lambda-red recombinase, prepared as previously described [157]. Transformations were allowed to recover for 3 hours in LB at 37°C and 150 μl of each transformation was plated on LB Kan and LB Cm plates. The remaining transformation was saved and replated when very few colonies were obtained using the shorter grow out period. Two transformants for each gene and each antibiotic resistance marker were twice colony purified, and stored in 30% glycerol at -80°C. The Kan^R mutants characterized here are available to the community.

Labeling Technique, Part I: DNA Isolation, Sonication and Polyadenylation

Our labeling protocol is outlined in **Figure 6**. Genomic DNA was prepared for the input and output pools of ~1000 mutants using the GenElute Bacterial Genomic DNA kit (Sigma). 4 μg of genomic DNA was fragmented by sonication using twenty pulses of two seconds in a Branson Sonifier 150 (Branson Ultrasonics Corp., Danbury, CT). PolyA tails were added to fragmented genomic DNA using terminal transferase (TdT) as follows: 1.5 μg of DNA fragments were incubated for 30 min at 37°C in a total reaction volume of 50 μl containing 40 U TdT (New England BioLabs.), $CoCl_2$ 0.25 mM, and dATP 0.4 mM. Terminal transferase was subsequently inactivated at 70°C for 10 min and the tailed product was purified using the QIAquick PCR purification kit (Qiagen).

Labeling Technique, Part II: PCR Amplification and Labeling of Fragments Adjacent the Deletion Location

Nested PCR was used to amplify the polyA-tailed DNA fragments containing the insert P_{T7} and the flanking inserted region. In the first PCR reaction, 50 ng of purified polyA-tailed DNA was used as template for a PCR reaction using primer FRT Out 3-1 (TTCCTATACTTTCTAGAGAA), and a primer designed to anneal to the polyA-tail ($CCT_{24}VN$). The reaction mixture consisted of 1X PCR buffer, 0.2 mM of dNTP, 1.5 mM $MgCl_2$, 0.05 U Taq polymerase (Promega, WI), and 0.2 μM of each forward and reverse primer in a total reaction volume of 25 μl. The PCR reaction was performed under the following conditions: initial denaturation at 94°C for 1 min followed by 30

cycles with denaturation at 94°C for 10 s, annealing at 50°C for 10 s, and extension at 72°C for 5 s. The last cycle was followed by a final extension for 3 min at 72°C. In the second amplification step, a nested PCR was performed using 1 μl amplified product from the initial PCR in a total volume of 50 μl. Internal primer FRT Out 3-2 (TAGGAACTTCGGAATAGGAA) and primer $CCT_{24}VN$ were used under identical cycling conditions as during the initial PCR reaction. PCR products were analyzed on 1% agarose gels.

An aliquot of 8 μl of the nested PCR reaction was used directly as template for a 20 μl *in vitro* transcription reaction using the AmpliScribe T7 transcription kit (Epicentre), following the manufacturer's protocol with some modifications. The RNA was labeled during the synthesis by including 2 μl of 5 mM Cy5- or Cy3-UTP (GE Healthcare) in the *in vitro* transcription reaction. Labeled RNA was treated with RNase-free DNase (Epicentre) and purified with the RNeasy Mini Kit (Qiagen).

NimbleGen Microarray Hybridization

Hybridizations were performed according to the manufacturer's protocols (NimbleGen Systems, Madison, WI, http://www.nimblegen.com/products/lit/lit.html, cgh_userguide_2008_05_27[1].pdf) with some modifications. For each hybridization, 4 μg of labeled RNA was mixed with alignment oligo, NimbleGen hybridization components and hybridization buffer. The arrays were hybridized at 42°C for 16 hours. Arrays were washed according to the manufacturer's protocol, and scanned using a GenePix 4000B laser scanner (Molecular Devices, Sunnyvale, California) at 5 μm

resolution. The signal intensities were quantified using NimbleScan software v2.4 (NimbleGen Systems). The data was normalized and analyzed using Webarray and WebarrayDB with quantile normalization [175]. The moving median intensity of five adjacent probes on the same strand in the genome was calculated and plotted against the genome annotation. Peaks were identified visually [171].

In-house Array Hybridization

The 933 65mer 3' oligos and over 1000 control oligos were printed at 10 μg/ml in 50% DMSO on Corning UltraGap II slides in triplicate arrays. Oligo sequences can be found in supplementary information [171] and the arrays deposited in GEO as platform GPL5687 and GPL5688. Array hybridization was performed in hybridization chambers (Corning Inc., Corning, NY), following protocols suggested by the manufacturer for hybridizations in formamide buffer for pre-hybridization, hybridization, and post-hybridization washes (http://www.corning.com/Lifesciences/technical_information/techDocs/gaps_ii_manual_protocol_5_02_cls_gaps_ 005.pdf). Immediately before hybridization, 2 μg of the labeled probes for input and 2 μg of each experimental sample were unified in 36.5 μl, mixed with 3.5 μl of 100 μM 27mer competitor oligo (CATATGAATATCCTCCTTAGGTCTCCC), and 40 μl of 2 X hybridization buffer (50% formamide, 10 X SSC [1 X SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 0.2% sodium dodecyl sulfate) and then denatured at > 96°C for 5 min. The probes were hybridized to the *Salmonella* microarray overnight at 42°C in a water bath.

After overnight incubation, slides were washed and scanned using the ScanArray 5000 laser scanner (Packard BioChip Technologies, Billerica, Mass.) with ScanArray 2.1 software. Fluorescence signal intensities were quantified using the QuantArray 2.0 software package (Packard BioChip Technologies). The data were subsequently analyzed using Webarray [175] (www.webarraydb.org), with quantile normalization.

Recombination Between FRT Sites Using FLP Recombinase

Antibiotic resistance cassettes were removed from targeted deletion mutants as previously described [157]. Briefly, each mutant was transformed with the temperature-sensitive plasmid pCP20, that encodes FLP recombinase [177]. Transformants bearing the plasmid were selected at 30°C on LB Ampicillin plates. Several colonies from each transformation were streaked twice and grown at 37°C on LB plates (without antibiotics). Loss of the antibiotic resistance cassettes was determined by patching candidate strains on LB containing the appropriate antibiotics (either Kan or Cm). The concomitant loss of plasmid pCP20 was confirmed by patching the candidate mutants on LB Amp plates.

In our mutant collection, the recombination event leaves a "scar" of ten codons from the 5' end of the original gene, a 39 codon region containing the P_{T7}, and the final ten codons of the original gene (**Figure 5**). Scarred derivatives of mutants Δ*STM0854*::*kan*, Δ*STM1402*::*kan*, Δ*STM2840*::*kan*, Δ*STM2884*::*kan*, Δ*STM2581*::*kan* and Δ*STM2581*::*cam* were generated to ensure the accuracy of FLP recombination. The presence of each scarred mutant allele was confirmed by PCR amplification using

primers flanking mutation, and the subsequent sequence of both strands of the PCR product was obtained by the conventional dye-terminating Sanger method. Non-resistant scarred derivatives of mutants Δ*leuX*::*kan*, Δ*sroA*::*kan*, Δ*istR*::*kan*, Δ*oxyS*::*kan*, Δ*STM1131*::*kan*, Δ*STM2303*::*kan*, Δ*STM3120*::*kan*, and Δ*STM3121*::*kan* were generated for further testing in mice.

Construction of Complementing Plasmids

STM2303 was amplified for complementation using the following primers: *STM2303* forward primer 5' GGCGTCTGGTACCAATTCAGTAT 3', and *STM2303* reverse primer 5' TGTTCTGGATCCGTGCGATAGC 3'. The resulting 947 base pair PCR product, in which the only full length ORF is *STM2303*, was purified using agarose gel electrophoresis and extracted from the gel using the Qiaquick gel extraction kit (Qiagen). *STM3121* was amplified for complementation using the following primers: *STM3121* forward primer 5' GCTATTTTCAGGGTACCGTTTGGTCG 3', and *STM3121* reverse primer 5' GCTCCGTTAGCGGATCCTTTAGACAC 3'. The resulting 1266 base pair PCR product was purified by agarose gel electrophoresis, and extracted from the gel using the Qiaquick gel extraction kit (Qiagen). The PCR product contains the full-length *STM3121* open reading frame (879 bp) with approximately 296 bp of upstream and 90 bp of downstream sequence.

Purified PCR products were digested with *Kpn*I and *Bam*HI and ligated into pWSK29 previously cut with the same enzymes and gel purified. *E. coli* DH5a was transformed with each ligation, and positive clones were selected on LB plates

containing Ampicillin. Plasmids bearing the correct insert from each cloning were found by identification of fragments of the proper size after digestion with *Pvu*I. pWSK29, pWSK29::*STM2303*, and pWSK29::*STM3121* were transformed into *S*. Typhimurium strain LB5000 (restriction-, modification+) [178]. Plasmid DNA was prepared from LB5000 plasmid-bearing transformants using the Qiaprep spin Miniprep kit (Qiagen), and purified plasmid DNA was used to transform *ΔSTM2303*::FRT and *ΔSTM3121*::FRT mutants using heat shock. Plasmid-bearing deletion mutants were selected on LB Amp plates, and were twice colony purified prior to use for infections.

Animal Studies in BALB/c Mice

The pools of ~1,000 mutants used as inocula were grown overnight at 37°C in LB with aeration and serially diluted in PBS to the proper concentration for inoculation, and the titer of the inoculum was determined by plating on LB containing Kanamycin. A group of 6 BALB/c mice (8-10 week old female) was infected intraperitoneally with 1 x 10^6 CFU of the pool in 100 μl PBS. Mice were monitored twice daily for signs of infection, and were humanely euthanized at 48 hours post infection. Immediately after euthanasia, the spleen was removed and homogenized in 5 ml sterile ice cold PBS. 100 μl of the spleen homogenate was used for serial dilution and titer on LB Kan plates. The bacteria in the remaining homogenate were grown in LB to stationary phase and total DNA was extracted.

Individual mutants with an apparent phenotype in the pools were chosen for confirmation in mixed infections with wild-type strain HA431 (ATCC14028 *phoN*

Nal^{R}), to determine the competitive index. Inactivation of *phoN*, encoding alkaline phosphatase, abolishes the ability to cleave 5-bromo-4-chloro-3-indolyl phosphate (XP), but does not reduce the ability of serotype Typhimurium to colonize organs or reduce fecal shedding of this organism in mice during competitive infections [32]. Growth on LB agar plates supplemented with XP thus provided an easy means to distinguish between individual mutants (PhoN^{+} blue colonies) and HA431 (PhoN^{-} white colonies). Prior to further analysis, the mutant allele present in each individual mutant to be confirmed was transferred to HA420 (ATCC14028 *Nal*^{R}) by P22 transduction. Mutant strains and our wild type strain HA431 were grown overnight at 37°C with aeration, mixed in a 1:1 ratio, and serially diluted in PBS to the proper concentration for inoculation and titer. Titer was used to determine the exact ratio of strains administered. Groups of 4-6 BALB/c mice (8-10 week old female) were infected intraperitoneally with 1 x 10^{6} CFU of mutant and wild-type virulent HA431 in 100 μl PBS. Mice were euthanized and spleens, livers, and ceca were recovered as above. Mutant and wild-type organisms were enumerated by serial dilution and plating on LB plates containing 20 mg/L of XP.

CHAPTER III

ABROGATION OF THE TWIN ARGININE TRANSPORT SYSTEM IN LEADS TO ATTENUATION

Introduction

Human infection with *Salmonellae*, both typhoidal and non-typhoidal serotypes, results in approximately 3 million deaths per year worldwide [1]. Infection with non-typhoidal serotypes, such as *Salmonella enterica* serotype Typhimurium, occurs after ingestion of contaminated food or water and leads to self-limiting gastroenteritis in otherwise healthy individuals.

S. enterica, like other gram negative organisms, is dependent upon secretion of proteins beyond the outer membrane via several different types of secretion systems for many functions. The Type Three Secretion Systems (TTSS) encoded in *Salmonella Pathogenicity Islands* (SPI) I and II are specialized secretion systems for the injection of effectors into eukaryotic cells. These systems secrete effectors through needle-like structures and enable the bacteria to invade epithelial cells (TTSS1) and promote survival within the vacuole of host cells (TTSS2) [36,137,138,179,180,181,182]. *Salmonellae* also utilize the general secretory pathway (GSP) for the secretion of many bacterial factors, including some virulence related proteins including MisL, an autotransporter protein [183]. The GSP requires the Sec translocase for secretion across the inner membrane and other secreton machinery for moving proteins from the periplasm across the outer membrane.

A third type of secretion system, the twin arginine transport (Tat) system is required in many organisms for the transport of folded proteins from the bacterial cytoplasm into the periplasm. The *Salmonella enterica* serotype Typhimurium twin arginine transport system encoded by the Tat operon has ~80% identity to the Tat operon in *E. coli*. This ubiquitous system transports substrates containing a positively charged N-terminus with the canonical signal sequence S/T-R-R-x-F-L-K N-terminal to a weakly hydrophobic core of approximately 38 residues and a "Sec avoidance" signal upstream of a peptidase cleavage site[184,185,186,187]. The Tat secretion system machinery includes 3 major components, TatA, B and C. TatA is found in molar excess and is responsible for formation of the pore through which folded proteins traverse the membrane [188]. TatB plays a role in recognition of the signal sequence of substrates and interaction of TatA with TatC [189]. TatC is the largest component of this transport apparatus [190,191,192] and is highly conserved, especially at the termini and other cytoplasmic domains [193,194,195]. Initial docking of the signal sequence occurs via TatC [196]. While many of the substrates of the Tat system are cofactor-containing redox enzymes, the particular substrates predicted to be transported by the Tat system in different organisms are quite variable [197]. The Tat system of *Salmonella* is predicted to transport approximately 30 proteins into the periplasm, including one substrate that lacks a twin-arginine that is *Salmonella* specific, TtrB [94,198].

We became interested in the Tat system in *Salmonella* when we identified Δ*tatC* mutants as selected against in a screen for mutants under selection during systemic infection [171]. In the current study, we explore the effects of deletion of *tatC* in

Salmonella enterica serovar Typhimurium ATCC14028 during infection. We confirm that Δ*tatC* mutants are impaired for colonization of systemic organs after intraperitoneal delivery in *Salmonella*-susceptible BALB/c mice, and they are internalized by J774-A.1 murine macrophages in lower numbers than the isogenic wild type parental isolate, ATCC14028. We also studied the ability of Δ*tatC* mutants to colonize the intestinal tract and spread systemically after oral delivery and we show that these mutants are highly sensitive to bile acids. Finally, we show that Δ*tatC* mutants have an unusual motility defect; they are unable to swim yet they swarm similar to wild type ATCC14028.

Results

Δ*tatC Mutants Are Attenuated in a Systemic Model of Infection*

In a previously performed genetic screen, we identified a large group of mutants that were not described as important for *Salmonella* growth during infection [171]. Deletion mutants in *tatC* were identified in this screen as potentially selected against during systemic infection in *Salmonella*-susceptible BALB/c mice. We confirmed that TatC is required for systemic infection after intraperitoneal inoculation using competitive infections between Δ*tatC* mutants (HA473 ATCC14028 Nal^R Δ*tatC*::Kan^R) and wild type (HA431 ATCC14028 Nal^R Δ*phoN*::Kan^R). In these experiments, Δ*tatC* mutants had a reduced ability to colonize the liver and spleen 48 hours after intraperitoneal infection of either *Salmonella*-susceptible BALB/c mice or *Salmonella* resistant CBA/J mice (**Figure 11**). Using intraperitoneal inoculation, the infecting strains bypass the intestinal tract, a niche that non-typhoidal *Salmonella* must survive during

most naturally acquired infections. Thus, the Δ*tatC* mutants are defective for colonization and replication in systemic sites even when they are delivered by a route that bypasses the intestine.

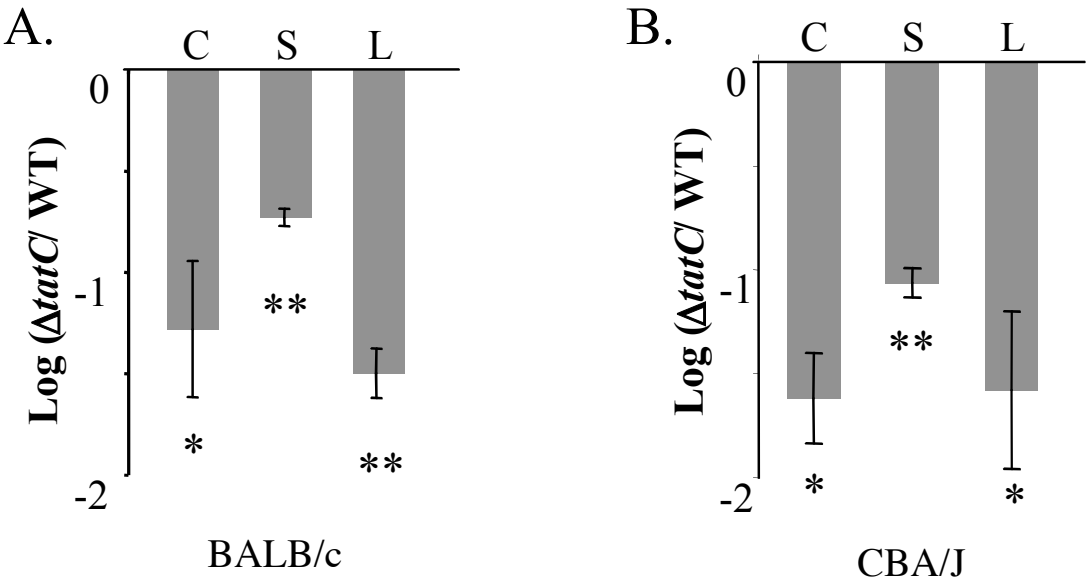


Figure 11: Δ*tatC* is Attenuated in *Salmonella*-Susceptible and Resistant Mice After Inoculation by the Intraperitoneal Route. Mice were infected with an equal ratio of HA473 (ATCC14028 Δ*tatC::* Kan^R) and HA431(ATCC14028 Nal^R Δ*phoN::* Kan^R) by intraperitoneal inoculation with $1x10^6$ bacteria for 2 days in BALB/c (A), or CBA/J (B). Analysis of CFU in the cecum (C), spleen (S), and liver (L) showed that Δ*tatC* mutants have reduced fitness in both *Salmonella*-sensitive (BALB/c) and *Salmonella*-resistant (CBA/J) mouse models, *$p<0.05$, **$p<0.005$.

ΔtatC Mutants Are Defective During Infection of J774-A.1 Macrophages

The ability to adhere to, invade and replicate in eukaryotic cells, notably macrophages, is intimately associated with the pathogenesis of ssp. I serotype Typhimurium [199], and is important for virulence and systemic colonization by this organism [60,200]. We postulated that the defect of Δ*tatC* mutants in systemic colonization after intraperitoneal infection might be due to a defect in internalization and/or survival within macrophages. To test this hypothesis we compared Δ*tatC* mutants, wild type ATCC14028 (HA420 Nal^R), and isogenic Δ*invA* mutants for adherence, internalization by and replication in J774-A.1 macrophages. In these assays, Δ*tatC* mutants were defective in cell association, invasion and intracellular replication when compared to wild type ATCC14028. The ability to adhere to cells, become internalized by them and replicate was restored when *tatC* was supplied *in trans* (**Figure 12**).

To rule out the possibility that the defects of the Δ*tatC* mutants association and internalization by macrophages were the result of an inability to come into close proximity with J774-A.1 cells, we repeated our experiments using low speed centrifugation to force the bacteria into close contact with J774-A.1 cells. Despite this treatment, the Δ*tatC* mutant was still defective for cell association, and was internalized more poorly than the isogenic wild type ATCC14028 (**Figure 12**).

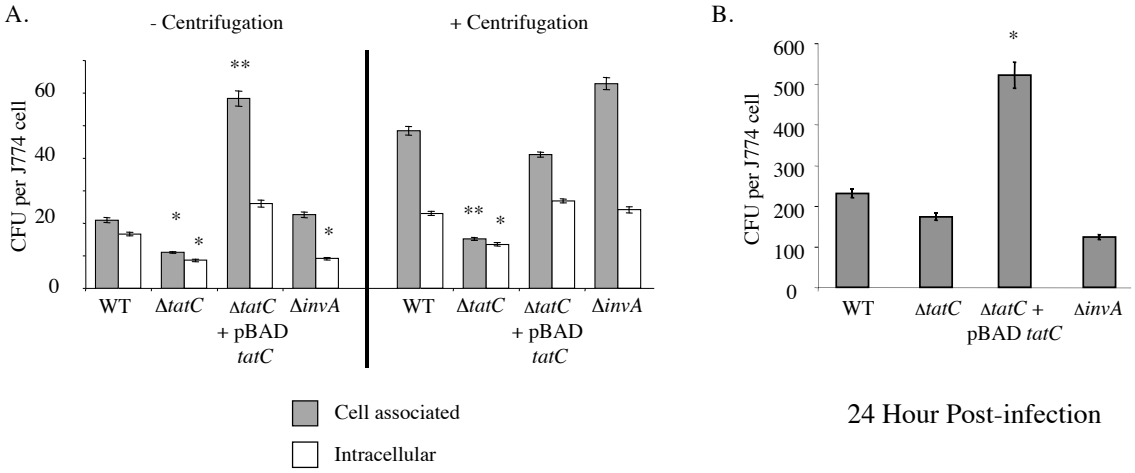


Figure 12: Δ*tatC* Mutants are Defective For Internalization and Replication within Macrophages. J774-A.1 macrophages were infected in a gentamicin protection assay in order to examine ability of Δ*tatC* mutants to associate with and be internalized by cultured macrophages. Δ*tatC* mutants had reduced cell association and internalization by J774-A.1 macrophages whether centrifugation was used or not (A). Mutants were somewhat defective after 24 hours (B). The phenotype was complemented by addition of a plasmid containing functional *tatC*. The data shown is a combination of three independent experiments. Data for each strain was averaged and compared to the corresponding wild type data point in a student's two-tailed T-test. *$p<0.05$, **$p<0.005$.

*Δ*tatC *Mutants Poorly Colonize the Intestinal Tract*

We also assayed the ability of Δ*tatC* mutants to colonize and persist in the intestinal tract of mice, both during short-term infections and for prolonged periods of time. In our short-term infections, we infected BALB/c mice orally with an equal mixture of wild type ATCC14028 and Δ*tatC* mutant and evaluated colonization by both strains at 5 days post-infection. Δ*tatC* mutants were highly defective for colonization of the intestinal tract and spread to systemic organs in this model (**Figure 13**).

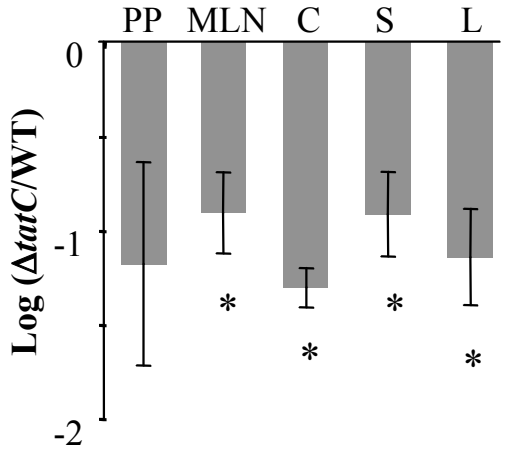


Figure 13: Δ*tatC* is Attenuated in *Salmonella*-Susceptible Mice After Inoculation by the Oral Route. *Salmonella*-susceptible mice were infected with an equal ratio of HA473 (ATCC14028 Δ*tatC*::Kan^R) and HA431 (ATCC14028 Nal^R Δ*phoN*::Kan^R) by oral gavage with 1x10^9 bacteria for 5 days. Analysis of CFU in the Peyer's patches (PP), mesenteric lymph nodes (MLN), cecum (C), spleen (S), and liver (L) showed that Δ*tatC* mutants are unfit in different methods of infection. Data may indicate extracellular bacteria, as well as intracellular bacteria, *$p<0.05$.

To evaluate colonization of the intestinal tract for longer periods of time, we followed fecal shedding of Δ*tatC* mutantsand the isogenic wild type ATCC14028 in *Salmonella*-resistant CBA/J mice that were infected by the oral route. We infected CBA/J mice orally with an equal mixture of Δ*tatC* mutants and the isogenic wild type and monitored fecal shedding in these mice for 40 days post infection. These mice possess a functional *NRAMP* allele, and do not develop systemic salmonellosis, although they are colonized by serotype Typhimurium in the gastrointestinal tract [7,8,9,11,201].

Δ*tatC* mutants had 10-fold reduced fecal shedding from CBA/J mice at early time points post infection, as compared to wild type ATCC14028 (**Figure 14A**),

consistent with our previous results showing reduced intestinal colonization by Δ*tatC* mutants in BALB/c mice. Furthermore, this defect became more severe with longer duration post infection (**Figure 14A**). At 40 days post infection Δ*tatC mutants* were not detectable in the feces of any of the infected mice (5/5 mice). In the organs of infected mice at the termination of the experiment, the majority of the bacterial load was found in the cecum and this population overwhelmingly consisted of wild type *Salmonella* (**Figure 14B**).

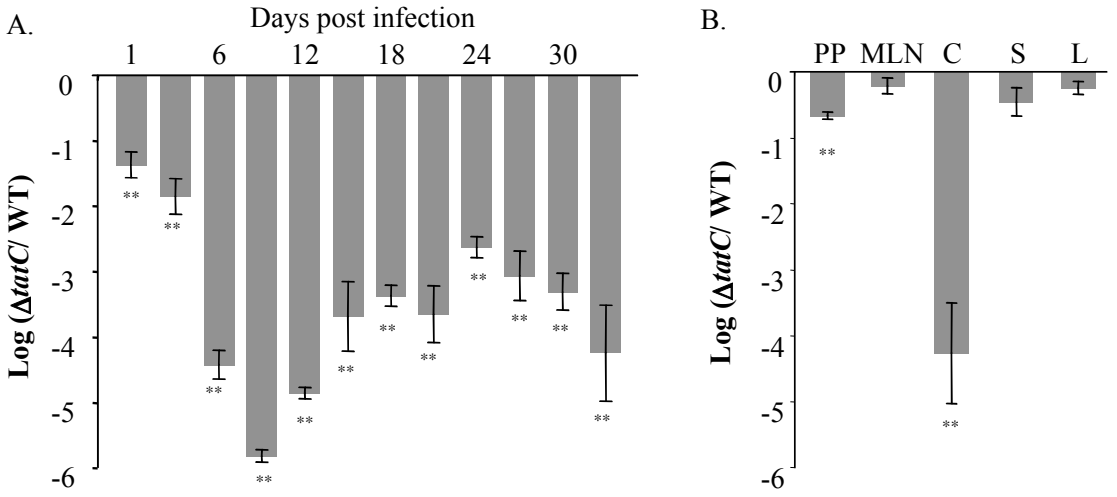


Figure 14: Δ*tatC* Mutants are Attenuated in *Salmonella*- Resistant Mice After Oral Infection. Mice were infected with an equal ratio of HA473 (ATCC14028 Δ*tatC*::Kan^R) and HA431 (ATCC14028 Nal^R Δ*phoN*::Kan^R) by oral gavage with 1x10^9 bacteria (A and B). Fecal samples of six mice were analyzed over 40 days (A) after which the Peyer's patches (PP), mesenteric lymph node (MLN), cecum (C), spleen (S), and liver (L) were collected (B). Δ*tatC* mutants are heavily attenuated compared to wild type even at early time points. Organ data may indicate extracellular, as well as intracellular bacteria, *p<0.05, **p<0.005.

ΔtatC Mutants Are Highly Sensitive to Bile Acids

Because Δ*tatC* mutants are defective for intestinal colonization in murine models at very early time points after oral infection, we explored sensitivity to bile acids as a possible reasons for this phenotype. In *E. coli*, Δ*tatC* mutants are susceptible to detergent due the inability to secrete *amiA* and *amiC* [202,203,204]. We hypothesized that Typhimurium Δ*tatC* mutants may also be more susceptible to detergent killing.

We tested this hypothesis by evaluating the susceptibility of our Δ*tatC* mutants to the bile acid deoxycholate *in vitro* (DOC, 1%) [205]. Bacteria were grown in rich media overnight, sub-cultured in LB broth, grown to mid-log phase, and serial dilutions were plated on LB plates with or without 1% DOC. Both the wild type and Δ*tatC* mutant grew equally well on LB plates without added DOC, yet the Δ*tatC* mutant had dramatically reduced survival (>99%) on DOC as compared to the isogenic wild type (**Figure 15**). We show that the presence of a functional Tat system is responsible for this defect, as returning an intact copy of the *tatC* gene *in trans* restores the ability of this strain to survive 1% DOC at levels similar to the isogenic wild type. On this basis, we suggest that Δ*tatC* mutants are simply poorly able to survive exposure to bile acids upon transit of the upper small intestine during infection. This poor survival in the upper small intestine results in a defect in colonization of the lower intestinal tract, and reduced levels of fecal shedding.

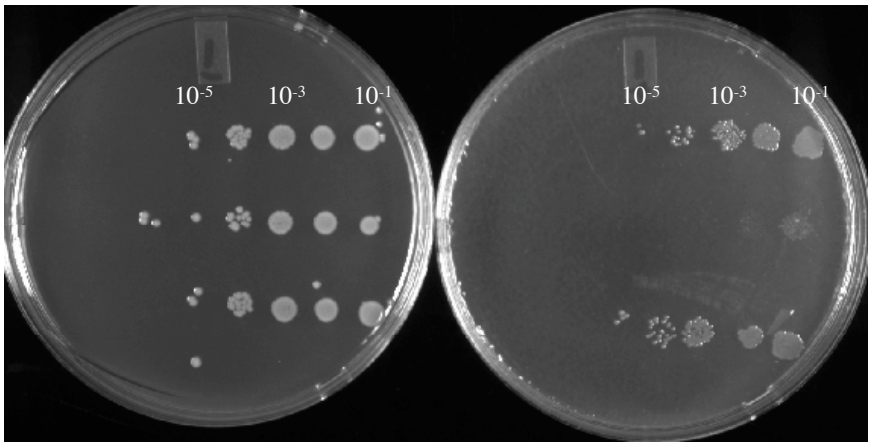


Figure 15: Δ*tatC* Mutants are Sensitive to Bile Acids. HA420 (ATCC14028) HA473 (ATCC14028 Δ*tatC*::Kan^R) and HA640 (Δ*tatC*::Kan^R bearing tatC *in trans*) were grown to stationary phase, subcultured and grown to mid-log phase before diluting and spotting on plates containing either 0% or 1% DOC. Δ*tatC* mutants have reduced viability after treatment with 1% DOC. The samples shown are representative of three independent experiments.

Altered Motility of ΔtatC Mutants Is a Result of Ability to Express Flagellins on the Surface of Salmonella

As part of a larger screen for motility defects, we also observed Δ*tatC* mutants had a very unusual phenotype. These mutants are defective for swimming motility, but are able to swarm well (**Figure 16**). Δ*tatC* mutants complemented *in trans* with the *tatC* open reading frame, under the control of an arabinose inducible promoter, restored the ability of these mutants to swim normally (**Figure 16A**). We examined the ability of these mutants incubated in various *in vitro* conditions to produce and elaborate flagellins, FliC and FljB, on the bacterial surface.

A. B.

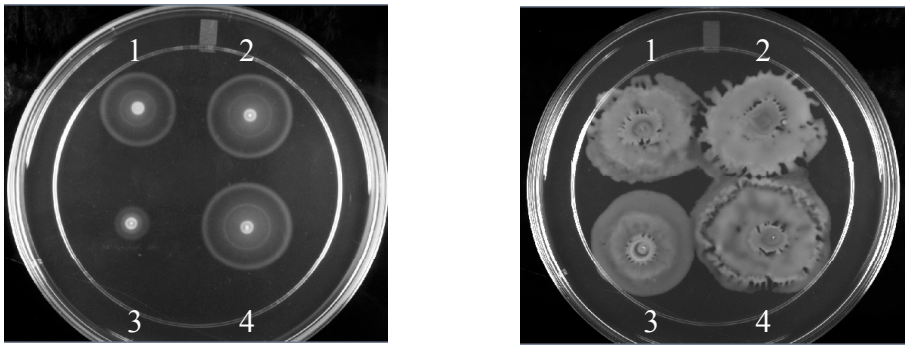


Figure 16: Δ*tatC* Mutants Swarm, but Do Not Swim. 1) HA630 (ATCC14028s Nal^R + pBAD vector $Carb^R$) 2) HA634 (ATCC14028s Nal^R + pBAD tatC $Carb^R$) 3) HA636 (ATCC14028s Nal^R Δ*tatC*::Kan^R + pBAD vector $Carb^R$) and 4) HA640 (ATCC14028s Nal^R Δ*tatC*::Kan^R + pBAD tatC $Carb^R$) were plated on (A) swimming (0.3% agar) and (B) swarming (0.6% agar, 0.5% glucose) motility plates and incubated at 37^0C for 5 and 7 hours respectively. Assays were performed in triplicate on three separate occasions.

Wild type ATCC14028, elaborates the flagellins on the bacterial surface during all three conditions we tested: at stationary phase in LB broth, after incubation on swimming motility agar, and after incubation on swarming motility agar (**Figure 17A, B, and C, lane 3**). The level of flagellins elaborated on the bacterial surface of Δ*tatC* mutants and isogenic wild type ATCC14028 incubated on swimming and swarming agar plates for 5-7 hours was compared. Δ*tatC* mutants grown in LB broth or incubated on swimming plates have very little of either flagellin, FliC or FljB on their surfaces (**Figure 17A and B, lane 4**). The level of flagellins on the surface of Δ*tatC* mutants was restored to the level of the isogenic wild type when the mutant was complemented *in trans* with an intact copy of *tatC* (**Figure 17A and B, lane 5**). Thus, Δ*tatC* mutants have

reduced motility on swimming motility agar as they likely produce fewer flagella in these conditions.

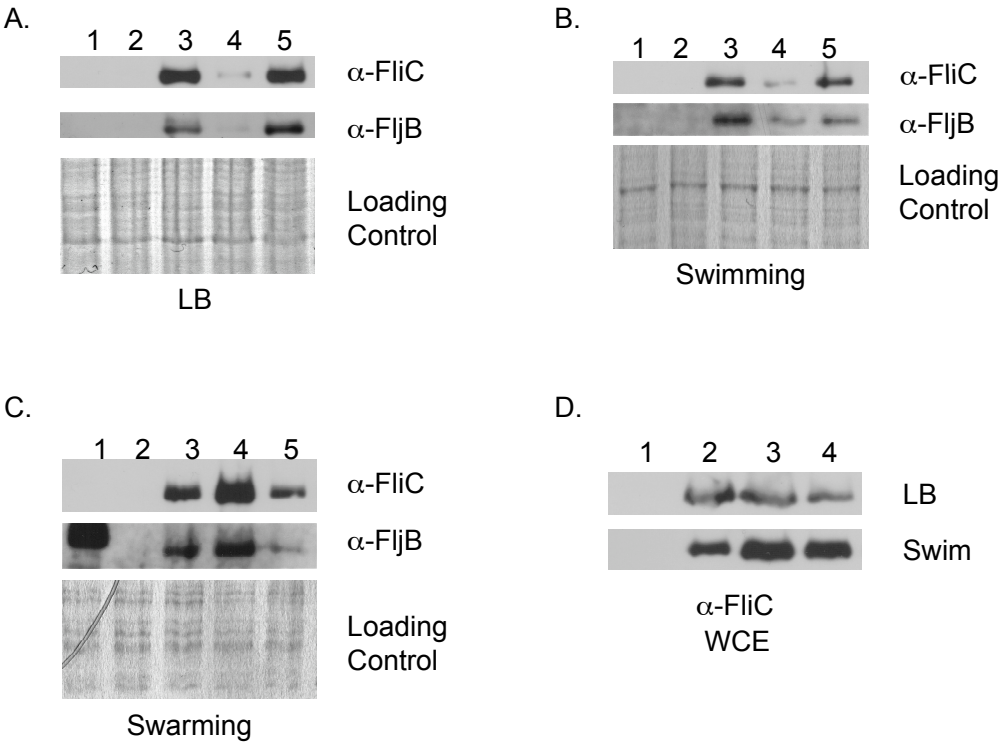


Figure 17: Flagellins in Δ*tatC* Mutants are Decreased Under LB and Swimming Conditions, but Increased Under Swarming Conditions. Bacteria from 1) HA478 (ATCC14028s Nal^R Δ*fliC*::Kan^R), 2) HA690 ATCC14028s Nal^R Δ*fljB*::Kan^R), 3) HA420 (ATCC14028s), 4) HA473 (ATCC14028s Nal^R Δ*tatC*::Kan^R), and 5) HA640 ATCC14028s Nal^R Δ*tatC*::Kan^R + pBAD tatC Carb^R) were harvested from LB broth, 0.6% agar plates used to assay swarming motility and 0.3% agar plates used to assay swimming motility. Flagellins were sheared from the surface for Western analysis. The sheared fractions from LB (A), swimming conditions (B), and swarming conditions (C) were probed with α-FliC and α-FljB. The whole cell lysate for each sample was stained with Coomassie Brilliant blue to control for loading of the sheared fractions. The whole cell fraction was probed with α-FliC (D) to determine whether Δ*tatC* mutants were defective in production of flagellins. The samples shown are representative of three independent experiments.

On swarming motility agar, Δ*tatC* mutants are motile (**Figure 16B, 3**), and they are able to elaborate flagellins on their surface when incubated on media for swarming motility (**Figure 17C, lane 4**). Thus, they appear to produce flagella when they are swarming. This data indicates that a general defect in the ability to export flagellins to the bacterial surface is unlikely to be the cause of the reduced amount of flagellins observed on the surface of Δ*tatC* mutants grown in LB broth or incubated on swimming motility agar. Our data show that Δ*tatC* mutants are able to elaborate flagellins on the bacterial surface under particular conditions, indicating that their lack of motility on swimming motility agar is not simply due to a blanket inability to produce flagellins and assemble flagella on the bacterial surface.

The Predicted Substrates of the Tat System in Two Closely Related Species Are Highly Varied

Utilizing a published genomic survey of prokaryotes we compared potential substrates of the Tat system in two closely related species, *Salmonella enterica* and *E. coli*. [197] Approximately 40% of the predicted substrates differ between the 2 organisms (**Table 4**), including 2 substrates encoded on *Salmonella pathogenicity island 2* (SPI-2) but not part of the type III secretion system, *ttrA* and *ttrB*. Three substrates in *E.coli* have homology to duplicate genes in *Salmonella*, possibly due to large conserved domains in these enzymes (**Table 4**). A few protein sequences contain non-canonical signal sequences that were either predicted to be transported or shown experimentally. By comparing these two species, we can infer that even though they are closely related,

the Tat system may transport proteins that fulfill different functions in different organisms for the same purposes in both organisms.

Table 4: Comparison of Putative Tat Substrates between *Salmonella* and *E. coli*. Based on Dilks et al.[197] Bold denotes differences between species, * denotes twin arginine present, but not recognized as a signal sequence, ** denotes no twin arginine, but demonstrated to be a substrate [198], and superscripted letter denotes gene duplication.

***Salmonella* gene #**	***Salmonella* gene name**	**Tat signal (y/n)**	***E. coli* gene #**	***E. coli* gene name**	**Tat signal (y/n)**	**Function**
STM0060	citE2[a]	y	b0616	citE	y	citrate lyase beta chain
STM0070	**caiD**	**n**	**b2919**	**scpB**	**y**	**methylmalonyl-CoA decarboxylase**
STM0084	**aslA**	**y**	**b3678**	**yidJ**	**n**	**putative sulfatase**
STM0107	thiP	y	b0067	thiP	y	thiamine transporter, ABC family
STM0168	cueO	y	b0123	cueO	y	multicopper oxidase
STM0193	fhuD	y	b0152	fhuD	y	hydroxamate dependent iron uptake
STM0611	ynfE[b]	y	b1587	ynfE	y	oxidoreductase
STM0622	citE [a]	y	b0616	citE	y	citrate lyase beta chain
STM0834	**ybiP**	**y**	**b0815**	**ybiP**	**n**	**putative integral membrane protein**
STM0964	dmsA	y	b0894	dmsA	y	dimethyl sulfoxide reductase, A
STM0996	ycbK	y	b0926	ycbK	y	putative outer membrane protein
STM1383	**ttrA**	**y**				**tetrathionate reductase (SPI-2)**
STM1385	**ttrB**	**y****				**tetrathionate reductase (SPI-2)**
STM1498	ynfF	y	b1588	ynfF	y	putative dimethyl sulfoxide reductase
STM1499	ynfE[b]	y	b1587	ynfE	y	putative dimethyl sulfoxide reductase
STM1539	hyaA[c]	y	b0972	hyaA	y	hydrogenase 1, small subunit
STM1570	fdnG	y	b1474	fdnG	y	formate dehydrogenase
STM1622	ydcG/mdoD	y	b1424	ydcG/mdoD	y	glucans biosynthesis
STM1710	**pgpB**	**n***	**b1278**	**pgpB**	**y**	**phosphatidylglycerophosphate phosphatase**
STM1786	hyaA[c]	y	b0972	hyaA	y	hydrogenase 1, small subunit
STM2064	**phsB**	**n**	**b1671**	**ydhX**	**y**	**putative oxidoreductase**
STM2065	**phsA**	**y**				**hydrogen sulfide production**
STM2099	wcaM	y	b2043	wcaM	y	putative colanic acid biosynthesis
STM2258	napG	y	b2205	napG	y	ferredoxin, electron transfer
STM2259	napA	y	b2206	napA	y	periplasmic nitrate reductase
STM2446		**n**	**b1019**	**efeB/ycdB**	**y**	**putative iron dependent peroxidase**

Table 4 Continued

Salmonella gene #	*Salmonella* gene name	Tat signal (y/n)	*E. coli* gene #	*E. coli* gene name	Tat signal (y/n)	Function
STM2450	amiA	y	b2435	amiA	y	N-acetylmuramoyl-L- alanine amidase I
STM2991	amiC	y	b2817	amiC	y	N-acetylmuramoyl-L- alanine amidase
STM3058	**pepP**	**y**	**b2908**	**pepP**	**n***	**proline aminopeptidase II**
STM3149	hybA	y	b2996	hybA	y	putative hydrogenase
STM3150	hypO	y	b2997	hybO	y	putative hydrogenase
STM3172	sufI	y	b3017	sufI	y	suppressor of ftsI
STM3377	yedY	y	b1971	yedY	y	putative nitrate reductase
STM3615	**yhjK**	**n**	**b1163**	**ycgF**	**y**	**putative phosphodiesterase**
STM3644	**bisC**	**n**	**b1872**	**torZ**	**y**	**biotin sulfoxide reductase 2**
STM3822	torA	y	b0997	torA	y	TMAO reductase
STM4037	fdoG	y	b3894	fdoG	y	formate dehydrogenase
STM4190	pepE	y	b4021	pepE	y	alpha-aspartyl dipeptidase
STM4279	nrfC	y	b4072	nrfC	y	formate dependent nitrate reductase
STM4557	holD	y	b4372	holD	y	DNA polymerase III, psi subunit
PSLT024		**y**				**hypothetical protein**
PSLT046		**y**	**b0126**	**can**	**n**	**putative carbonic anhydrase**
PSLT067		**n**	**b0249**	**ykfF**	**y**	**hypothetical protein, CP4-6 prophage**
			b0286	**yagT**	**y**	**putative xanthine dehydrogenase**
			b0324	**yahJ**	**y**	**putative deaminase**
			b0705	**ybfL**	**y**	**pseudogene**

*Deletion of RcsB Partially Restores Motility to Δ*tatC* Mutant*

It has recently been shown that the master operon for flagellar synthesis, *flhDC*, is negatively regulated by RcsB [206]. The *Rcs* regulon is up-regulated in Δ*tatC* mutants in response to cell wall defects [207] that can occur in these mutants. In order to determine whether *Rcs* is involved in the motility defect seen in Δ*tatC* mutants under swimming conditions, *rcsB* was deleted in a Δ*tatC* background. We hypothesized that if

RcsB repression of *flhDC* is responsible for the motility defect, then deletion of *rcsB* should lead to a rescue of both swimming and surface flagellins. We compared wild type parental isolate, the Δ*tatC* mutant, and a double Δ*tatC* Δ*rcsB* mutant on swimming motility agar. We observed that deletion of *rcsB* partially rescued the motility phenotype we observed in Δ*tatC* mutant (**Figure 18**). Furthermore, deletion of *rcsB* also partially rescued export of flagellins to the bacterial surface in the Δ*tatC* Δ*rcsB* double mutant.

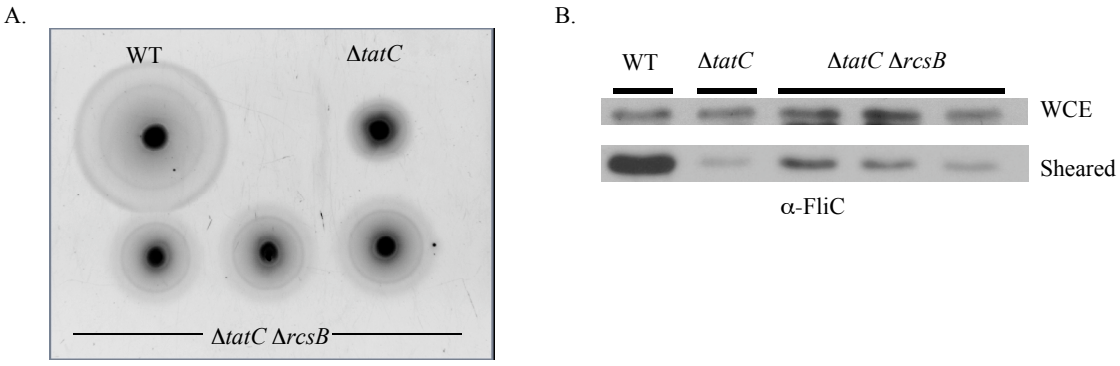


Figure 18: Deletion of *rcsB* Partially Rescues Δ*tatC* Defects in Motility and Flagellin Secretion. RcsB, an inhibitor of the master flagellar regulator, was deleted in a Δ*tatC* background and tested for swimming motility (A) and ability to elaborate FliC flagellins on the surface of *Salmonella* (B).

Materials and Methods

Bacterial Strains and Plasmids

The strains used in this study were derived from *Salmonella enterica* serovar Typhimurium ATCC14028. We generated a spontaneous nalidixic acid resistant derivative of ATCC14028, HA420, that is virulent and persistent in murine models [150]. All deletion mutants were generated by the lambda red method of Datsenko and Wanner [157]. Deletion mutants that were studied in animal models were first moved into a clean genetic background by P22 transduction.

Strains were routinely cultured in Luria-Bertani (LB) broth and plates, supplemented with 50 mg/L nalidixic acid, 100 mg/L carbenicillin and 50 mg/L Kanamycin or 20 mg/L Chloramphenicol where appropriate. For the detection of *phoN* expression, 20 mg/L of 5-bromo-4-chloro-3-indolyl-b-D-phosphate (XP) was added to LB agar plates. Strains were grown in the presence of 0.02% arabinose for over-expression of TatC by arabinose induction. 0.5% glucose was used for catabolite repression.

For infections in murine models, all strains were grown at 37^0C with aeration to stationary phase in Luria-Bertani (LB) broth containing the appropriate antibiotics. Strains used for invasion assays were grown statically for 16 hours at 37^0C in LB broth containing 0.3 M NaCl, these conditions have been described previously to promote SPI-1 expression [208,209]. For deoxycholate (DOC) sensitivity assays, strains were grown overnight in LB, and sub-cultured at a dilution of 1:100 in LB until log phase was reached (OD=0.3-0.4). Cultures were incubated in the presence or absence of 1% DOC

[205] for 4 hours with aeration at 37^0C, serially 10-fold diluted and plated on LB agar plates.

We complemented our Δ*tatC* mutant *in trans* by cloning an intact *tatC* open reading frame into pBAD TOPO (Invitrogen). A fragment containing the *tatC* open reading frame was amplified by PCR using the following primers: *tatC* Forward 5' GGGACCGTAAACATGGCTGTA 3' and *tatC* Reverse 5' CGGTTGTGTAAAGTCTTCAGT 3'. The 780 base pair PCR product was ligated in frame with the pBAD promoter as well as a C-terminal fusion to a V5 epitope and polyhistidine region by altering the stop codon (see underlined base pairs). The orientation and frame of the cloned fragment were determined by dideoxy sequencing using the following primers: pBAD Forward (5' ATGCCATAGCATTTTTATCC 3') and pBAD Reverse (5' GATTTAATCTGTATCAGGCT 3').

Systemic Infection of Mice by Intraperitoneal Infection

Salmonella enterica serotype Typhimurium strains used as inocula were grown to stationary phase at 37^0C with aeration and mixed in a 1:1 ratio of Δ*tatC* mutant to HA431 (Nal^R Δ*phoN*::Kan^R), and then diluted to $1x10^7$ CFU/ml in PBS. Inocula were serially diluted and titered for bacterial CFU to determine the exact ratio of both strains in the competitive infection.

Groups of four to six mice were inoculated intraperitoneally with approximately $1x10^6$ bacteria in 100 μl of PBS. Two days post infection mice were euthanized and livers, spleens, and ceca of infected mice were excised and homogenized in 5 ml ice cold

PBS. Organ homogenates were serially diluted and plated to determine the ratio of Δ*tatC* mutant CFU versus wild type HA431 CFU from the collected tissues of infected animals. Data are expressed as the ratio of Δ*tatC* mutant CFU versus the wild type HA431 CFU, and were normalized to the input ratio, converted logarithmically, displayed graphically. Statistical significance was determined using a Student's *t* test and a P value of < 0.05.

Cell Association, Invasion and Intracellular Replication

The ability of Δ*tatC* mutants to associate with, be internalized by and replicate within J774-A.1 macrophages was tested using the following method. J774-A.1 cells were propagated in DMEM (Cellgro) supplemented with10% Fetal Bovine Serum (PAA), and plated at a density of $3.5x10^5$ cells per well in tissue-culture treated 24 well dishes for all infections. Bacteria used for infecting J774-A.1 macrophages were grown to stationary phase without aeration in LB broth supplemented with 0.3M NaCl [208,209]. The titer of the inoculum in each experiment was determined by serial dilution and plating on appropriate bacteriologic media.

J774-A.1 Cells were infected with *Salmonella* at a multiplicity of infection of 50:1 (bacteria:J774-A.1), and bacteria were spun onto the cells at 750 rpm for 5 minutes where noted (Eppendorf 5804R). Infected cells were incubated for 1 hour at 37^0C with 5% CO_2 in a humidified tissue culture incubator. J774-A.1 monolayers were washed three times with 1 ml sterile PBS prior to lysis, to enumerate cell associated bacteria, or

treated with 100 μg/ml gentamicin sulfate for 1 hour at 37^0C with 5% CO_2 in a humidified tissue culture incubator.

For enumeration of intracellular bacteria that were internalized by J774-A.1 cells, gentamicin was removed and monolayers were washed with 1 ml sterile PBS three times. Infected monolayers were lysed in 1% Triton X-100, and intracellular CFU were enumerated by serial dilution and plating.

For assessment of intracellular growth, infected, gentamicin treated monolayers were washed with sterile PBS, and fresh DMEM supplemented with 10% FBS and 10μg/ml Gentamicin were incubated 24 hours post Gentamicin treatment, washed with sterile PBS three times, lysed and intracellular CFU were enumerated. At each stage when infected cells were lysed, the number of viable J774-A.1 cells in duplicate infected monolayers was assessed by 0.4% Trypan Blue (Cellgro) exclusion and counting viable cells. Each experiment evaluated samples in triplicate, and each experiment was performed on three separate occasions.

Organ Colonization in Salmonella-Susceptible BALB/c Mice

Mutants in *tatC* were tested for virulence in 8 - 10 week old female BALB/c mice (Jackson Labs) in competitive infections with virulent *Salmonella enterica* serotype Typhimurium 14028 using the following protocol. *Salmonella enterica* serotype Typhimurium strains used as inocula were grown to stationary phase at 37^oC with aeration, diluted 1:10 and mixed in a 1:1 ratio of mutant to HA431 (ATCC14028

Nal^R Δ*phoN*::Kan^R) . Inocula were serially diluted and titered for bacterial CFU to determine the exact ratio of both strains in the competitive infection.

Groups of four to six mice were inoculated intragastrically by gavage with approximately $2x10^7$ bacteria in 200 μl of LB. Infected mice were observed daily for signs of illness and were euthanized after the development of signs, usually in 4-5 days (inactivity and reluctance to move, ruffled fur, crouching together). Immediately after euthanasia, livers, spleens, Peyer's patches, and ceca of infected mice were excised and homogenized in 5 ml ice cold PBS. Organ homogenates were serially diluted and plated to determine the ratio of Δ*tatC* mutant CFU versus wild type HA431 from all of the collected tissues of infected animals. Data are expressed as the ratio of Δ*tatC* mutant CFU versus WT CFU, and were normalized to the input ratio, converted logarithmically, displayed graphically. Statistical significance was determined using a Student's *t* test and a P value of < 0.05.

Persistence in Salmonella Resistant CBA Mice

Δ*tatC* mutants (HA473) were tested for the ability to persist in the intestine of *Salmonella*-resistant CBA/J mice in competitive infections with virulent ATCC14028 derivative HA431 (Δ*phoN*). *Salmonella enterica* serotype Typhimurium strains were grown to stationary phase at 37^0C with aeration, and were mixed 1:1 prior to inoculation and the precise titer of this mixture was determined by serial dilution and plating. 8-10 week old CBA mice were infected intragastrically by gavage with an equal mixture of Δ*tatC* mutant and HA431, approximately $2x10^9$ in 100 μl LB in groups of 4-6 mice.

Approximately 100 mg of feces were collected every three days for 40 days, resuspended in 5 ml of sterile PBS, serially diluted and plated for enumeration of CFU of Δ*tatC* mutant vs. WT (HA431). Data are expressed as the ratio of Δ*tatC* mutant CFU versus wild type HA430, were normalized to the input ratio, converted logarithmically, and displayed graphically. Statistical significance was determined using a Student's *t* test and a P value of < 0.05.

DOC Sensitivity

Sensitivity to bile acids was estimated by assaying sensitivity to 1% deoxycholic acid [205]. Strains were grown to stationary phase at 37^0C with aeration and each strain was diluted 1:100 into fresh LB broth and grown at 37^0C with aeration to exponential phase. Exponentially growing cultures (OD_{600}= 0.3-0.4) were serially diluted and spotted in 3 μl spots on LB agar or LB agar supplemented with 1% DOC. Plates were incubated overnight at 37^0C.

Motility Assays

Strains were tested for ability to swim on 0.3% agar Luria-Bertani (LB) plates or swarm on 0.6% agar LB plates supplemented with 0.5% glucose, as previously described [210]. Strains to be tested were spotted in equal amounts on the appropriate agar and incubated at 37^0C for 5 or 7 hours, respectively and examined for motility.

Flagellin Precipitation and Western Analysis

Flagellin production was analyzed under a variety of conditions. Strains were initially grown in LB broth to stationary phase. A portion of the culture was used for swimming and swarming motility assays, while the remaining cells from the 5 ml culture were collected by centrifugation for 10 minutes at 4300 rpm in an Eppendorf 5804R centrifuge. Swarming bacteria were collected from swarming motility plates by swabbing the surface using a sterile cotton swab, resuspended in sterile PBS and collected by centrifugation after determining the bacterial concentration by OD_{600}. Organisms were collected from plates used to assess swarming motility after 8 hours of incubation. Bacteria were collected from swimming motility plates by collecting agar plugs from inoculated swimming motility plates and the agar solids were removed by filtering using a sterile porous paper (Kimax) and gentle pressure. The number of bacteria in the filtrate was estimated by reading the OD_{600} and bacteria were collected by centrifugation.

The pelleted bacteria from all growth conditions were resuspended in 1 ml of sterile PBS and subjected to high- speed vortex for 5 minutes in order to shear the flagellins from the bacterial surface [211]. After centrifugation, the supernatant was removed and subjected to TCA (6% final concentration) precipitation on ice for 15 minutes [211,212]. TCA precipitated proteins were collected by centrifugation at 4^0C for 10 minutes at 13,000 rpm in an Eppendorf 5415R centrifuge and washed twice with 300 μl of acetone. Sheared precipitated proteins were resuspended in SDS-sample buffer. The bacterial pellet was also treated with 100 μl of SDS-sample buffer.

Sheared, TCA precipitated samples and whole cell lysates were boiled for 10 minutes in SDS-PAGE loading buffer, separated on SDS-PAGE (7.5% running gel, 4.5% stacking gel), and transferred to PVDF membrane using standard Western blotting protocols [213]. Membranes were blocked with 5% (wt/vol) non-fat dry milk in PBS containing 1% Tween (PBST) for 1 hour at 25^0C and probed with rabbit α-FliC (Becton Dickson, Difco *Salmonella* H Antiserum i) at a dilution of 1:10,000 or α-FljB (Becton Dickson, Difco *Salmonella* H Antiserum Single Factor 2) at a dilution of 1:1,000 overnight at 4^0C in blocking solution. Blots were washed three times in sterile PBST for 10 minutes. Alkaline phosphatase-conjugated secondary α-Rabbit IgG (Sigma) was used at 1:10,000 in 5% (wt/vol) non-fat dry milk in PBST on blots for 1 hour at 25^oC. Blots were washed three times in PBST followed by addition of the Immune-Star AP substrate (Bio-Rad) for 5 minutes.

CHAPTER IV

SUMMARY AND CONCLUSIONS

Summary

ABACUS

Targeted deletion by lambda-red recombination was previously used to construct a library of specific gene deletion mutants for *E. coli* [166]. Here, we use this approach for *Salmonella*, and modify it by adding a T7 RNA polymerase promoter to the insert for the generation of unique transcripts from each mutant. These transcripts are used to identify and measure the relative abundance of each mutant from a pool containing all of our mutants. Furthermore, we introduce several technical innovations to improve the throughput of library construction, and the labeling and detection of transcripts from each mutant on an inexpensive, customized microarray. We use a well-studied model, systemic infection of BALB/c mice, to validate our assay and identify additional candidate genes that affect the fitness of *Salmonella* in that infection model.

Our targeted deletion strategy has several important advantages over screening of pools of mutants made by random transposon mutagenesis. Screening targeted deletion pools reduces the complexity of the bacterial pool necessary to cover all genes of interest by at least 10-fold over random transposon mutant pools; mathematically, 40,000 random transposon mutants cover about 90% of all non-essential *Salmonella* genes, whereas 4,000 specific mutants in each non-essential gene provides 100% coverage of the targeted genes. This less complex pool is highly advantageous for forward genetic

strategies in circumstances where population sizes drop during the selection process, such as in animal models [163,214,215,216,217]. For *Salmonella*, this "bottleneck" is particularly severe in the transition from the intestinal tract to the systemic circulation [218,219] and perhaps in different parts of the gastrointestinal tract where niche conditions can vary considerably. These bottlenecks cause random loss of mutants from the pool if the population falls to numbers close to the complexity of the interrogated pool. For example, if a population falls transiently to 40,000, then a pool of 40,000 mutants will lose about 37% of its members at random whereas a pool of 4,000 should not lose any mutants ($P<0.01$ for loss of one mutant). Furthermore, a pool of lower complexity may be used at a lower infectious dose, allowing a more physiologically relevant infection. The smallest possible pool that includes each mutant of interest is therefore highly desirable.

In any particular experimental system the size of the inoculum needed to avoid bottlenecks will have to be determined empirically. It is possible to determine the optimum size of the inoculum and ascertain whether or not there is a bottleneck by including a "neutral" mutant carrying a different antibiotic marker at the same CFU as the average mutant CFU in the pool in each experiment. If the representation of this marked strain varies dramatically (either highly over- or under-represented) in the recovered samples in this "fluctuation test", then the inoculum is of insufficient size to prevent random loss from a pool of that complexity (McClelland et al., unpublished data).

Random transposons can generate pronounced downstream effects that contribute to selection. Our targeted in-frame deletions are engineered to minimize or eliminate these effects. Desirable existing features of the cassette inserted by lambda-red recombination [157], such as an internal ATG start codon at the end of the inserts to allow downstream translational coupling, and the inclusion of FRT sites for the removal of the antibiotic cassette by FLP recombination, remain intact. In our construct, FLP recombination results in the production of an in-frame mini-protein, while retaining the $P_{T7,}$ that uses the original start and stop codons of the deleted protein. The first and last 30 bases of each targeted gene were maintained to reduce the chance that targeted deletions disrupt adjacent genes, overlapping genes, and *cis*-acting sequences thus further minimizing the risk of polar effects. Subsequent analyses should consider that in rare cases the remaining mini-protein may retain some function. Finally, an advantage of this collection of specific deletion mutants is that confirmation of mutants identified by genetic screening requires the generation of targeted mutations. A targeted deletion library allows independently generated mutants in the gene of interest to simply be picked from the ordered collection of mutants for further analysis.

Earlier labeling strategies for T7 transcripts from transposon libraries use restriction digestion to fragment the genomic DNA, which has the effect of producing shorter labeled transcripts that are easily mapped near the P_{T7} [160,161,163,168]. This approach results in non-uniform labeling of transcripts, because those transposons that are not in the ideal proximity to a restriction site are poorly sampled. Our labeling

strategy uses randomly sheared ends, resulting in more uniform and consistent labeling efficiency for every mutated gene.

The presence of a P_{T7} promoter in each targeted mutant allows the presence and the level of each individual mutant to be tracked when mutants are pooled, using the mutant-specific transcript produced from this promoter. This innovation eliminates the need to introduce a different 'signature' sequence in each of the mutants in the collection. The P_{T7} facilitates efficient RNA synthesis only from those DNA fragments that contain P_{T7}. Any artifacts that might be generated during the tailing and PCR steps are not transcribed. Note that our P_{T7} procedure generates primarily single stranded labeled RNA, minimizing competition for hybridization to the oligonucleotide array. The performance of this protocol was consistently superior to direct labeling of PCR products as measured by microrray (data not shown).

The presence of the T7 RNA polymerase promoter in each mutant construct allowed us to check all candidate mutants simultaneously for the location of a functional P_{T7}. Using a NimbleGen tiling array of 387,000 oligonucleotides, we confirmed 933 of 1031 mutants in the Kan^R pool as present in exactly the correct place, and functional, simultaneously. Similarly, 892 of 972 Cm^R mutants were confirmed using this technique. Thus, we bypassed much more expensive and laborious traditional PCR confirmation. We also confirm the activity of the P_{T7}, which is not possible using PCR.

For routine hybridizations of the pool, we constructed an inexpensive oligonucleotide microarray using the same oligonucleotides that we used to construct the mutants, containing 45 bases of homology to the *Salmonella* genome. This array could

also be used to confirm the presence of nearby active P_{T7} in a pool. However, unlike the NimbleGen array, this minimal array does not prove the location of the inserted cassettes, or find extraneous insertions that occur elsewhere in the genome. Fortunately, the more expensive NimbleGen arrays need only be used once to determine which mutations are in the correct location and have an active P_{T7}. After the library is characterized, the less expensive in-house arrays can be used for further experiments without ambiguity.

In order to test our targeted deletion collection and novel protocols, we used systemic infection of BALB/c mice, a model system that has been studied extensively using transposon libraries [126,129,136,137,162]. All the mutants in our collection that were reported to have attenuated growth in these previous transposon assays were confirmed in our assay, including those in SPI-2 and lipopolysaccharide biosynthesis genes. In addition, among the 120 mutants showing the strongest fitness phenotype we identified 51 candidates with no previously known phenotype in this model (**Table 2**), although many of these new candidate phenotypes are generally more subtle than those seen for SPI-2.

We retested sixteen of the novel mutants, including Δ*tatC*, with at least a 2-fold change in representation between the input and output pool ($P<0.0001$) in individual competitive infections, and eleven were confirmed in mutants containing the antibiotic cassette. We retested eight of these mutants (in *STM1131*, *STM2303*, *STM3120*, *STM3121*, *sroA*, *istR*, *leuX* and *oxyS*) after removal of the antibiotic resistance cassette, and the phenotype remained in all eight of these mutants (**Figure 9** and **Table 3**). We

complemented two of these novel mutants, in *STM2303* (*pmrM*) and *STM3121* (a LysR-type transcriptional regulator), with the corresponding intact open reading frames *in trans*. We observed reversal of the mutant phenotype for both of these mutants in competition assays with wild type (**Figure 10**), thus directly and unambiguously linking the observed phenotypes to these genes. These results suggest that most of the remaining 25 top mutants of the original 40 novel candidates that we identified may have a phenotype in this BALB/c acute systemic infection model, and there may be additional mutants yet to be discovered.

Using our method, the overall false negative rate (failure to observe phenotypes for mutants already known to be under strong selection in this model) was zero. Among 120 mutants showing a phenotype with $P<0.0001$ and a greater than two-fold change, more than half are already known to have a phenotype and among the new candidates, eleven out of fifteen were confirmed. Thus, the false discovery rate is very low. However, the false discovery rate for new mutants in a system that is already well studied is invariably high. When most genes with a role in a process are known, only a few phenotypes remain to be discovered among the thousands of genes that have no phenotype in that process. The future rate of discovery in the intraperitoneal model in BALB/c mice, is unlikely to be better than the rate of 11 out of 15 that we experienced. The rate of confirmation of mutants that show a weaker statistical confidence and lower fold-difference in fitness will likely be very poor. In addition, those mutants with subtle phenotypes in an animal, that in the past have included important genes such as TTSS effectors will require a larger number of biological replicates in order to establish the

phenotype with confidence. Other phenotypes that will be missed by using a pool of mutants are any that are suppressed by the presence of wild-type bacteria.

Of the mutants we confirm in individual competitive infections, four were in small RNA molecules. Mutations in *leuX*, encoding a minor tRNA-Leu, were previously known to reduce the expression of Type I fimbriae due to codon usage, and reduce bladder epithelial invasion and intracellular proliferation of Uropathogenic *E. coli* [220,221]. The attenuation of a *leuX* deletion during *Salmonella* infection was previously unknown. Mutants in the regulatory sRNAs IstR, OxyS and SroA showed small, but reproducible, phenotypes in our *in vivo* experiment, and all have orthologs in *E. coli*. In *E. coli,* IstR inhibits the translation of *tisAB*, an SOS-induced toxic peptide, and may arrest growth allowing DNA repair [222,223,224,225]. SroA is a known regulator of the *thiBPQ* operon in *E. coli*, that encodes an ABC transport system for the import of thiamine and thiamine pyrophosphate (TPP) into the cell [226]. TPP is an essential cofactor for key enzymes in carbon metabolism.

The *oxyS* mutant was one of the few examples of a mutant that displayed an increase in fitness *in vivo* in our screen. OxyS is a member of the OxyR regulon expressed during oxidative stress. This sRNA is a pleiotropic regulator of about 40 genes in *E. coli* including *rpoS*, and an anti-mutator that may inhibit alternate stress adaptation pathways when OxyR is activated [225,227]. Our observations for OxyS, SroA, and IstR, are the first examples of phenotypes for small non-coding RNA mutants during *Salmonella* infection, other than the tmRNA system that targets proteins for degradation [228]. The targets of these sRNAs in *Salmonella* are not fully known, and given, for

example, the recently reported variable composition of the PhoP regulon across related organisms [229], may differ significantly from their targets in related organisms.

Of the seven remaining confirmed mutant phenotypes, four are in genes that encode putative membrane proteins (*STM1131, STM2120, STM2215 and STM2303*), and a fifth gene (*STM1760*) encodes a putative secreted protein. *STM1131* encodes a member of the KdgM superfamily (oligogalacturonate-specific porin). A paralog of this gene, *STM4016* (*yshA*, 26% identity, 40% similarity) is involved in O-Antigen capsule production and environmental persistence [230]. Mutants in a second membrane protein, *STM2120* (*asmA*), are highly sensitive to gastric contents of swine, but otherwise little is known of the function of this gene in *Salmonellae* [151]. Mutants in the *E. coli* orthlog of *asmA* are extragenic suppressors of outer membrane protein assembly and have less LPS on the bacterial surface [231]. A third gene encoding a predicted membrane protein, *STM2215,* is one of at least eight proteins encoded by Typhimurium that contain cyclic-di-GMP phosphodiesterase, or EAL domains (named after the amino acids in the conserved domain). Unlike several other EAL-containing proteins, mutations in *STM2215* do not influence a multicellular behavior known as the rdar morphotype [232]. Our work reports the first phenotype of any kind for *STM2215*.

Finally, *STM2303* (*pmrM*; *pbgE3*) is the terminal gene in the *pmr* operon (polymyxin resistance) and also encodes a predicted membrane protein. This operon is involved in 4-aminoarabinose addition to Lipid A, a modification that confers resistance to polymixin [233]. Paradoxically, PmrM does not appear to be necessary for this function [233,234], but may be important for resistance of *Salmonella* to high iron

concentrations *in vitro* [235,236]. Recent studies show that mutants in *STM2303* have reduced expression of *hilA*, an important regulator of virulence genes in *Salmonella*, and reduced invasion in T84 human colonic epithelial carcinoma cells [237].

Mutants in *STM1760*, encoding a protein containing a classical N-terminal secretion signal sequence, were also confirmed as selected against in our competitive infection assays. The protein encoded by this gene contains multiple Tricopeptide Repeats (TPR) of the Sel-1 superfamily, a motif that mediates protein-protein interactions [238]. Nothing further is reported in the literature about this protein and its potential role during systemic infection by *Salmonella*.

The final genes we confirmed to be important during systemic infection in BALB/c mice, *STM3120* and *STM3121*, are neighboring in the genome. *STM3121* is a LysR family transcriptional regulator neighboring *STM3120* but is transcribed in the opposite direction. Interestingly, expression of *STM3121 in trans* appeared to increase fitness during infection (**Figure 10**). *STM3121* has not previously been reported to be important during Typhimurium infection, and determination of its regulatory targets may reveal clues as to its role during infection.

STM3120 and several adjacent genes (cluster *STM3120-STM3117*) are present in serotypes Typhimurium, Enteritidis, and Gallinarum [159] and in some *Yersinia* [239], but are not present in the typhoidal *Salmonellae* or other enterobacterial species studied to date. A second gene in this group, *STM3119*, was also selected against during our ABACUS screen. Proteomic and transcriptional data indicates that the proteins encoded in this region are highly up-regulated in macrophages [119,120,240]. In serotype

Enteritidis, these genes are essential for growth in chicken macrophages and mutants in some of these genes in Gallinarum are defective for systemic colonization after oral infection in chickens [159,241].

Although *STM3120* is a predicted *citE* (citrate lyase) homolog, the other two subunits (*citD* encoding the a-subunit, and *citF* encoding the g-subunit) that normally make up bacterial ATP-dependent citrate lyase are not encoded in the region containing this gene. This suggests that *STM3120* may have a different function than a traditional citrate lyase. In *Yersinia pestis*, homologs of *STM3120-3117* are necessary for survival in macrophages that are activated with IFN-γ after infection. These genes appear to function in *Y. pestis* by lowering the level of nitric oxide in macrophages without affecting iNOS levels in g-interferon post-treated macrophages [239]. It is possible that these genes play a similar role in *Salmonella*, but this hypothesis remains to be investigated.

To summarize, we have designed a collection of novel technologies that permit easy generation and confirmation of specific deletion mutants for genetic screening, both individually and in pools. Use of our collection, designed to minimize the effects of population bottlenecks on screening, will allow relevant animal models and more relevant doses to be efficiently used to identify *Salmonella* mutants with altered fitness *in vivo*. During our testing of this approach, eleven of such mutants were confirmed in individual competition assays, including three sRNAs and *leuX*. The tools used here are a first step to a more complete description of *Salmonella* genes involved after systemic delivery in mice, particularly those genes with milder phenotypes that were difficult to

identify with confidence in previous studies. These tools are also of wide applicability to identify genes involved in other aspects of *Salmonella* biology. Finally, similar strategies can be applied in other genetically malleable bacteria.

TatC

Gram negative organisms secrete folded proteins across the inner membrane using the twin arginine transport (Tat) system [184,192,242,243,244,245,246,247,248,249]. Disruption of the Tat system results in a range of defects in diverse processes including transport of virulence determinants, cell division, motility and chemotaxis. In *E. coli* 0157:H7, Tat mutants have attenuated toxicity to Vero cells, and they have motility defects [244]. In *Yersinia pseudotuberculosis*, a functional Tat system is necessary for virulence, motility and acid resistance [243]. In *Vibrio cholerae*, mutants in the Tat system are impaired for intestinal colonization in the infant mouse model and have reduced ability to form biofilms [249], but do not have motility defects. In *Pseudomonas aeruginosa*, an intact Tat system is necessary for transport of virulence determinants, such as phospholipases, and is also involved in iron uptake, stress defense, motility and biofilm formation [245,246]. Some gram-negative organisms, such as *Mycobacterium tuberculosis*, rely on a functional Tat system for the secretion of beta-lactamases for antibiotic resistance [247,248]. The number and complement of proteins predicted to be secreted by the Tat system is highly variable between organisms [197], and this may be one factor that influences the multiple and varied phenotypes of Tat mutants.

Tat mutants in *Salmonella enterica* serotype Typhimurium ATCC14028 also display multiple defects. We determined that Δ*tatC* mutants in *Salmonella* are defective for growth in systemic organs after intraperitoneal infection, a route of infection that bypasses the intestinal tract. Because macrophages are an essential niche for serotype Typhimurium during systemic infection in mice, we tested the ability of Δ*tatC* mutants to associate with and become internalized by macrophages. The ability of Δ*tatC* mutants to associate with, be internalized by, and replicate in J774-A.1 was reduced as compared to the isogenic wild type.

Tat mutants in other pathogenic bacteria also associate poorly with or replicate to lower numbers in eukaryotic cells. In *Vibrio cholera*, mutants in the Tat system attach poorly to HT-29 cells [249]. *Legionella pneumophila* Tat mutants have defects in intracellular replication within differentiated U937 cells during later stages of growth [250]. In *E. coli*, Δ*tatC* mutants have been have a mutant cell septation phenotype and thus form long chains of bacteria [202]. The reduced internalization of *Salmonella* Tat mutants by cultured macrophages may actually be difficulty in phagocytosis, as long chains of bacteria may not be easily phagocytosed by macrophages.

We also show that Δ*tatC* mutants are defective for intestinal colonization after oral infection of both *Salmonella*-susceptible BALB/c mice and *Salmonella*-resistant CBA/J mice. Similar defects in intestinal colonization have previously been shown for Tat mutants in other gram negative pathogens including *Vibrio cholera* [249], *Yersinia pseudotuberculosis* [243], and *Camplyobacter jejuni* [251] We further show that Δ*tatC* mutants are extremely sensitive to 1% Deoxycholic acid (DOC) *in vitro*, an assay

commonly used to test for increased sensitivity to bile acids [205]. This sensitivity is likely a contributing factor for the reduced ability of *Salmonella* Δ*tatC* mutants to colonize the murine intestine. Tat mutants in *E. coli* and *C. jejuni* have also been shown to have increased sensitivity to detergent and choleate [202,203,204,251,252].

Finally, similar to Tat mutants in other organisms [243,244,245,251], *Salmonella* Δ*tatC* mutants have a strong defect in swimming motility, as a result of failing to export flagellins under conditions that allow swimming motility. We show that Tat mutants are able to export flagellins to the surface and that these mutants are motile under conditions that promote swarming motility, unlike Tat mutants in *Pseudomonas* [245]. Thus *Salmonella* Tat mutants are not non-motile under swimming conditions because they do not export flagellins to the bacterial surface under these conditions. Similarly, Tat mutants in several other organisms, including *Agrobacterium* and *Pseudomonas*, were shown to be non-motile but to be able to produce flagella in a small proportion of the population [245,253].

The mechanism responsible for the inability of Tat mutants to produce flagella has not yet been determined, but several hypotheses have been proposed. In Δ*tatABC* mutants of *E. coli* O157:H7 the inability to export FliC is hypothesized to be due to impaired insertion of FliP into the outer membrane [244]. The *FliOPQR* operon encodes class 2 flagellar proteins that make up part of the MS ring of the flagellar export system, and they are necessary for the export of flagellins[254]. FliP contains a cleavable signal sequence (M**RR**LLFLSLAGLWLFSPAAAA) with a twin arginine motif, necessaryfor its insertion into the cytoplasmic membrane [255,256] . However, the twin arginine

motif in the FliP signal sequence is not recognized by either the TatP or TATFIND signal recognition software and FliP has not been shown experimentally to be secreted via the Tat system [197,257].

Also, elimination of FliP secretion when the Tat system is inactivated seems an implausible mechanism for the defects in swimming motility and flagellar export of *Salmonella* Tat mutants under swimming conditions, because we show that these mutants are still motile and able to secrete flagellins in swarming conditions. FliR, which acts in conjunction with FliP for the export of flagellin [256] was previously shown to be up regulated in Δ*tatC* mutants in *E. coli* under anaerobic conditions [207]. The effect on flagellins appears to be coordinated with environmental cues, and is likely to be more complex than was previously hypothesized.

The integrity of cell envelope of Δ*tatC* mutants has been shown to be impaired in *E. coli* [204], *P. aeruginosa* [245], and we show that *Salmonella* Tat mutants are sensitive to the detergent DOC (**Figure 4**). In Δ*tatC* mutants in *E. coli*, the Rcs regulon (regulator of capsule synthesis) is up-regulated in response to cell envelope defects. This regulon has also been shown to inhibit *flhDC* leading to impairment of motility [206]. It is possible that the defects in motility of Δ*tatC* mutants are an indirect effect of increased Rcs activity. We show that deletion of *rcsB* in a Δ*tatC* background is able to partially rescue both the swimming defect and the flagellar export defect. One possible interpretation of this data is that under swimming conditions a signal is necessary to remove *rcsB* from the promoter region of *flhDC* and this signal is not necessary under swarming conditions.

In this work, we show that Δ*tatC* mutants do not survive well in the intestinal tract or in systemic sites during experimental infections. Defects in the ability of Δ*tatC* mutants to survive antimicrobial defenses, such as bile, affect survival during intestinal infection, and inability to survive within macrophages during systemic infection contribute to the survival of Δ*tatC* mutants during infection. Though not yet shown experimentally, there may also be a link between the ability to express flagellins on the surface of *Salmonella* under different conditions with the ability to survive in those conditions, for example, perhaps escaping innate immune detection during infection. These factors, along with the ubiquitious nature of the Tat system, make the twin arginine transport system a viable target for drug development.

Conclusions

We have developed an array-based method, which we used to identify mutants defective during systemic infection with *Salmonella enterica* serotype Typhimurium. Novel data generated from this screen will further our understanding of virulence mechanisms and potentially lead to development of increasingly well-defined drug targets. Future work will include completion of the deletion library consisting of single mutants, as well as large island deletions. Our lab will continue to follow novel data generated from the systemic model, but will also focus on mutants defective in intestinal persistence in orally infected animals. The tools developed during this work provide great promise for the future of our understanding of *Salmonella* pathogenesis.

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