NOVEL MECHANISMS USED BY *SALMONELLA* TYPHIMURIUM TO COLONIZE THE INTESTINE

A Dissertation

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JOHANNA REBECCA ELFENBEIN

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Chair of Committee,	Helene Andrews-Polymenis
Committee Members,	Noah Cohen
	Ann Kier
	Sara Lawhon
Head of Department,	Linda Logan

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ABSTRACT

Non-typhoidal salmonellae are zoonotic pathogens that cause the largest number of cases of bacterial foodborne gastroenteritis in the United States annually. Food products of animal origin contaminated with *Salmonella* are major sources for human infection in the United States with food products of bovine origin responsible for up to 30% of all outbreaks of human disease. Salmonellae are heavily studied pathogens due to the ease of genetic manipulation and rapid growth rate. However, despite thorough investigation of *Salmonella* virulence mechanisms over the past three decades, few studies have used relevant animal models to study the gastrointestinal phase of infection. In addition, the vast majority of inquiry has focused on few virulence loci, leaving approximately half of the genome poorly explored.

We used the calf, a natural host of *Salmonella* and model that most closely recapitulates early enteric infection, to identify new *Salmonella* genes needed for infection and further characterized a subset of these genes. We identified more than 20 genes never previously implicated in enteric infection and confirmed the necessity of two genes, *STM3846* (retron reverse transcriptase) and *STM3602* (transcriptional regulator), during infection of the calf. Additional exploration using both mouse models and *in vitro* experiments showed that the *STM3846* reverse transcriptase produces a RNA-DNA hybrid molecule called multicopy

single-stranded DNA. This molecule regulates protein abundance during anaerobiosis, leading to poor colonization of mutants unable to produce this molecule. Further characterization of *STM3602* showed that this putative transcriptional regulator is involved in regulating multiple processes that are necessary for *Salmonella* to thrive within the complex microbial community of the intestine. Thus, through the use of a carefully orchestrated genetic screen in a relevant animal host, novel genes were identified and their functions for colonization characterized. These genes and the processes in which they participate are potential targets for development of novel therapeutics to combat this increasingly antibiotic resistant pathogen.

DEDICATION

To Pepper, Tiny, Michelle, and Rich for teaching me to appreciate life.

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Salmonella enterica is zoonotic pathogen of substantial concern to global human and animal health. It is a leading cause of morbidity and mortality in people worldwide [1, 2]. Infection with *Salmonella* arises from consumption of contaminated food or water or direct contact with an infected individual or animal [3, 4]. Infected individuals and animals may shed billions of organisms per gram of feces and represent a major source of environmental contamination, particularly in areas where sanitation is poor.

There are two major disease syndromes associated with *Salmonella* infection, enteric disease and systemic disease, although these disease syndromes are not mutually exclusive. Enteric disease caused by non-typhoidal *Salmonella* (NTS) persists for as long as one week and is characterized by fever, abdominal cramping, diarrhea, vomiting, and weight loss [5-8]. It is estimated that more than 90 million cases of NTS occur worldwide each year [9]. Uncomplicated NTS infection generally resolves without specific treatment in immunocompetent hosts. Immunosuppressed individuals, children under the age of 5, and the elderly may develop life-threatening bacteremia as a sequel to enteric disease [5]. Pathologic lesions of NTS are observed primarily within the ileum, cecum, and colon. Enteric disease is characterized histologically by mucosal edema and ulceration, neutrophilic inflammatory infiltrates, hyperplasia

of Peyer's patches and mesenteric lymph nodes, and crypt degeneration and abscessation [10-12]. Regardless of the severity of disease, one important characteristic of *Salmonella* infection is subclinical carriage with intermittent fecal shedding of organisms [8]. This subclinical persistence represents an important reservoir for continued environmental contamination.

Typhoid fever is caused by the host-adapted serotypes of *Salmonella*: Typhi, Paratyphi A, B, and C for humans [13]. A conservative estimate of the global burden of Typhoid and Paratyphoid fever is more than 13 million cases annually [14]. Typhoid fever is characterized by fever, headache, anorexia, and general malaise with symptoms lasting as long as 3 weeks [13, 15]. Only approximately one-third of patients with this systemic disease develop gastrointestinal manifestations. Treatment with antibiotics is required for successful resolution of systemic disease.

The pathologic lesions seen in Typhoid fever differ from those of *Salmonella* enterocolitis. Typhoid fever is characterized by granulomatous inflammation with mononuclear cell infiltrates in the intestine particularly centered over Peyer's patches, and diffusely throughout the liver and spleen [16, 17]. There is a relative paucity of neutrophil involvement as compared with the disease syndrome created by NTS. As with NTS, chronic carriage develops in up to 5% of patients leading to intermittent fecal shedding and chronic carriers are considered the main reservoirs of the typhoidal serotypes [18].

Salmonellae are facultative intracellular pathogens, capable of colonizing and causing disease in all species of mammals and birds. However, salmonellae may also live on abiotic surfaces and can remain in soil for more than 300 days in permissive conditions (reviewed in [19]). Even plants cultivated in *Salmonella*-infested soil may become culture-positive via a variety of mechanisms [20]. Studies on the biology of the organism in the laboratory environment poorly replicate the environmental pressures to which the organism is exposed. It is, therefore, essential to study this pathogen in its natural environment, during infection of a host, in order to understand pathogenic mechanisms. A recent World Health Organization report suggests that multidrug resistant (MDR) salmonellae are an emerging food-borne threat with some areas reporting more than 90% of human infections caused by MDR salmonellae [21]. This fact, as well as the emergence of *Salmonella* strains with increasing potential to cause deadly disease [22], drive the need for further research to develop new antibacterial strategies and improved vaccines.

In the work presented here, I will discuss enteric salmonellosis, particularly that caused by *Salmonella* Typhimurium (STm). I will briefly discuss the epidemiology of NTS from animal sources and describe two complementary animal models of enteric disease. I will also describe the accepted strategies used by *Salmonella* to colonize the mammalian intestine. Finally, I will provide the framework for the discussion of two novel strategies of intestinal colonization that are detailed in the following chapters.

Animal sources of salmonellae

Animals of agricultural importance are frequently colonized with salmonellae. These animals are a source for environmental contamination by infected animal waste and are a direct route to contamination of the food supply. There is also an animal welfare concern for those that develop clinical disease. In a survey of 403 food-borne *Salmonella* outbreaks in the United States (US), products of the poultry industry (eggs, chicken, and turkey) accounted for 50.6% of outbreaks, bovine products (beef and dairy) accounted for 12.2% of outbreaks, and pork products for 9.1% of outbreaks [23]. The absolute number of non-outbreak cases attributable to a given food source varies but the trends are similar [3, 24]. It is apparent from these data that food products of animal origin are major sources of *Salmonella* cases and outbreaks.

However, the relative contribution of different agricultural animals to total numbers of human enteric salmonellosis cases in the US annually is difficult to estimate. Humans may become infected from direct contact with animals that are shedding *Salmonella* [4]. Animal waste may be released into the environment where infectious agents may gain access to growing crops and/or the water supply. In addition, rodents, raccoons, birds, and insects may gain access to production animals or their waste products when animals are reared in systems without strict isolation from the external environment. Both waste contamination of the environment and potential colonization of wild animals may lead to dissemination of salmonellae and other infectious agents from the point

of animal production, and the relative impact would be difficult to monitor. Thus, it is possible that agricultural animals are important sources of contamination of non-animal food products that may be point sources for outbreaks in the human population. There is a complex interplay between animals, the environment, and humans for transmission of *Salmonella* and for generation of outbreaks with potential devastating consequences.

Animal models of disease

Using animals to model infectious disease is a common practice with the overall goal of understanding the disease process in order to create interventions that will treat or prevent the development of disease. When modeling disease, there are some important points to consider. Desirable characteristics of an animal model are that the disease resembles human disease in the organ affected, histologic appearance of lesions, and immune response to the invading organism. Other desirable characteristics include the ready availability of animals with defined husbandry and an affordable cost of animal purchase and housing. In order to study the host response to disease, models that have a sequenced genome and are genetically tractable are preferred. Finally, the animal's capacity to metabolize xenobiotic compounds and immune responses must be considered in order to test the efficacy of new therapeutics and vaccines. It is not possible to fulfill all of these requirements for each animal model, but these characteristics must be considered and any lack

of concordance recognized when choosing an animal species to use as a model system.

Murine colitis model

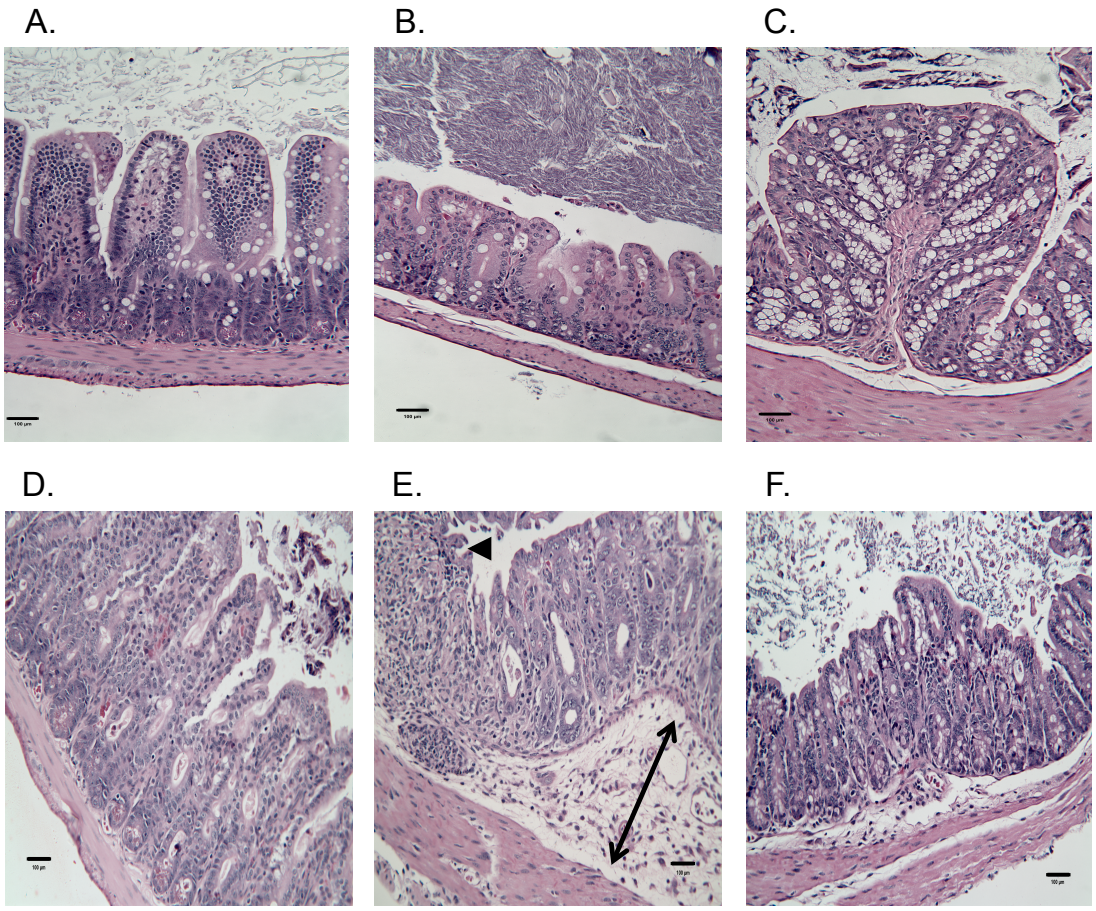
Mice are the most highly utilized animal to model salmonellosis. Mice of the C57B6 lineage do not develop diarrheal disease after oral infection with NTS, but instead the organisms disseminate systemically and colonize the internal organs (murine typhoid model). Pathology in the intestine is mild, and is characterized by infiltrates of mononuclear cells [25]. Colonization resistance mediated by the microbiota, makes the mouse a poor model to study enteric salmonellosis (reviewed in [26, 27]).

The murine colitis model was developed in order to overcome these limitations. In this model, mice are treated with streptomycin to reduce the intestinal microflora prior to infection with *Salmonella* [28, 29]. *Salmonella* Typhimurium colonizes the cecum in these streptomycin-pretreated mice with high efficiency, and induces a severe inflammatory response (reviewed in [30-32]). The inflammatory response is characterized by neutrophil and mononuclear cell infiltration, submucosal edema, crypt abscessation, villous blunting and fusion, and epithelial damage. These histologic indicators of disease are similar to those observed in cases of enteric salmonellosis in people. However, the primary histologic abnormalities found in this model are within the cecum, with relatively little inflammation evident within the ileum or colon 2 to 4 days post

infection (Figure 1.1 and reference [33]). Thus, this model might be more appropriately termed the murine typhlitis model to describe the severe cecal inflammation and relative paucity of inflammatory changes in the colon. While the cecum is affected in human cases of enteric salmonellosis, a more diffuse enterocolitis is also observed [10-12]. The difference in disease localization is important to note as the different portions of the gastrointestinal tract have highly diverse functions in digestion, microbial community complexity and composition, and may have different responses to infection.

Inbred C57BL/6 mice are the most heavily used for the murine colitis model [28]. These mice are highly susceptible to infection with intracellular pathogens because they lack a functional Natural Resistance Associated Macrophage Protein-1 (*NRAMP-1*) gene (*SLC11A1*), encoding an exporter of divalent cations from the phagosome [34]. Cells lacking *NRAMP-1* permit growth of pathogens within phagosomes [34]. In mice, the *NRAMP* gene is highly expressed within cells of the myeloid lineage in numerous organs including the intestine [35]. This protein is important for adequate innate immune response to *Salmonella* in mice as mortality rates after *Salmonella* infection are substantially greater in $NRAMP^{-/-}$ mice as opposed to those that are $NRAMP^{+/+}$ after intravenous *Salmonella* challenge [36].

Figure 1.1: Intestinal histopathology from the murine models of salmonellosis. Representative images of intestinal segments from mice euthanized four days after oral infection with 10^8 colony forming units of virulent *Salmonella* Typhimurium. (A-C) Murine typhoid model showing no observed inflammatory changes in (A) ileum, (B) cecum, or (C) large intestine. (D-F) Murine colitis model with extreme variation in the inflammatory response between intestinal segments. (D) Ileum with mild mononuclear leukocyte infiltration and epithelial damage. (E) Cecum with submucosal edema (arrow), severe neutrophilic inflammation (arrowhead) and mononuclear leukocyte infiltration, and severe epithelial damage. (F) Large intestine with mild mononuclear leukocyte infiltration. Horizontal bars are 100 μm.



Interestingly, *NRAMP*$^{+/+}$ mice rarely succumb to infection and may live for more than 1 year harboring the pathogen [37]. The murine colitis model has been described in *NRAMP*$^{+/+}$ mice and they develop similar disease with similar histologic abnormalities observed in *NRAMP*$^{-/-}$ mice [38]. The major advantage of this model is that mice survive the acute infection, which allows for monitoring the development of protective immunity, epithelial restitution following acute disease, and for evaluating therapeutic efficacy. This model may have utility for the study of factors responsible for chronic shedding of NTS, a factor contributing to the contamination of the food supply.

The murine colitis models have substantial value and have contributed to our knowledge of the pathogenesis of enteric disease. Mice are inexpensive, do not require specialized facilities for housing, handling skills are easily learned, the genome sequence is available, and there are invaluable genetic tools available to study the host response. However, the major limitation of mice as model organisms for the study of enteric disease is the necessity to disrupt the microflora prior to infection. This fact makes it impossible to use this model to assess microbiota-mediated factors *Salmonella* encounters during infection of a natural host. Secondly, the use of inbred animals to model host response fails to encompass the range of disease responses in an outbred population naturally acquiring infection as has been shown for other bacterial and viral pathogens using outbred mouse strains [39, 40]. In addition, there has been recent debate as to the utility of the mouse to model human inflammatory disease [41]. While

much valuable information regarding the pathogenesis and host response to *Salmonella* infection has been elucidated using murine models of infection, there is no replacement for the study of disease in a natural host.

Bovine enteritis models

Cattle are natural hosts of salmonellae and are a source of contamination of the environment and food products [23, 24]. As natural hosts of this organism, cattle are susceptible to all of the syndromes of salmonellosis: acute or chronic diarrheal disease, bacteremia and systemic disease, or subclinical carriage and prolonged fecal shedding [42]. Cattle are susceptible to all non-typhoidal serotypes and serotype Dublin is host-adapted to cause bacteremia and systemic disease in cattle.

Susceptibility to disease is age-dependent as younger calves are more prone to severe disease and death from *Salmonella* infection [43], as is the case for human infection. However, older animals will also become infected and can develop diarrheal disease and serve as a reservoir for continual environmental contamination [42]. Enteric disease is characterized by high fever, inappetence, signs of abdominal discomfort, and diarrhea [44]. Clinical signs persist for up to 10 days and infected animals may consistently shed the organism in feces for as many as 20 days and intermittently for as many as 7 weeks [44]. The tissues with the largest bacterial burden are the aboral small intestine and colon [44]. Histologically, disease is characterized by a neutrophilic exudate, disruption of

the epithelial cell layer, submucosal edema, and villous blunting [45, 46]. Each of these features of clinical disease is similar to those observed in human disease.

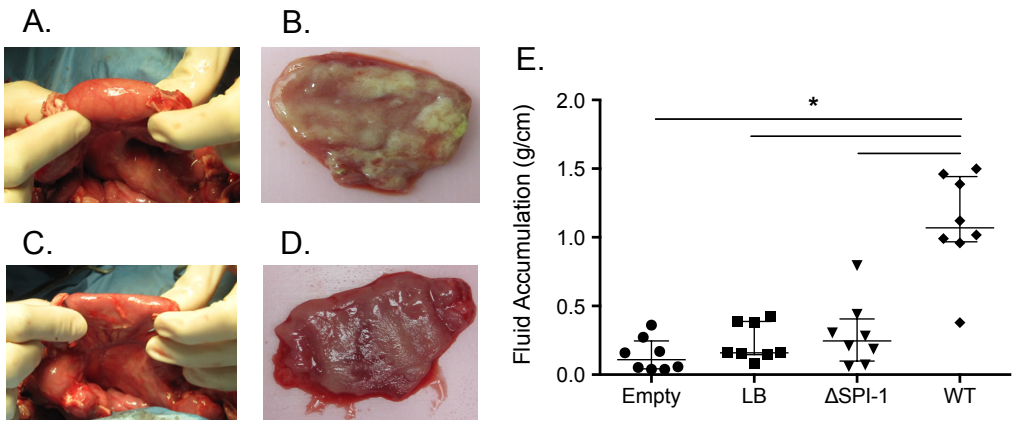
Numerous bovine infection models have been described with variation in the age of animals and route or duration of infection. Oral infection models have the benefit of recapitulating the natural route of infection and allow monitoring of the host response for the duration of infection as well as evaluation of efficacy of vaccination or treatment for modulation of disease. Calves are most susceptible to development of enteric disease between 3 and 8 weeks of age and the proportion of animals that develop diarrhea increases with infectious dose [43]. Most animals 3 to 8 weeks of age will develop diarrhea if given an oral dose of at least 10^{10} colony forming units (CFU). Calves younger than 3 weeks of age commonly develop bacteremia upon oral infection with non-typhoidal serotypes, making younger animals poor models for the study of enteric disease pathogenesis. The limitations of the calf oral infection model include obvious animal welfare concerns as the animals develop diarrheal disease, the relatively large cost of animal purchase and housing, and the relative inconsistency in disease development unless large infectious doses are delivered.

Surgical models of infection have proven useful to study *Salmonella* factors necessary for colonization of the intestine as well as the early response of the epithelium to *Salmonella*. The calf is particularly well suited for surgical models because bovine Peyer's patches are diffuse and extend the entire length of the ileum and aboral portion of the jejunum, making more than 1 meter of

intestine available to study colonization and epithelial response to infection. Both short- (up to 12 hours) and long-term (5 days) models have been described [45-47]. In the short-term ligated ileal loop model, 10^9 organisms are injected directly into the lumen of ligated intestinal segments in anesthetized calves (reviewed in [48-50]). Detailed kinetic studies have revealed STm invasion of the epithelial layer beginning within 5 minutes of infection and neutrophil influx beginning within 15 minutes of infection [45, 46]. By 8 to 12 hours post-infection, the mucosal surface is denuded and a pseudomembrane forms, similar to the pathology in natural infection [42]. The volume of fluid accumulation within the lumen is correlated with the neutrophilic inflammatory response (Figure 1.2 and reference [51]) reducing the necessity for histologic evaluation of each loop to determine host response. The host response has been cataloged for infection with both wild-type organisms and mutants lacking the TTSS-1 [52] laying the groundwork for further interrogation of the host response in this model.

Calf models have made great contributions to the knowledge of *Salmonella* pathogenesis. First, as a natural host for *Salmonella*, calves develop disease with similar clinical course and histologic abnormalities observed in people infected with NTS. Second, calf models allow hypothesis testing in an outbred population, with all of the potential genetic variation that exists in nature. Finally, the study of enteric disease in calves allows for development of interventions that may be useful for both cattle and humans to reduce the

Figure 1.2: Fluid accumulation in calf ligated ileal loops correlates with the inflammatory response. (A) *In situ* ileal loop 12 hours post-infection with 10^9 CFU virulent *Salmonella* Typhimurium filled with inflammatory fluid and (B) pseudomembrane covering the epithelial surface of ileal tissue. (C) *In situ* ileal loop infected with an avirulent STm mutant lacking the type-3 secretion system-1 (ΔSPI-1) with very little fluid accumulation and (D) normal appearance of the epithelial surface. (E) The volume of fluid that accumulates in loops infected with virulent WT STm is significantly greater than that of loops infected with an avirulent mutant (ΔSPI-1), those inoculated with sterile media alone, or uninoculated loops. * P < 0.05 using Student's t-test.



incidence of disease globally. The short-term ligated ileal loop model has the benefit of allowing for up to 35 experiments in a single animal, thereby reducing the intra-animal variation in disease response observed in an outbred population. Additionally, calves are anesthetized for the duration of the experiment and therefore do not experience the discomfort associated with enteric disease and development of endotoxemia. However, ligated ileal loops are considered isolated compartments, precluding the evaluation of the effects

of progressive motility of ingesta and microbes on *Salmonella* colonization. Additionally, this model requires highly trained investigators with surgical expertise, a surgical suite large enough to accommodate a 50-kg animal, and trained staff to monitor the animal during the anesthetic period. Calves are expensive as compared with conventional laboratory animals and require specialized housing prior to the experiment. Finally, as natural hosts of the organism, cows may shed *Salmonella* subclinically, acting as a potential source of infection of calves. Thus, it is essential to ensure the animal has not been exposed to *Salmonella* prior to the experiment. In spite of these shortcomings, the calf surgical model has undeniable power in the study of the early stages of intestinal colonization, a stage of infection that has been under-interrogated and has great potential for discovery.

Mechanisms of *Salmonella* colonization of the host

Huge advances have been made in recent decades to elucidate the virulence mechanisms of *Salmonella* using various animal models. Salmonellae gain entry into the host through ingestion and must rapidly respond to a wide variety of environmental conditions in the digestive tract: acidic pH in the stomach, bile salts within the small intestine, host-derived antimicrobial peptides, and high osmolarity of luminal fluid (reviewed in [53]). In addition, *Salmonella* must compete with the host and resident microflora for continuously changing nutrient sources. Once within the intestinal lumen, motility mediated through

peritrichous flagella, is critical for contact with the epithelium and virulence of the organism [54-57].

Anaerobic metabolism and gut colonization

The intestine is a specialized and highly diverse niche. Oxygen tensions in the lumen decline from the stomach to the colon [58, 59]. In addition, there is an increasing gradient of oxygen tension from the center of the lumen towards the epithelium [60]. *Salmonella* must replicate within these anaerobic conditions and begin expression of virulence programs including the type-3 secretion system-1 (TTSS-1). *Shigella flexneri*, a related enteric pathogen, uses the oxygen gradient within the intestinal lumen to induce the expression of a functional TTSS in order to invade the intestinal mucosa directly linking virulence to oxygen sensing [61].

As facultative anaerobes, *Salmonella* and *E. coli* share the core metabolic pathways for anaerobic lifestyle: nitrate and fumarate respiration and fermentation [62]. The transcriptional and protein profiles of anaerobically-grown *Salmonella* mutants deficient in *fnr* and *arcA*, the two known master regulators of anaerobic metabolism, have already been established [63-65]. One striking difference in the *fnr*-dependent transcriptome between *Salmonella* and *E. coli* is the *fnr*-dependent regulation of the *Salmonella* TTSS-1 and other virulence genes absent in *E. coli* [64, 66]. These important findings suggest that anaerobic gene expression has evolved since *Salmonella* diverged from *E. coli* and that

further mechanistic study of *Salmonella* in anaerobic conditions is essential to understand the behavior of this pathogen in the intestine.

Exploitation of the host response

Salmonella senses environmental cues such as osmolarity of luminal fluid [67, 68], composition and concentration of microbial-derived short chain fatty acids [69-71], oxygen tension [67, 72, 73], and likely many other signals to induce the expression of the TTSS-1 encoded within *Salmonella* pathogenicity island-1 (SPI-1). Through this system, protein effectors are injected directly into the host cell cytosol in order to induce uptake of the organism [47, 51, 74-79]. An alternate TTSS-1 independent mechanism of crossing the epithelial barrier is through dendritic cell luminal sampling [75, 80]. The key to successful infection is passage across the epithelial layer, a process that also requires the TTSS-2 [33, 47, 81] in order to access the lamina propria and induce a strong host inflammatory response.

Pro-inflammatory cytokines and chemokines are induced in response to the TTSS-1 during infection that result in neutrophil trafficking to the intestine (reviewed in [82]). Bovine neutrophils release a wide array of compounds including but not limited to reactive oxygen species (ROS; superoxide anion [O_2^-], hydrogen peroxide [H_2O_2], hypochlorous acid [HOCl], and chloramines), β-defensins, serine proteases, metalloproteinases (collagenase and gelatinase), cathelicidins, and calprotectin when activated [83, 84]. *Salmonella* encounters

neutrophil-derived antimicrobial products both within the lumen of the intestine and during transient residence within neutrophils [85]. However, it is relatively resistant to the toxic byproducts of neutrophils through a variety of mechanisms including resistance to ROS and antimicrobial peptides (reviewed in [86, 87]).

The host inflammatory response gives *Salmonella* a competitive advantage over resident microflora. Within the intestinal lumen, the product of the neutrophilic oxidative burst generates tetrathionate from oxidation of thiosulfate [88]. *Salmonella* uses tetrathionate as a terminal electron acceptor within the anaerobic conditions of the intestinal lumen to gain a competitive advantage over resident microflora. Effectors of the TTSS-1 may directly activate epithelial production of inducible nitric oxide synthase (iNOS) thereby creating nitrate, an additional terminal electron acceptor [76]. The relative importance of nitrate during infection is illustrated by the fact that it is a powerful chemoattractant for *Salmonella* during anaerobiosis [89]. In addition, *Salmonella* uses host-derived nutrients such as ethanolamine [90], microbial-liberated host-derived nutrients such as 1,2-propanediol [91], and is capable of scavenging transition metals zinc and iron [92-94] during intestinal inflammation. It is clear that NTS have developed numerous mechanisms to benefit from the host inflammatory response in order to gain a competitive advantage within the complex microbial niche of the intestinal tract.

Genetic screens to identify novel mechanisms to colonize animal hosts

Despite many eloquent studies describing virulence mechanisms of *Salmonella*, the number of cases of human enteric salmonellosis has remained unchanged for more than 15 years [95]. This fact as well as the emergence of MDR salmonellae drives the need for further studies to elucidate the mechanisms used by this bacterium to survive within different niches in the host. Genetic screens are invaluable resources used for decades to understand the biology of bacteria and other organisms. Invaluable information can be gleaned from well-designed genetic screens. For example, the regulation of the lactose and maltose operons were described as a result of meticulous, well-designed screens and the regulation of these operons have become paradigms for bacterial gene regulation upon which large volumes of data have been based (reviewed in [96]).

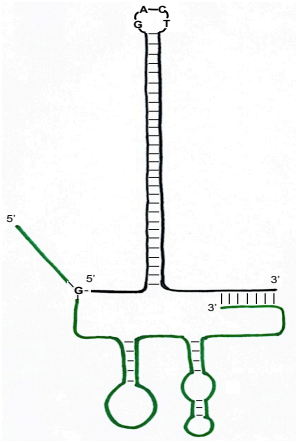
By far the majority of genetic screens have been completed in mouse models of infection, primarily using *Salmonella*-sensitive mice lacking the *NRAMP-1* gene [97-103]. This has resulted in a wealth of knowledge on *Salmonella* pathogenesis but the murine response to enteric infection with *Salmonella* is a poor representation of enteric salmonellosis in natural hosts. Recently genetic screens using animals that are natural hosts of the organism have been published [104-107]. These studies have served two major purposes. First, they have highlighted the obvious fact the genes necessary for systemic colonization differ from those for enteric colonization. Second, they show that

Salmonella has evolved numerous mechanisms for colonization of different hosts that are most likely related to the biology of the host organism. These studies illuminate the importance of studying disease in a natural host in order to interrogate the mechanisms needed by the pathogen to colonize the host organism. Using a calf model of infection, we show two novel mechanisms of colonization of the intestine: production of a hybrid RNA-DNA molecule called multicopy single-stranded DNA and phosphonate utilization.

Retron reverse transcriptases

The reverse transcriptase, an enzyme that transfers genetic information from RNA to DNA, was first described in 1970 by David Baltimore and Howard Temin in retroviruses [108, 109]. Bacterial reverse transcriptases were first identified in 1984 in *Myxococcus xanthus* [110]. Investigators consistently found a fast-migrating band of nucleic acid during isolation of genomic DNA that they named multicopy single-stranded DNA (msDNA). A gene encoding a reverse transcriptase neighbored the sequence of msDNA and msDNA was a covalently linked hybrid RNA-DNA molecule (Figure 1.3). The operon containing the sequence of msDNA and the reverse transcriptase was termed a 'retron.' Further work showed that a clinical isolate of *E. coli* produced msDNA [111] and since that time numerous species of eubacteria and archaea have been found to produce these molecules [112-128]. *Salmonella* Typhimurium encodes a reverse transcriptase and produces msDNA [112, 122, 129, 130].

Figure 1.3: Predicted secondary structure of msDNA St-85 from STm. RNA component is shown in green and DNA component in black. The RNA and DNA portions are covalently linked by a 2' 5' phosphodiester linkage at a guanine encoded by *msr*. Figure adapted from [112].



All retrons contain three essential components for the production of msDNA: *msr* (RNA primer), *msd* (template sequence for reverse transcription) and the reverse transcriptase [117]. Retrons of pathogens may also encode an additional open reading frame of unknown function [112]. There has been extensive study of the mechanism of msDNA production (reviewed in [117]). The operon is transcribed from a single promoter and a series of inverted repeats located both within the sequence of *msd* and located 5' to *msr* and 3' to *msd* allow the transcript to fold on itself. This temporary structure places a guanine residue of *msr* (present in all msDNAs) in contact with the sequence of *msd*. This guanine is the priming residue whereby the reverse transcriptase adds an

msd sequence-specific deoxyribonucleotide to the 2' hydroxyl group to create a 2' 5' phosphodiester bond between the guanine and incoming DNA [131]. It is an absolute requirement that the sequence of *msr* and the reverse transcriptase originate from the same retron [132]; however the possibility that additional mRNAs sharing the sequence of *msr* can be turned into cDNA has been suggested [133]. There are specific residues and structures within *msr* that are required for the production of msDNA [134]. For production of a mature msDNA, RNase H is required to remove the RNA encoded by *msd* [135] and it is possible to produce msDNA *in vitro* with purified *msr/msd* transcript, reverse transcriptase, and RNase HI [136]. It has been reported that some mature msDNA are missing the RNA-portion encoded by *msr* [112, 114, 121, 122], although the available data are not convincing in all cases. Despite the detailed information on the mechanism of msDNA production, the function of this molecule remains elusive.

The lack of an observed phenotype in mutants unable to make msDNA has been a major roadblock to elucidating the function of this unique molecule. Two hypotheses of function have been explored using artificial systems. One hypothesis was that msDNA with mismatched base pairs in the *msd*-encoded stem increases the rate of mutation of the host bacterium through sequestration of mismatch repair proteins [137-139]. The other hypothesis is that msDNA acts in a regulatory fashion through an unknown mechanism [140, 141]. In all published studies, msDNA from one organism or strain was highly over-

expressed in a heterologous organism or strain that lacks msDNA. Thus, it is not possible to determine whether the results indicate the natural function of the molecule or result from massive (>10,000 fold) over-expression of a foreign nucleic acid species. Therefore, the natural function of this molecule remains elusive, despite intensive study over a period of 30 years.

Phosphorus homeostasis within the microbial community

Salmonellae live within microbial communities during all stages of life, whether within the intestine of an animal host or within the soil or bodies of water. In order to replicate within these communities, salmonellae are capable of withstanding microbial-derived antimicrobial products and have strategies for gaining nutrients within these diverse environments. Numerous strategies such as host cell invasion, benefitting from the products of the host inflammatory response, and preferential utilization of nutrients have already been discussed.

One under-studied aspect of *Salmonella* metabolism in the host is phosphorus acquisition and utilization. Phosphate anion (PO_4^{2-}; Pi) is important for numerous cellular processes including signal transduction, energy production, gene regulation, and many more. The phosphorus content in the intestinal lumen varies with diet, location, and species [142]. The majority of intestinal phosphate is absorbed in the small intestine of monogastrics [143, 144] and ruminants [145], although the actual location along the small intestine differs between species. Hindgut fermenters also absorb phosphate in the small

intestine but also secrete phosphorus into the large colon, likely to serve as a buffer in this environment [146]. These data suggest that there are locations within the host infestinal tract where phosphorus will be in limited supply and that these locations will likely differ between host species.

In *E. coli*, phosphorus homeostasis is regulated by the two-component regulatory system PhoBR (reviewed in [147]), where PhoR is the sensor and PhoB the transcriptional regulator. During phosphorus starvation (<4 μM Pi), PhoR phosphorylates PhoB to induce expression of genes of the Pho regulon. The Pho regulon contributes to virulence mechanisms in both enterohemorrhagic *E. coli* [148] and *Salmonella* [149], although the relative importance of this regulon in the metabolism of *Salmonella* within the intestine of the host has not been explored.

One method some microorganisms employ to survive in the face of phosphorus limitation is to produce phosphonates, molecules characterized by stable carbon-phosphorus bonds. The relative importance of phosphonates has been best characterized in marine ecosystems where Pi is limiting and phosphonates contribute approximately 25% to the total organic phosphorus content of oceans [150]. Bacteria and some marine invertebrates produce phosphonates to store phosphorus [151, 152]. In addition to a metabolic utility of these molecules, some of these compounds have potent antimicrobial activity [153, 154] and may be produced by microbes for interspecies competition.

With only one known exception, the synthesis of all phosphonates begins with the same initial steps [154]. The carbon-phosphorus bond is produced from phosphoenolpyruvate by the action of the coupled enzymes phosphoenolpyruvate phosphomutase (PepM) and phosphonopyruvate decarboxylase (Ppd) to generate phosphonoacetaldehyde, which is converted to 2-aminoethylphosphonate (2-AEP) by AEP transaminase [151, 154]. 2-AEP was the first phosphonate to be identified and was found in the sheep rumen [155]. The concentration of 2-AEP in ruminal and duodenal contents was higher in sheep with undisturbed microflora as compared with defaunated sheep [156]. 2-AEP has been identified in feed as well as various tissues from mammals [156-158]. Mammals lack genes necessary for biosynthesis of 2-AEP so it has been hypothesized that they utilize phosphonates from dietary sources [154]. The genes for biosynthesis and utilization of various phosphonates have been identified in many phyla of eubacteria and archaea, including γ-proteobacteria, isolated from diverse sources including animal sources [151, 152, 159].

Although *Salmonella* lacks genes for the biosynthesis of phosphonates, it has the capability to utilize these compounds and at least one of the loci (for 2-AEP utilization) is under control of the Pho regulon [160, 161]. Phosphonates are present within the intestine and in other locations within animal hosts and *Salmonella* may encounter these molecules during infection. However, no studies to date have elucidated the role of phosphonates in the virulence strategies of *Salmonella* or its metabolic profile during infection of a host.

Conclusion

Approximately half of the genome of *Salmonella* Typhimurium is of putative function or unknown function [129]. This organism undoubtedly possesses strategies for colonization of the intestine that have yet to be described. The following chapters will describe my work to identify new genes needed for *Salmonella* Typhimurium to colonize the intestine. In chapter 2, a genetic screen performed using the calf ligated ileal loop model is described. In chapter 3, a study to identify the function of multicopy single-stranded DNA is described. Finally, in chapter 4, the relationship of a transcriptional regulator needed for colonization of the intestine with phosphonate metabolism is described. Each of these newly described strategies for intestinal colonization were identified during infection of a natural host and each represent new targets for development of vaccine or antimicrobial strategies, critically needed to combat the global *Salmonella* burden.

CHAPTER II

NOVEL DETERMINANTS OF INTESTINAL COLONIZATION OF *SALMONELLA ENTERICA* SEROTYPE TYPHIMURIUM IDENTIFIED IN BOVINE ENTERIC INFECTION[*]

Overview

Cattle are naturally infected with *Salmonella enterica* serotype Typhimurium and exhibit pathologic features of enteric salmonellosis that closely resemble those in humans. Cattle are the most relevant model of gastrointestinal disease resulting from non-typhoidal *Salmonella* infection in an animal with an intact microbiota. We utilized this model to screen a library of targeted single deletion mutants to identify novel genes of *Salmonella* Typhimurium required for survival during enteric infection. Fifty-four candidate mutants were strongly selected, including numerous genes known to be important for gastrointestinal survival of salmonellae. Three mutants with previously unproven phenotypes in gastrointestinal infection were tested in bovine ligated ileal loops. Two of these mutants, in *STM3602* and *STM3846,* recapitulated the phenotype observed in

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[*] Reprinted with permission from "Novel determinants of intestinal colonization of Salmonella enterica serotype Typhimurium identified in bovine enteric infection" by Johanna R. Elfenbein, Tiana Endicott-Yazdani, Steffen Porwollik, Lydia M. Bogomolnaya, Pui Cheng, Jinbai Guo, Yi Zheng, Hee-Jeong Yang, Marissa Talamantes, Christine Shields, Aimee Maple, Yury Ragoza, Kimberly DeAtley, Tyler Tatsch, Ping Cui, Katharine D. Andrews, Michael McClelland, Sara D. Lawhon, and Helene Andrews-Polymenis, 2013. *Infection and Immunity,* Volume 81, Pages 4311-20,

the mutant pool. Complementation experiments successfully reversed the observed phenotypes, directly linking these genes to the colonization defects of the corresponding mutants. *STM3602* is a putative transcriptional regulator that may be involved in phosphonate utilization and *STM3846* is a retron reverse transcriptase that produces a unique RNA-DNA hybrid molecule called multicopy single-stranded DNA. The genes identified in this study represent an exciting new class of virulence determinants for further mechanistic study to elucidate the strategies employed by *Salmonella* to survive within the small intestine of cattle.

Introduction

Non-typhoidal salmonellae (NTS) are the leading cause of bacterial foodborne gastroenteritis in people worldwide [1, 2], and are responsible for hundreds of millions of cases of gastroenteritis and bacteremia annually [5]. In humans, gastrointestinal disease caused by NTS is characterized by neutrophilic infiltrates within the ileum and symptoms of inflammatory diarrhea [6].

Cattle are naturally susceptible to infection with NTS and develop inflammatory diarrhea histologically characterized by neutrophilic inflammation [46, 48]. Cattle either clear the organism after resolution of disease or become persistently infected and continually shed *Salmonella* in the feces [162]. Approximately 30% of human cases of enteric salmonellosis originate from bovine sources [24]. Therefore, knowledge of factors important for survival of

Salmonella within the gastrointestinal tract of cattle allows not only extrapolation to human disease, but also the opportunity for creation of new strategies to reduce bovine colonization and thus reduce the contamination to the food supply and environment. Additionally, use of calves as a model organism makes discoveries in this model directly applicable to farm animal populations.

Although there are many tractable animal models of salmonellosis, the majority of screening and the development of mechanistic understanding of NTS infection have historically been done in small animal models that do not naturally develop inflammatory diarrhea upon infection with NTS. These models include mice of the BALB/c, C57B6, 129SvJ, and CBA/J lineages [49, 98, 103]. To more closely resemble human disease, mice can be treated with antimicrobials to eliminate natural microflora prior to infection with *Salmonella*. These pre-treated animals do develop neutrophilic inflammation (commonly known as the murine colitis model) [28, 30, 31, 38]. The murine colitis model is attractive because it requires minimal technical expertise and allows study of host factors involved in *Salmonella* pathogenesis through use of widely available immunological reagents and genetically altered mice. However, the lack of an intact microbiota precludes full evaluation of the strategies used by *Salmonella* to survive in the complex microbial ecosystem of the gastrointestinal tract. Thus the use of an animal that is a natural host of *Salmonella* is optimal to understand the biology of *Salmonella* during infection.

The current animal model with intact microbiota that most closely resembles gastrointestinal salmonellosis in people in both clinical presentation and histopathology is the calf model of infection [45, 48, 79]. This model, although expensive and complex to use, has become very useful for identification of bacterial factors necessary for NTS to thrive in the complex environment of the gastrointestinal tract [51, 57, 88, 163]. Bovine ligated ileal loops have been used to elucidate the absolute requirement of the type III secretion system (TTSS) and effectors encoded by *Salmonella* pathogenicity island-1 (SPI-1) for development of neutrophilic enteritis [46, 51, 52, 79]. They have also been used to study the importance of flagella for virulence [57], and to understand the mechanism by which *Salmonella* employs the host inflammatory response to gain a survival advantage by the use of tetrathionate as a terminal electron acceptor [88]. An additional benefit of the calf model is that it reliably replicates enteric salmonellosis in cattle, a population that contributes to the maintenance of *Salmonella* in the food supply and environment, allowing for development of novel pre-harvest interventions for this important zoonotic pathogen [44, 48-50, 164]. However, because of its complexity this model has not previously been used in an unbiased approach to study novel virulence factors.

Ligated ileal loops in calves provide a unique environment for the study of *Salmonella* pathogenesis where virulence factors necessary for establishing early infection may be identified in the presence of intact microbiota. We

previously constructed a library of targeted single gene deletion (SGD) mutants of *Salmonella* Typhimurium that we used to discover novel genes required for survival during systemic infection in BALB/c mice [97]. In the work described here, we used this library of targeted single gene deletions in combination with the calf ligated ileal loop model to identify novel genes used by *Salmonella* during enteric infection of a natural host. Using this strategy, we identified 54 mutants under selection. Over 20 of these genes have not previously been described as under selection in this model. We tested three mutants (in *STM3602, STM3846*, and *STM4602*) and confirmed two in individual competitive infections, in addition to testing and confirming Δ*phoP* and Δ*phoQ* mutants (for a total of 5 mutants tested). Complementation *in trans* restored the ability of mutants in *STM3602* and *STM3846* to colonize ligated ileal loops. The genes we reveal here to have roles in colonization represent an exciting group for further study to elucidate the mechanisms that *Salmonella* use to survive within and cause disease in the complex environment of the small intestine of cattle.

Methods

Ethics statement

The Texas A&M University Institutional Animal Care and Use Committee approved all animal experiments, and all experiments were performed in accordance with the PHS "Guide for the Care and Use of Laboratory Animals",

and USDA Animal Welfare Regulations. Texas A&M has AALAC accredited animal facilities.

Bacterial strains

All bacterial strains are isogenic derivatives of virulent *Salmonella enterica* serotype Typhimurium ATCC14028. The SGD mutant library was constructed as previously described [97]. All bacteria were grown in Luria Bertani (LB) broth or LB agar supplemented with kanamycin (50 mg/L), nalidixic acid (50 mg/L), carbenicillin (100 mg/L), or streptomycin (100 mg/L) where appropriate.

Construction of complementing plasmids

PCR products were generated by colony PCR using *Pfu* polymerase (Agilent Technologies). To obtain a 1.1 kb PCR product for *STM3602* we used an annealing temperature of 45˚C for 5 cycles, 58˚C for a further 25 cycles, and the following primers: 3602forward 5' GTTGAATTCTTCCGCCTCGATCATTTC 3' and 3602reverse 5' GTCAAGCTTTCATACGGTAAACCGTATTTTATC 3'. To clone *STM3846*, a PCR reaction using an annealing temperature of 45˚C for 5 cycles, and 57˚C for a further 25 cycles, generated a 1.7 kb product using the following primers: 3846forward 5' GTCGAATTCAAGTCTCATCCTCTGTTGTAATCTATC 3' and 3846reverse 5' GTCAAGCTTTATCTACAGCGTTCTGTCTGC 3'. The appropriate length of the

PCR products was ensured by agarose gel electrophoresis. A poly-A tail was added to the 3' end of the product using Taq polymerase (New England Biolabs) for 9 minutes at 72˚C. PCR products were then ligated into pCR2.1 (TOPO TA cloning; Invitrogen) and transformed into chemically competent One Shot *E. coli* (Invitrogen) using heat shock, following manufacturer's instructions. Plasmids were isolated using the Qiagen Miniprep kit (Qiagen), and the insert was removed by digestion with *Eco*RI (New England Biolabs). The insert was isolated from the plasmid backbone by agarose gel electrophoresis and gel purified using a Qiaquick gel purification kit (Qiagen). The insert was then ligated into *Eco*RI digested and gel purified pWSK29 [165]. Ligations were performed overnight at 14^{o}C, using T4 DNA ligase (New England Biolabs). Ligation reactions were transformed into chemically competent *E. coli* XL-1 blue (p*STM3846*) or Mach One *E. coli* (p*STM3602*, Invitrogen). Transformants were obtained by selection on LB agar supplemented with carbenicillin, and were streaked twice to single colonies. Plasmids were isolated using the Qiagen miniprep kit (Qiagen), and correct inserts were verified by restriction digestion of plasmids using *Bam*HI or *Bst*XI (New England Biolabs; p*STM3846* and p*STM3602*, respectively). The desired sequence was confirmed by sequencing. Complementing plasmids were transformed into chemically competent *S*. Typhimurium LB5000 (restriction -, modification +) [166], and transformants were obtained by selection on LB with carbenicillin. Plasmids were then isolated as above and transformed into Δ*STM3846* and Δ*STM3602* mutants using heat

shock or electroporation, respectively. Mutants bearing complementing plasmids were purified by streaking twice for single colonies prior to use in competitive infection experiments.

Calves and ligated ileal loop surgery

Angus-cross calves were obtained from a breeding herd at the Veterinary Medical Park at Texas A&M University. A total of 12 calves were used in this study; three for screening the mutant library and 9 for competitive infection and complementation analysis. Calves were separated from the dam at 1 day of age and adequate passive transfer estimated by measurement of serum total protein. Calves were housed in an AALAC approved barn, fed milk replacer twice daily and were provided with free choice water and grass hay. Selective fecal cultures were performed at least once weekly to ensure calves remained negative for *Salmonella spp*. [46, 167].

At 3-6 weeks of age, calves were anesthetized for ligated ileal loop surgery as previously described [45, 46]. Briefly, calves were placed in left lateral recumbency and a right flank incision was made. Twenty-four to thirty eight 4-6 cm loops were tied within the ileum within grossly visible Peyer's patches, leaving 1 cm spacers between adjacent loops. Loops were infected individually with 3 mL of LB containing approximately 10^9 *Salmonella* Typhimurium. The intestine was returned to the abdomen, the incision closed, and the calves were monitored under inhalant anesthesia for the duration of the

experiment. At 12 hours post-infection the incision was opened and each loop individually excised. Calves were euthanized by barbiturate overdose (pentobarbital) administered intravenously.

SGD pool preparation, inoculation and recovery from ligated ileal loops

The pool of ~1000 SGD mutants prepared and described previously [97], was grown overnight at 37˚C with agitation in LB supplemented with kanamycin. Overnight cultures were subcultured 1:100 into LB with kanamycin and incubated for 3 hours at 37˚C with agitation. Cultures were washed twice in sterile LB broth, and the concentration of organisms was adjusted to 10^9 CFU in 3 mL LB. A wild type strain marked with streptomycin resistance in a neutral location, HA697 (Δ*phoN::strep*) [168], was added at a ratio of 1:500 (HA697:total inoculum) to measure random loss of mutants in the pool. Eight ligated ileal loops were inoculated with the SGD library in 3 total calves. Inoculum titers were determined by serial dilution and plating. Following excision of the infected loops, intestinal fluid, mucus, and tissue were harvested and processed separately. Fluid volume, which is correlated with inflammatory response [51], was calculated by weighing each loop before and after removal of fluid. Data from only those loops with similar fluid accumulation to a WT-infected control loop were used. Mucus was gently scraped from the epithelial surface and diluted in 3 mL PBS. The remaining tissue was diluted in 5 mL PBS. These specimens were subsequently homogenized and were serially diluted in PBS

and plated for enumeration of CFU. The remaining homogenates were grown to stationary phase in LB supplemented with kanamycin and washed in PBS prior to extraction of total DNA.

Mutants with an observed phenotype during the library screen were selected for competitive infection experiments against the wild type (WT), HA420 (ATCC 14028 Nal^R). Mutations were moved into a clean genetic background using P22 transduction [169]. Mutant strains and the isogenic WT were grown in LB with kanamycin and nalidixic acid (mutant) or with nalidixic acid alone (WT) as described above. The inoculum was prepared by mixing SGD mutant and WT at a 1:1 ratio. Ligated ileal loops from 3 to 8 calves were infected with the prepared inoculum and the WT to mutant ratio of the inoculum was determined by serial dilutions and plating. Intestinal fluid, mucus, and tissue were processed as described above and the ratio of WT to mutant determined by differential plating. Competitive index (CI) was determined by dividing the output ratio of WT to mutant by the inoculum ratio.

Microarray analysis

The protocol used to prepare transcripts from input and output pools for microarray analysis was essentially as previously described [97]. Briefly, total DNA of input or output mutant pools was sonicated, poly-A tailed, and PCR amplified with a primer targeting the shared portion of each mutant and a primer including oligo dT at the 3' end [97]. PCR products were subjected to reverse

transcription from a T7 RNA polymerase promoter located inside each mutant and a mixture of NTPs that included a fluorescently labeled UTP. The RNA was purified using the RNeasy Mini Kit (Qiagen), and approximately 4 micrograms of labeled RNA was hybridized to a NimbleGen tiling array of 387,000 50-mer oligonucleotides at 42°C for 16 hours. The 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. Data was uploaded into WebArrayDB [170-172] and data analyzed for peak height in the DNA directly downstream of each mutant location. The relative signal of each mutant was compared to the relative signal in a corresponding array of the same library prior to selection. All large changes in mutant representation were manually inspected and converted into a numerical score between -1 (strongly under-represented in the output pool) and 1 (over-represented in the output pool).

Data analysis

The SGD library was screened in a total of 8 loops: four in one calf, and two loops each in two further calves. A mean score for mutants in each calf was determined by calculating the mean score from multiple loops. The overall score for each mutant was the mean of data from all loops in the 3 calves. Mutants that were not represented in the input pool in all of the loops were excluded from further analysis. The inter-calf variation was defined as the absolute value of the

standard deviation of the mean scores from each calf. Mutants under selection in our screen of the SGD library were defined as those mutants with scores outside the 90% confidence interval of the mean scores and with an inter-calf variation less than the calculated mean score.

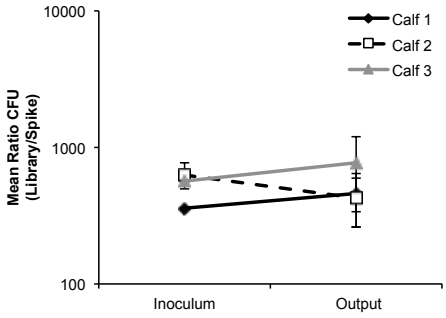
For competitive infection experiments, the competitive index was defined as the ratio of WT to mutant in output normalized to the input ratio. Statistical significance was determined using Student's two-tailed t-test with significance set at $p < 0.05$.

Results

Screen for mutants under selection during enteritis in calves

In order to assess the fluctuation of mutant representation in the pool, we added a derivative of the wild type marked with streptomycin resistance cassette, HA697 (*ΔphoN*::Strep^R), in a neutral location to the input pool [168]. In the input pool, HA697 was present in a ratio relative to the total inoculum that approximated the representation of each individual mutant in the pool. By enumerating the representation of this mutant in the output pool relative to the total recovery of the pool, we observed less than 1.4 fold fluctuation of HA697 in intestinal tissue samples as compared to the input pool (Figure 2.1). As HA697 was inoculated into and recovered from loops in approximately the same proportion relative to the total pool, there appears to be only minimal random loss of mutants occurring during the incubation of our pool in ligated ileal loops.

Figure 2.1: Mutant representation in the pool remains stable. The 'input' pool of kanamycin-marked mutants was spiked with HA697 (Δ*phoN::strep*) at a ratio of 1:500 (HA697:total pool). Comparison of the representation of this mutant in the inoculum to the representation of this mutant in the output pools indicated less than 1.4 fold change in HA697 representation over the duration of the incubation of the pool in ligated ileal loops.



In order to identify candidate mutants under selection in ligated ileal loops, both the input pool and the output pool were used to prepare labeled transcripts unique to each mutant. Representation of mutants in the input versus the output pools was performed by analysis of labeled transcripts using a Nimblegen tiling array. The resulting data are presented by genome position of each deleted gene represented in the SGD library in Figure 2.2. We identified 54 mutants under selection in our screen with a mean score outside a 90% confidence interval and with an inter-calf variation smaller than the mean (Table 2.1). We chose to exclude mutants with high inter-calf variation because we found numerous mutants with a strong phenotype in only a single calf. These

mutants had a mean score outside the 90% confidence interval but are considered outliers and not reported here.

Figure 2.2: Loss of targeted mutants from the pool after inoculation into ligated ileal loops shown by genomic position. 8 ligated ileal loops from 3 different calves were infected, collected and analyzed. Scores from multiple loops in a single calf were averaged and then an overall mean score was generated from all 3 animals. The X-axis represents mutants by genomic position with the mean score plotted on the Y-axis. The horizontal dotted lines indicate the 90% confidence interval. Mutants under selection were outside the 90% confidence interval with an inter-calf standard deviation less than the absolute value of the given score. Labeled mutants are in known virulence factors and those that were confirmed by individual competitive infection are in black.

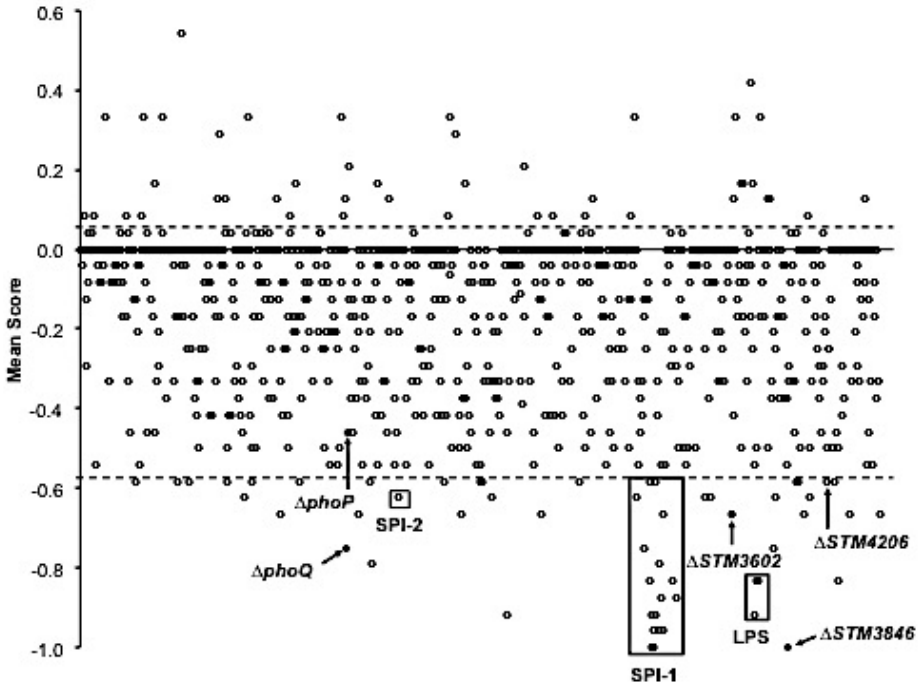


Table 2.1: Mutants under selection, grouped by pathogenicity island or clusters of orthologous groups (COG). Asterisk (*) indicates the mutant was under selection during library screen in systemic infection of BALB/c mice [97] and pound ([#]) [105] or dollar sign ([$]) [104] indicates the mutant was identified during screen of a transposon library in oral infection of a single calf.

Gene number	Name
***Salmonella* pathogenicity island-1**	
*STM2867**	*hilC*
STM2875[#$]	*hilD*
STM2883[#$]	*sipD*
STM2884[#$]	*sipC*
STM2885[$]	*sipB*
*STM2886**[#$]	*sicA*
STM2887[*$]	*spaS*
*STM2888**[#$]	*spaR*
STM2889[$]	*spaQ*
STM2892[$]	*invJ*
*STM2893**[$]	*invI*
STM2896[$]	*invA*
STM2897[#$]	*invE*
STM2898	*invG*
***Salmonella* pathogenicity island-2**	
*STM1441**[#]	*ssaK*
***Salmonella* pathogenicity island-4**	
STM4261[#$]	
***Salmonella* pathogenicity island-6**	
STM0296	
Cell envelope biogenesis	
*STM0719**	
STM1737	*tonB*
*STM3719**[#$]	*rfaB*
*STM3722**[#$]	*rfaG*
*STM3723**[$]	*rfaQ*
Cell motility and secretion	
STM3975[*$]	*tatC*
DNA replication, recombination, repair	
*STM3846**	*rrtT*
Post-translational modification	
STM3342[$]	*sspA*
STM4067	

Gene number	Name
Metabolism	
STM0522[$]	*allP*
STM1636	
STM2437	
STM3781	
Signal transduction	
STM0398	*phoR*
*STM1230**[$]	*phoQ*
*STM1947**	*sirA* or *uvrY*
*STM2958**[$]	*barA*
Transcriptional regulation	
STM0031	
*STM0552**[$]	*fimW*
STM1588[$]	*yncC*
STM3245[$]	*tdcA*
STM3602[#]	
*STM4417**[$]	*iolR*
Unknown	
STM0278	
STM0285[$]	
*STM1258**	
STM1329	
STM1331	
STM1785	
STM1861	
STM2209	
STM3026	
STM3954	*yigG*
*STM4030**	
STM4206[$]	
STM4302	
STM4596[$]	

Among the mutants with reduced fitness, fourteen mutants under selection were in genes located in *Salmonella* pathogenicity island-1 (SPI-1), and three were in genes needed for LPS biosynthesis, all known to be important virulence determinants of *Salmonella* during enteric infection [47, 51, 77, 173-175]. Numerous genes previously identified as virulence factors in animal hosts were also identified in our screen: *ssaK*, a gene within SPI-2 encoding a portion of the TTSS apparatus [75, 81]; *phoQ*, the sensor in the two-component regulatory system *phoPQ* responsible for regulation of virulence genes [176, 177]; *barA* and *sirA*, the sensor and regulator in a two-component regulatory system that regulates SPI-1 [69, 178, 179]; *tatC*, a sec-independent transport protein responsible for resistance to bile salts [180]; and *tonB*, a transport protein necessary for iron acquisition in the intestine [181].

We have already used this library to screen for mutants under selection during systemic infection of BALB/c mice [97]. We found nineteen mutants to be under selection in both models (Table 2.1). Not surprisingly, of the genes not previously implicated to be important in enteric disease, only seven mutants were under selection in both models. These results confirm the necessity of different genes of *Salmonella* for survival in different niches during infection and show that our library is useful for identification of new virulence factors in different animal models.

Thirty-one mutants under selection were not previously proven to be essential for colonization of the bovine host. Of these genes, six are transcriptional regulators, four are involved in metabolism, two each in protein modification and cell envelope biogenesis, and one each in DNA modification and cell motility and secretion. Sixteen of our new mutants under selection have unknown function or are not assigned a group based on clusters of orthologous group assignments [182, 183]. Ten of these genes had predicted phenotypes in screening of a library of transposon mutants during oral infection of a single calf, but no further characterization was performed to validate the results of either screen [104, 105].

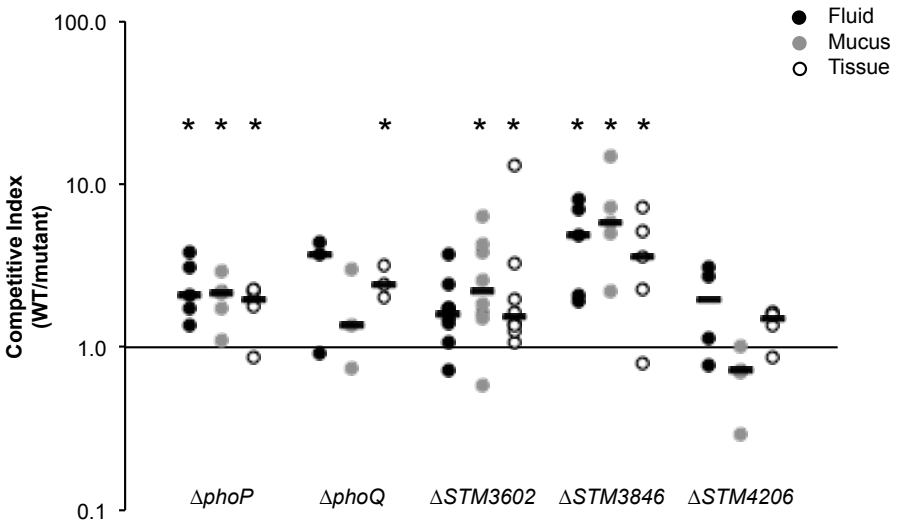
Confirmation of fitness defects of candidate mutants in the calf

We chose four mutants for confirmation by individual competitive infections with a derivative of the isogenic parental wild type ATCC14028. These mutants, Δ*phoQ*, Δ*STM3602*, Δ*STM3846* and Δ*STM4206,* were transduced to a clean genetic background by P22 transduction, and tested in competitive infections in ligated ileal loops in at least 3 animals.

A Δ*phoP* mutant, although it was just outside the stringent cut-off of our screen, was also studied in competitive infection experiments because of its known function in resistance against host-derived antimicrobial peptides. We determined that the Δ*phoP* mutant has a statistically significant survival disadvantage relative to the wild type organism in ligated ileal loops in calves, as

was expected (Figure 2.3). We also confirmed that the Δ*phoQ* mutant, a candidate mutant from our screen, has statistically significant survival defects in this model (Figure 2.3).

Figure 2.3: Individual competitive infection experiments confirm requirement for three genes during infection of ligated ileal loops. Ligated ileal loops were infected with a 1:1 mixture of WT and an isogenic mutant, either Δ*phoP* (n=5), Δ*phoQ* (HA1501; n=3), Δ*STM3602* (HA1473; n=8), Δ*STM3846* (HA1444; n=5), or Δ*STM4206* (HA1482; n=4), for 12 hours. Infected loops were harvested and the fluid contents, mucus layer, and intestinal tissue were processed for enumeration of bacteria and determination of competitive index. Solid bars indicate the median. (*) Asterisks indicate statistically significant differences between the ratio of the mutant and wild type in the inoculum versus in that ratio in the material collected at the termination of the infection ('output' ratio). Significant difference ($p<0.05$) in the output to input ratio was determined using a two-tailed Student t-test.



Using competitive infections we confirmed that deletion of *STM3602* and *STM3846,* genes not previously linked to virulence during enteritis, colonize poorly during competitive infections in ligated ileal loops (Figure 2.3). It was surprising to us that the Δ*STM3846* mutant is more severely affected in the calf intestine than the mutants in *phoP* and *phoQ,* genes with a previously defined role in pathogenesis that only have very modest phenotypes in the calf model (Figure 2.3).

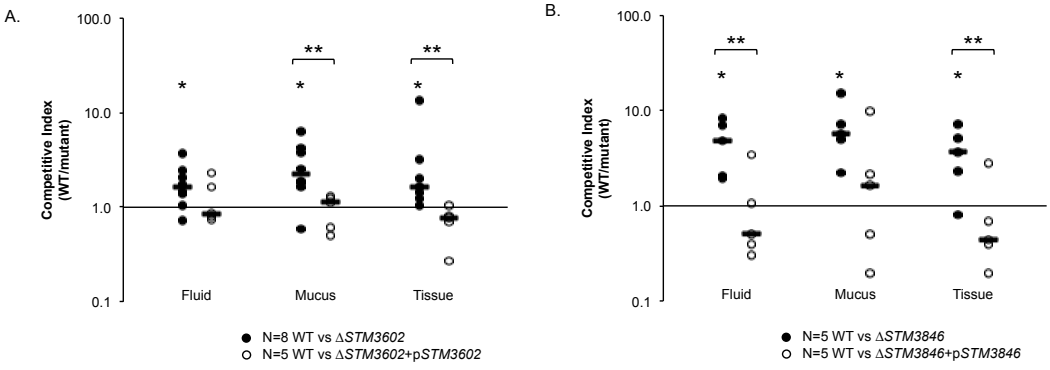
We also attempted to confirm the phenotype of a deletion in *STM4206* during competitive infection in 4 calves. Although we were unable to confirm the phenotype of this mutant, the calf model has high variability between loops and between genetically non-identical animals. Thus we cannot exclude the possibility that the predicted phenotype of the Δ*STM4206* mutant could be confirmed if competitive infection experiments are performed in additional animals. To summarize, we were able to confirm the phenotypes of three (*STM3602, STM3846*, and *phoQ*) of four candidate mutants that met the stringent inclusion criteria of our screen, in ligated ileal loops in calves.

Complementation analysis

In order to link the observed phenotypes to disrupted genes definitively, we chose to complement the two mutants with confirmed phenotypes *in trans,* and re-test these complemented mutants during infection of ligated ileal loops in calves. We cloned *STM3602*, a putative transcriptional regulator, and

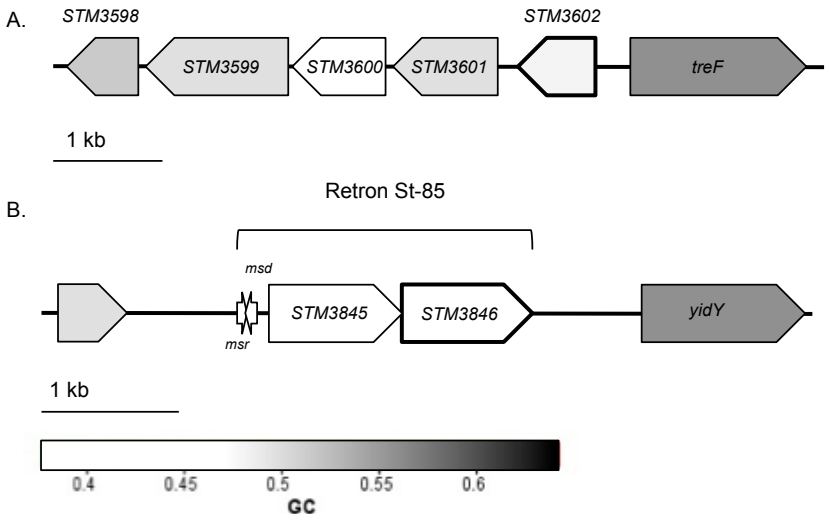
STM3846, a putative reverse transcriptase, onto a stable, low copy number plasmid and transformed these constructs into the corresponding deletion mutants. Complemented deletion mutants were tested in competitive infections with the isogenic wild type organism in ligated ileal loops. In both cases, the fitness defect of the deletion mutant was reversed by placement of the corresponding gene *in trans* (Figure 2.4).

Figure 2.4: Complementation *in trans* reverses the observed phenotypes of mutants in Δ*STM3602* and Δ*STM3846*. Ligated ileal loops from 5 calves were inoculated with the WT and the targeted deletion mutant complemented with an intact copy of the corresponding open reading frame *in trans* (open symbols). Data from the competitive infection experiments between WT and targeted deletion mutant (closed symbols), and statistical significance (*Figure 2.3) allow direct comparison to complementation data. (A) Competitive index for wild type versus the complemented Δ*STM3602* mutant. (B) Competitive index for wild type versus the complemented Δ*STM3846* mutant. In all panels, solid bars indicate the median. (**) Double asterisks indicate statistically significant differences between the ratio of the mutant and wild type in the inoculum versus in that ratio in the material collected at the termination of the infection ('output' ratio); ($p<0.05$) between groups as determined by a two-tailed Student t-test.



The genomic contexts of *STM3602* and *STM3846* are shown in Figure 2.5 [129, 184]. *STM3602* is located between *STM3601*, a putative phosphosugar isomerase, and *treF*, a trehalase. *STM3602* encodes a putative GntR family regulator and shares a conserved domain with *phnF*, a regulator of phosphonate utilization [161]. *STM3846* encodes a reverse transcriptase that catalyzes the formation of an RNA-DNA hybrid molecule called multi-copy single-stranded DNA, or msDNA [112, 116, 185].

Figure 2.5: Schematic representation of the genomic regions containing *STM3602* and *STM3846* - retron St-85. (A) Schematic diagram of the region surrounding *STM3602*. (B) Schematic diagram of the region surrounding *STM3846*. This gene is located on a 2.13 kb retron at position 4,051,144 on the chromosome between *STM3844*, a pseudogene encoding an integrase with a truncation at amino acid residue 164, and *yidY*, a putative multidrug efflux system protein. The orientations of *msr* and *msd* are indicated with arrows. Figure adapted from Colibase [184].



Discussion

We used a highly relevant model of enteric salmonellosis, bovine ligated ileal loops, to identify mutants under selection from our library of targeted deletion mutants in *Salmonella* Typhimurium [97]. Our work is the first example of a screen of a mutant library in ligated ileal loops in calves, a technically challenging model that is highly relevant to human enteric salmonellosis. In addition, this work is the first to confirm predicted phenotypes in the bovine model.

The ligated ileal loop model is ideal for screening of a library of mutants to identify the early strategies utilized by *Salmonella* to survive within the small intestinal environment. Using this model, the 'input' pool is administered directly into the ileum reducing random loss of mutants traveling through the upper gastrointestinal tract, and ensuring that all mutants arrive at the small intestine at the same time. The short duration of infection allows reliable invasion of the epithelium and development of a robust host neutrophilic inflammatory response [45, 46]. Thus, we are able study the early factors responsible for survival of *Salmonella* within the lumen of the intestine and those factors necessary for creation of and survival during the host inflammatory response. In addition, this model provides an opportunity to dissect the genetic strategies required for survival in the different microenvironments within the small intestine – intestinal fluid, mucus, and tissue layers.

In this study we evaluated the representation of mutants in the output pool isolated from intestinal tissue of 3 separate calves because this site might include all mutants under selection in the intestinal lumen and mucus layers in addition to those mutants defective for invasion or survival within epithelial cells. We show that mutants in our pool experience a very low level of random fluctuation during infection of loops, removing a significant barrier to screening in this model. We screened our mutant library in eight loops in three calves, the first screen in any bovine model to use multiple calves to identify candidate mutants under selection. We repeated our screen in numerous animals because each of our outbred animals may respond to infection differently thus placing different selection pressures on our library. To develop a list of candidate mutants for further study that had the strongest phenotypes and the highest probability of being true positives we used stringent criteria to define a mutant as under selection, and this may have excluded some candidate mutants with relevant phenotypes from further analysis. One example of a mutant we know to be under selection in competitive infection that did not meet our defining criteria for significance in our library screen is Δ*phoP*.

Only two other screens have been performed in calves [104, 105]. In both cases, randomly generated transposon libraries were screened after oral infection, and phenotypes were assigned to mutants from infection of a single calf. No further characterization of mutant phenotypes was performed in the bovine model. Despite the excellent genome coverage obtained by transposon

mutagenesis, such studies provide only a list of candidate genes needed in the bovine host. They lack an estimated true positive rate of discovery making it difficult to determine how many of the candidate genes one would expect to have a relevant biological effect in the bovine host. This drawback is a critical roadblock to the design of future studies evaluating the importance of candidate *Salmonella* genes in the bovine host, a model that requires specialized housing, technical expertise, and great expense as compared with conventional small animal models of disease.

Fourteen of the fifty-four genes under strong selection in our screen were located within SPI-1, and a single gene was located within SPI-2. Among these genes were regulators of expression of SPI-1 (*hilC* and *hilD*) and proteins encoding portions of the TTSS-1 apparatus (*sipD, sipC, sipB, sicA, spaS, spaR, spaQ, invJ, invI, invA, invE,* and *invG*) *[186].* The requirement of the TTSS-1 and associated effectors for invasion of epithelial cells and creation of a host neutrophilic inflammatory response has been previously described using bovine models of enteric disease [47, 51, 77, 173] and has been replicated in the murine colitis model [30, 74]. We also predict a phenotype for a single gene (*ssaK*) encoding a portion of the TTSS-2 apparatus. The TTSS-2 and associated effectors are necessary for virulence during systemic disease [187] and for induction of an inflammatory response in the intestine [47, 188]. However, it is possible that 12 hours infection was not long enough to show a more pronounced phenotype for the remainder of the SPI-2 genes. These data show

that our screen appropriately identifies virulence factors known to be important in both bovine and murine models of enteric salmonellosis.

Both *sirA* and *barA* mutants have predicted phenotypes in the calf model. These genes comprise a two-component regulatory system that senses short-chain fatty acids within the intestine, causing activation of invasion gene expression via *hilA*, the master regulator of SPI-1 [69, 179, 189]. Mutants in each of these genes have reduced virulence during oral infection of BALB/c mice [69], but have not previously been proven to have a role during enteric infection in the bovine host.

In order to survive within the gastrointestinal tract, bacteria have mechanisms to resist antimicrobial peptides produced by the host. Within the small intestine, numerous antimicrobial peptides are constitutively produced by Paneth cells and are concentrated in the mucus covering the mucosa [190-192]. One response of the mucosa to pro-inflammatory cytokines released as a result of *Salmonella* infection is to increase the production of defensins [193]. Polymorphonuclear cells also contain numerous classes of antimicrobial peptides within cytoplasmic granules [194, 195]. PhoP and PhoQ comprise a two-component regulatory system that responds to antimicrobial peptides to regulate genes for LPS biosynthesis and virulence [176, 196-199].

Therefore, we tested the phenotype of Δ*phoQ* and Δ*phoP* mutants in competitive infection, even though the latter gene did not meet the stringent cut off of our screen. We confirmed the predicted phenotype of our Δ*phoQ* mutant in

intestinal tissue and found that a Δ*phoP* mutant also has a phenotype in bovine ligated ileal loops (Figure 2.3). The Δ*phoQ* mutant was tested in competitive infection in only 3 calves and the lack of an observed phenotype in intestinal mucus, the location with the greatest concentration of antimicrobial peptides, may be due to small number of calves used in the study. The phenotypes we observed for each of these mutants in ligated ileal loops were mild but statistically significant (CI of ~2). These mild phenotypes are likely due to the short duration of infection or the variable production of antimicrobial peptides as a result of the variation in ages of calves used in this study (3-6 weeks). Recent reports indicate that 3-week old Holstein-Friesian calves may not constitutively express much ß-defensin in the gastrointestinal tract but that this expression increases with age [200]. However, it is not known whether antimicrobial peptide production in intestinal tissue occurs in response to bacterial infections in calves of this age. Our data are the first to directly support the roles of the *phoPQ* regulatory system during survival of *Salmonella* in the inflamed intestinal tract.

STM3602 encodes a putative transcriptional regulator [129], and we show that this gene is necessary for survival in fluid, mucus, and tissue in ligated ileal loops (Figure 2.3 and 2.4A). *STM3602* was predicted to be under selection in a signature-tagged mutagenesis screen of transposon mutants during oral infection of a calf [105], but the predicted phenotype was never confirmed. This gene belongs to the GntR (gluconate operon repressor) family of regulators [201] and shares conserved domains with *phnF* (phosphonate utilization, e-value

1.29e-68) [202, 203], the regulator of the phosphonate-utilization operon in *E. coli* [161].

Phosphonates are stable carbon-phosphorus bonds produced by bacteria and some marine invertebrates as a means of storage of phosphate [151, 204]. *Salmonella* Typhimurium has a complete operon containing two genes for metabolism of phosphonate (*phnVUTSRWX*, *STM0426-0432,* GC content 56-60%) that is activated by inorganic phosphate during periods of phosphate starvation [161] and an additional locus involved in phosphonate metabolism (*phnOBA*, *STM4287-4289*, GC content 49-55%) [160]. *STM3602* is located at a different chromosomal site (Figure 2.5A), and has a much lower GC content (49.3%) than the *phnVUTSRWX* operon. Whether STM3602 is involved in regulating phosphonate metabolism, and whether this is related to the phenotype we observe during enteric infection is not yet clear. *STM3602* is a very interesting bacterial regulatory protein that merits further study to elucidate its precise function during enteric infection.

The second deletion mutant that we studied in this work, Δ*STM3846,* is deleted for a putative reverse transcriptase [129]. This gene is encoded on a bacterial retro-element termed a retron [112, 116, 124, 185, 205]. Bacterial retrons may be both horizontally and vertically acquired and produce a small multicopy single-stranded DNA molecule called msDNA, a unique RNA-DNA hybrid [124, 185, 205]. *STM3846* is encoded on the St-85 retron (Figure 2.5B) containing two open reading frames (*STM3845* and *STM3846*) and a small

segment of DNA upstream of these open reading frames that encodes the primer and the template (*msr* and *msd*) used by the reverse transcriptase to produce the msDNA [112]. Bacterial reverse transcriptases produce msDNA by using a leader RNA encoded by *msr* to prime the reaction and produce a 2', 5' phosphodiester linkage between an RNA (encoded by *msr*) and DNA (encoded by *msd*) molecule [131].

The msDNA produced by *STM3846* is 85 base pairs in length, has a predicted stem-loop structure with no mismatched base pairs in the stem [112] and may have lost the RNA template [122]. The *STM3846* reverse transcriptase is present in the genomes of all 19 serotype Typhimurium isolates that we have sequenced (McClelland *et al.*, unpublished data). No role for the St-85 retron has been established despite several previous studies of genes in this region [122, 130]. Furthermore, other entero-virulent Gram-negative organisms, including *Vibrio spp.* and virulent *E. coli,* produce msDNAs [112] yet no phenotypes have been identified for mutants unable to produce any of these msDNAs. We are the first to unambiguously show a phenotype for a mutant lacking a bacterial reverse transcriptase and this phenotype is for virulence in a highly relevant model of disease.

In the work we report here, we have used a library of targeted single gene deletion mutants to identify novel colonization and virulence determinants of *Salmonella* Typhimurium during infection of bovine ileal loops, a technically challenging model highly relevant to human gastrointestinal salmonellosis. The

bovine ileal loop model has not previously been used for unbiased screening of *Salmonella* mutants, although it closely replicates early events of enteric salmonellosis in humans. We identified more than 30 genes not previously proven to be important for survival of *Salmonella* in this model, and we confirmed 3 of these mutants individually in competitive infections. Complementation analysis linked the observed phenotypes directly to the disrupted genes for mutants in a putative regulator STM3602 and a reverse transcriptase STM3846. We show that the reverse transcriptase encoded by *STM3846* is essential for virulence, and we show the first phenotype of any kind for a bacterial reverse transcriptase located on a retron. Finally, we report an exciting group of genes for further study to elucidate the mechanisms utilized by *Salmonella* for survival in the complex niche of the host small intestine during the inflammatory response.

CHAPTER III

MULTICOPY SINGLE-STRANDED DNA DIRECTS INTESTINAL COLONIZATION OF ENTERIC PATHOGENS

Overview

Multicopy single-stranded DNAs (msDNAs) are unique hybrid RNA-DNA molecules produced by retron reverse transcriptases. Retrons are widespread in bacteria but the natural function of msDNA has remained elusive despite 30 years of study. We report that msDNA of the zoonotic pathogen *Salmonella* Typhimurium is necessary for colonization of the intestine. Similarly, we observed a colonization defect in an enteropathogenic *E. coli* mutant lacking its retron reverse transcriptase. Under anaerobic conditions in the absence of msDNA, proteins of central anaerobic metabolism needed for colonization of the intestine are dysregulated. The msDNA mutant can utilize nitrate but not other electron acceptors in anaerobic conditions. Consistent with the availability of nitrate in the inflamed gut, a neutrophilic inflammatory response partially rescued the ability of a mutant lacking msDNA to colonize the intestine. We conclude that regulation of protein abundance is a natural function of msDNA, the first attributable function for an msDNA.

Introduction

Retron reverse transcriptases (RT) in bacteria were first described in *Myxococcus xanthus* [110] and *E. coli* [111] in the 1980s and are now known to be widely distributed in eubacteria and archaea (reviewed in [205]). The product of the 'retron' is a small covalently linked RNA-DNA hybrid molecule called multicopy single-stranded DNA (msDNA) that is predicted to form complex secondary structures [114]. msDNA is produced by retrons in many pathogenic and non-pathogenic organisms. The predicted secondary structures of msDNAs from enteric pathogens are closely related to each other [112] but the reverse transcriptase amino acid sequence from these enteric pathogens share little identity and the location of the retron as well as the number of retrons in each species varies. These observations suggest that retrons have been horizontally acquired by convergent evolution to function in a fashion that is specific to the biology of the host bacterium.

All retrons contain three regions essential for production of msDNA: *msr* (RNA primer for reverse transcription), *msd* (template sequence), and a reverse transcriptase (RT). The retrons of pathogens, such as *Salmonella* Typhimurium (STm), may also encode an additional ORF of unknown function [112]. The predicted secondary structures of msDNA from enteric pathogens including STm, enteropathogenic *E. coli* and *Vibrio spp*. are similar [112]. Although the molecular details of the production of msDNA have been heavily studied, no

natural function is ascribed to this mysterious molecule despite 30 years of study (reviewed in [205]).

We have shown that the retron reverse transcriptase encoded by *STM3846* is essential for colonization of the calf intestine [106], a natural model of enteric salmonellosis that recapitulates the earliest stages of human non-typhoidal *Salmonella* (NTS) infection. NTS are threats to global animal and human health and are the most prevalent cause of bacterial food-borne gastroenteritis in people in the United States. Enteric salmonellosis is characterized by inflammatory diarrhea containing primarily neutrophils. NTS use the type 3-secretion system 1 (TTSS-1) encoded on *Salmonella* Pathogenicity Island-1 (SPI-1) to invade the intestinal epithelium [51, 173] and to promote the characteristic neutrophilic inflammatory response. Utilization of energy sources and alternate electron acceptors produced as a result of this neutrophilic influx provide a key competitive advantage to NTS over resident microflora [76, 88, 90].

In the work described here, we report that mutants lacking msDNA produced by the *STM3846* reverse transcriptase are defective for colonization of the intestine using murine models of salmonellosis. Similar colonization defects are observed for an enteropathogenic *E. coli* lacking its retron reverse transcriptase. This colonization defect is due, in part, to a growth defect for these mutants in anaerobic conditions. We show that mutants lacking msDNA have altered abundance of over 200 proteins in anaerobiosis, many of which are

known to be required for growth in anaerobic conditions and pathogenesis of STm in enteric infection. Inappropriate abundance of proteins encoding alternate terminal electron acceptor reductases results in an inability of mutants lacking msDNA to utilize these compounds. The mutants lacking msDNA can only utilize nitrate as an anaerobic terminal electron acceptor. Finally, we show that mutants lacking msDNA only fail to colonize portions of the intestine lacking neutrophilic inflammation, likely due to an ability to utilize nitrate during profound tissue inflammation. Thus, we report a role in regulating protein abundance for msDNA, the first reported natural function for any msDNA.

Methods

Bacterial strains

All *Salmonella* strains are derivatives of ATCC 14028s. Enteropathogenic *E. coli* O127:H6 strain E2348/69 [206], a generous gift of M. Donnenberg, is the genetic background for EPEC mutants. Mutants were constructed using a modification of the lambda-red recombination technique and antibiotic resistance cassettes removed as previously described [97, 207]. All *Salmonella* mutations were moved into a clean genetic background by P22 transduction [169]. Standard cloning protocols were used to generate complementing plasmids [208].

All bacterial cultures were grown at 37˚C aerobically with vigorous agitation or standing in an anaerobic chamber with internal atmosphere of 5% H_2, 5% CO_2, and 90% N_2 (Bactron I, ShelLab). Alternate electron acceptors (Sigma-Aldrich) sodium nitrate, sodium fumarate, sodium thiosulfate, and sodium tetrathionate were added to LB to a final concentration of 40mM. Sodium chloride (Sigma-Aldrich) at a final concentration of 40 mM served as a negative control. DMSO (Sigma-Aldrich) was added to LB to a final concentration of 0.1% (v/v). Bacteria were grown in Luria-Bertani (LB) broth or LB or MacConkey (Difco) agar supplemented with the following antibiotics as appropriate: kanamycin (50 mg/L), nalidixic acid (50 mg/L), carbenicillin (100 mg/L), streptomycin (100 mg/L), and chloramphenicol (20 mg/L).

All experiments were performed on at least three separate occasions. Bacterial generation number was calculated using the following equation: $[\log_{10}(\text{CFU final}) - \log_{10}(\text{CFU start})]/\log_{10}(2)$.

Mouse infections

The Texas A&M University Institutional Animal Care and Use Committee approved all animal experiments. All experiments that utilized mice were performed using 10-12 week old female C57BL/6J mice (Jackson Laboratories). For competitive infection experiments, mice were infected by gavage with an equivalent ratio of WT and mutant bacteria. The competitive index was

determined by dividing the ratio of WT to mutant bacteria in the selected organ by that ratio in the inoculum. For single infections, mice were infected with either WT or mutant bacteria. The harvested tissue was weighed, homogenized, and CFU was determined per gram of tissue collected.

Salmonella infections were performed as previously described [28]. For the murine colitis model, mice were administered 20 mg streptomycin in 75 µL sterile water by gavage. Twenty-four hours after treatment, mice were infected with approximately 10^8 CFU of *Salmonella* in 100 µL volume by gavage. Feces were collected 24 hours after infection. Mice were euthanized 96 hours post-infection and organs harvested, homogenized, serially diluted, and plated on LB agar with appropriate antibiotics for enumeration of CFU. For the murine typhoid model, mice were treated with 75 µL sterile water by gavage. Mice were then infected and euthanized as above.

EPEC mouse infections were performed essentially as previously described [209]. Mice were infected with approximately 10^8 CFU in 100 µL volume by gavage. Feces were collected every other day for 9 days. Mice were euthanized 10 days post-infection. The aboral 5 cm of small intestine, the entire cecum, and the entire colon were collected. Intestinal contents were exposed through a longitudinal incision. The intestinal segment was placed into sterile PBS and vigorously agitated to remove intestinal contents. Intestinal tissue was washed in sterile PBS to remove remaining ingesta. Intestinal contents and

tissue were homogenized separately, serially diluted, and plated on MacConkey agar and LB agar with appropriate antibiotics to enumerate CFU.

Histopathology

Samples from mouse ileum, cecum, and transverse colon were collected 96 hours post-infection and fixed in formalin. All tissues were routinely processed and stained with hematoxylin and eosin. All histologic analyses were performed by a blinded veterinary pathologist. Tissues were scored (0-4) for each of the following parameters: polymorphonuclear cell (PMN) infiltration, mononuclear leukocyte infiltration, crypt abscess, submucosal edema, villus blunting, and epithelial damage as described [10, 28, 90, 210].

msDNA isolation

msDNA was isolated from aerobic late log phase cultures normalized by OD_{600}. Bacteria were lysed as for plasmid isolation (Qiagen Mini-prep) and msDNA isolated from the filtered fraction and subsequent ethanol precipitation. msDNA was visualized using a native polyacrylamide gel with in-gel ethidium bromide staining.

Invasion assays

Cell lines were purchased from American Type Culture Collection (ATCC) and used within 15 passages. HeLa cells (human cervical adenocarcinoma epithelial, ATCC CCL-2) were grown as recommended by ATCC. HeLa cells were seeded in 24-well plates at 5 x 10^4 cells/well approximately 24 h prior to infection.

Late-log phase cultures were prepared by inoculating 10 ml LB broth with 0.3 ml overnight shaking culture. Flasks were grown at 37°C with agitation for 3 hours. Bacteria were collected by centrifugation at 8000 x g for 90 seconds, resuspended in an equal volume of Hanks' buffered saline solution (HBSS, Mediatech) and added directly to mammalian cells seeded in 24-well plates for 10 minutes. The multiplicity of infection was approximately 50. Non-internalized bacteria were removed by aspiration. Monolayers were washed three times in HBSS and were then incubated in growth media until 30 min post-infection. Thereafter, gentamicin was added at 50 µg/ml from 30-90 min post-infection to kill extracellular bacteria and reduced to 10 µg/ml from 90 min post-infection. For enumeration of intracellular bacteria, monolayers were washed once in phosphate-buffered saline, and then solubilized in 0.2% sodium deoxycholate and serial dilutions were plated on LB agar.

Data analysis

Statistical analysis was performed using GraphPad Prism 6. All data were log transformed prior to analysis. Statistical significance was set at $P < 0.05$ and was determined using a t-test or ANOVA where indicated.

Proteomic analysis

Wild type and the Δ*STM3846* and Δ*msd* mutants were incubated either aerobically or in an anaerobic chamber (Coy) for 4 hours on three independent occasions. Bacteria were pelleted and supernatants discarded. Cell pellets were resuspended in 100 mM NH_4HCO_3, pH 8.0 and lysed by vigorous vortexing in the presence of 0.1 mm silica/zirconia beads. Proteins were denatured and reduced with 8M urea and 5 mM dithiothrietol, respectively, for 30 minutes at 60°C. The proteins underwent enzymatic digestion for 3 hours at 37 °C with 1/50 enzyme/protein (w/w) ratio of sequencing-grade trypsin. The resultant peptides were desalted for mass spectrometric (MS) analysis using C18 solid phase extraction cartridges (50 mg, 1 mL, Discovery, Sulpelco). The cartridges were activated with methanol, followed by equilibration with 0.1% TFA before loading the samples. The cartridges were then washed with 5% acetonitrile (ACN)/0.1% TFA and eluted with 80% ACN/0.1% TFA. Eluted peptides were concentrated in the vacuum centrifuge and diluted to a concentration of 0.5 mg/mL with water for the MS analysis.

Digested peptides were loaded into capillary columns (75 µm x 35 cm, Polymicro) packed with C18 beads (3 µm particles, Phenomenex) connected to a custom-made 4-column LC system [211]. The elution was performed using the following gradient: equilibration in 5% B solvent, 5-8% B over 2 min, 8-12% B over 18 min, 12-35% B over 50 min, 35-60% min over 27 min and 60-95% B over 3 min. (solvent A: 0.1% FA; solvent B: 90% ACN/0.1% FA) and flow rate of 300 nL/min. Eluting peptides were directly analyzed either on an Orbitrap (LTQ Orbitrap Velos, Thermo Scientific, San Jose, CA) mass spectrometer using chemically etched nanospray emitters [212]. Full scan mass spectra were collected at 400-2000 m/z range and the ten most intense ions were submitted to low-resolution CID fragmentation once (35% normalized collision energy), before being dynamically excluded for 60 seconds.

Tandem mass spectra were searched with MSFG+ against *Salmonella enterica* serovar Typhimurium 14028s and common contaminant sequences (downloaded from NCBI, all in forward and reversed orientations), using the following parameters: (i) partial tryptic digestion, (ii) 50 ppm parent mass tolerance, (iii) methionine oxidation as a variable modification. The peptides were filtered with a MSGF probability score [213] ≤ $1x10^{-9}$. Peak areas for each peptide were retrieved using the MultiAlign tool [214], and to ensure the quality of peptide-to-peak matching, the data was filtered with a Statistical Tools for AMT tag Confidence (STAC) score ≥ 0.7 and uniqueness probability ≥ 0.5 [215]. Additionally, proteins were required to have at least 2 peptides and at least one

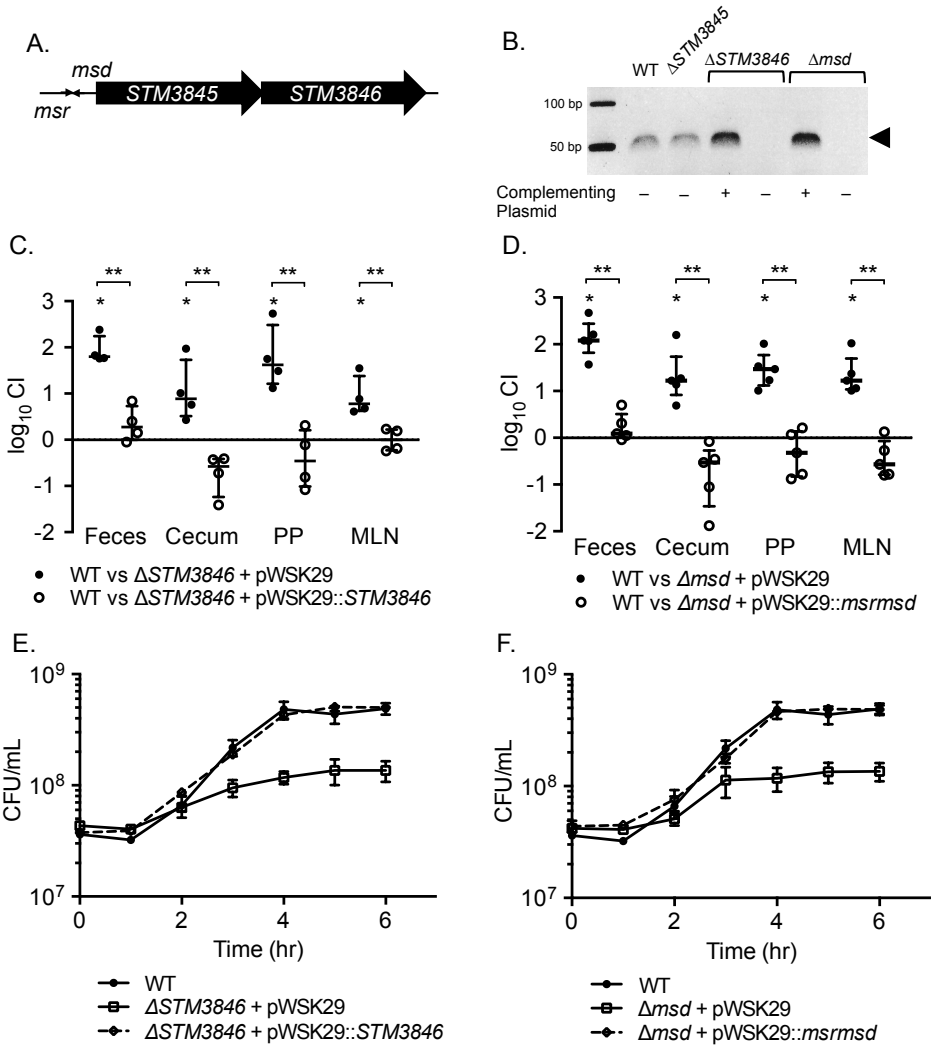
peptide with STAC ≥ 0.9. Peptide abundance values were rolled-up into proteins using Qrollup tool, available in DAnTE [216]. Abundance values for each protein in all conditions (WT, mutants, anaerobic, aerobic conditions, biological replicates, and technical replicates), 32 in all, were used to calculate a Z-score for each measurement. The Z-score transformation enables comparisons of trends across conditions and proteins to identify relevant abundance changes.

Results

Retron reverse transcriptases, including the *STM3846* reverse transcriptase of the St-85 retron, use *msr* to prime reverse transcription of the *msd* template sequence to produce msDNA [117]. In order to establish that this msDNA, and not some other potential product of the STM3846 RT, mediates STm colonization of the intestine, we generated a non-polar deletion of *msd* (Figure 3.1A). Neither the Δ*STM3846* mutant nor the Δ*msd* mutant produce msDNA and its production can be restored in both mutants by complementation *in trans* (Figure 3.1B). The additional ORF, *STM3845*, is dispensable for msDNA production.

We used the murine colitis model [28], which responds to NTS infection with profound neutrophilic inflammation in the cecum, to dissect the function of the retron in intestinal colonization. We confirmed the requirement for *STM3846* colonization of the inflamed intestine in this model (Figure 3.1C). In addition,

Figure 3.1: msDNA is produced by *msd* and STM3846 and is essential for *Salmonella* to colonize the intestine and grow in anaerobic conditions. (A) Genomic context of the retron St-85 (adapted from [106]). (B) Native polyacrylamide gel with in-gel ethidium bromide staining depicting msDNA (arrowhead). (C) Competitive index (CI WT/mutant) of WT vs Δ*STM3846* bearing empty vector (closed circles; n=4) or WT vs complemented Δ*STM3846* mutant (open circles; n=4) in the murine colitis model. (D) CI of WT vs Δ*msd* bearing empty vector (closed circles; n=5) or WT vs complemented Δ*msd* mutant (open circles; n=5) in the murine colitis model. Statistical significance was determined by a t-test with * P < 0.05 WT vs mutant and ** P < 0.05 between infection groups. (E and F) Anaerobic growth of WT (closed circles), Δ*STM3846* (E) or Δ*msd* (F) with empty vector (open square), and complemented Δ*STM3846* (E) or complemented Δ*msd* (F) mutant (open diamond) in LB broth. Each data point is the mean +/- SEM of three independent experiments. MLN – mesenteric lymph node; PP – Peyer's patches.

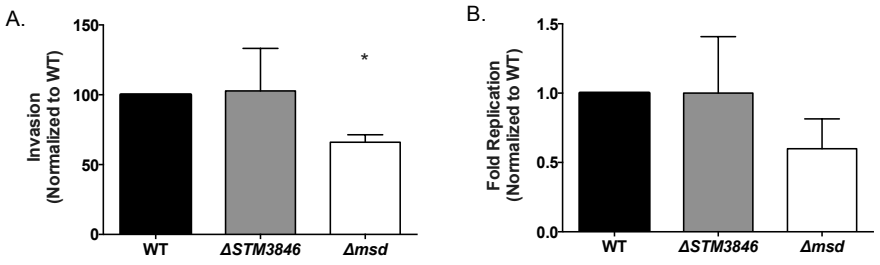


both the Δ*msd* and Δ*STM3846* mutants have indistinguishable phenotypes, suggesting that the effect of deletion of the RT is mediated by the msDNA itself. The ability of each of these mutants to colonize the intestine is rescued by complementation *in trans* (Figures 3.1C and 3.1D). In cell culture, the Δ*msd* mutant invades epithelial cells at a level only mildly reduced compared to the isogenic wild type (Figure 3.2A and 3.2B) suggesting that reduced tissue invasion is not the likely cause of the phenotype we observed during infection of animal models. Our findings definitively link msDNA to the ability of *Salmonella* to colonize the intestine.

The intestine is a specialized and highly diverse niche. Oxygen tensions within the lumen decline from the stomach to the colon [58, 59] and there is a gradient of increasing oxygen tension from the center of the lumen towards the epithelium [60]. Enteric pathogens must replicate in this hypoxic setting using both aerobic and anaerobic metabolic pathways [217, 218] and express genes necessary for virulence in order to compete with resident microflora and colonize the host efficiently. To determine whether the intestinal colonization defect of the STm msDNA mutants is due to an inability to grow in oxygen limited conditions, we measured the growth of our mutants in the absence of oxygen. Both mutants unable to produce msDNA have severe growth defects in anaerobic conditions (Figure 3.1E and 3.1F), while the growth of these mutants in the presence of oxygen is similar to the isogenic WT (Figure 3.3A-D). The necessity for msDNA

during anaerobic growth is consistent with the inability of msDNA-deficient mutants to efficiently colonize the intestine.

Figure 3.2: The Δ*msd* mutant has only a mild invasion defect in tissue cultured epithelial cells. (A) Invasion efficiency of Δ*STM3846* and Δ*msd* mutants into HeLa cell monolayers normalized to the efficiency of the WT at 1 h post-infection. (B) Fold-replication of Δ*STM3846* and Δ*msd* mutants 7 h post-infection/1 h post-infection normalized to WT fold-replication. Bars represent the mean +/- SD. Invasion was measured on five and intracellular replication on three separate occasions. * P<0.05.



We hypothesized that msDNA might act as a *trans* regulator of gene expression for two reasons. First, small RNAs are well known to have regulatory properties through base pairing with DNA or mRNA transcripts [219]. Second, substantial over-expression of msDNA from one strain of *E. coli* in a heterologous strain lacking a retron resulted in small changes in the proteome [140]. To determine whether the msDNA produced by the St-85 retron might have regulatory properties, we evaluated the proteome of WT and msDNA-deficient mutants (Δ*STM3846* and Δ*msd*) at late exponential phase, a time at

which the retron is expressed and msDNA is produced (Figure 3.1B and [220]), in both the presence and absence of oxygen. Of the 1505 proteins identified, there were no significant differences in protein abundance between the WT and mutants in the presence of oxygen (Figure 3.4A). This finding is not surprising given that mutants lacking msDNA have no known phenotypes when grown in standard laboratory conditions (Figure 3.3A-D). In addition, very few proteins differed in abundance between the two mutants, consistent with the hypothesis that the reverse transcriptase and msDNA operate in the same biological pathway.

Figure 3.3: The aerobic growth kinetics of msDNA mutants is equivalent to WT in rich and minimal media. Aerobic growth of WT and Δ*STM3846* mutant in (A) LB and (B) M9 minimal media and Δ*msd* mutant in (C) LB and (D) M9 minimal media. Each data point is the mean +/- SEM of three independent experiments.

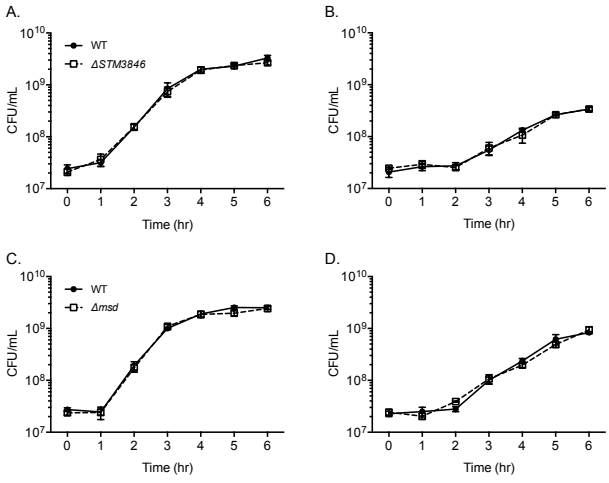
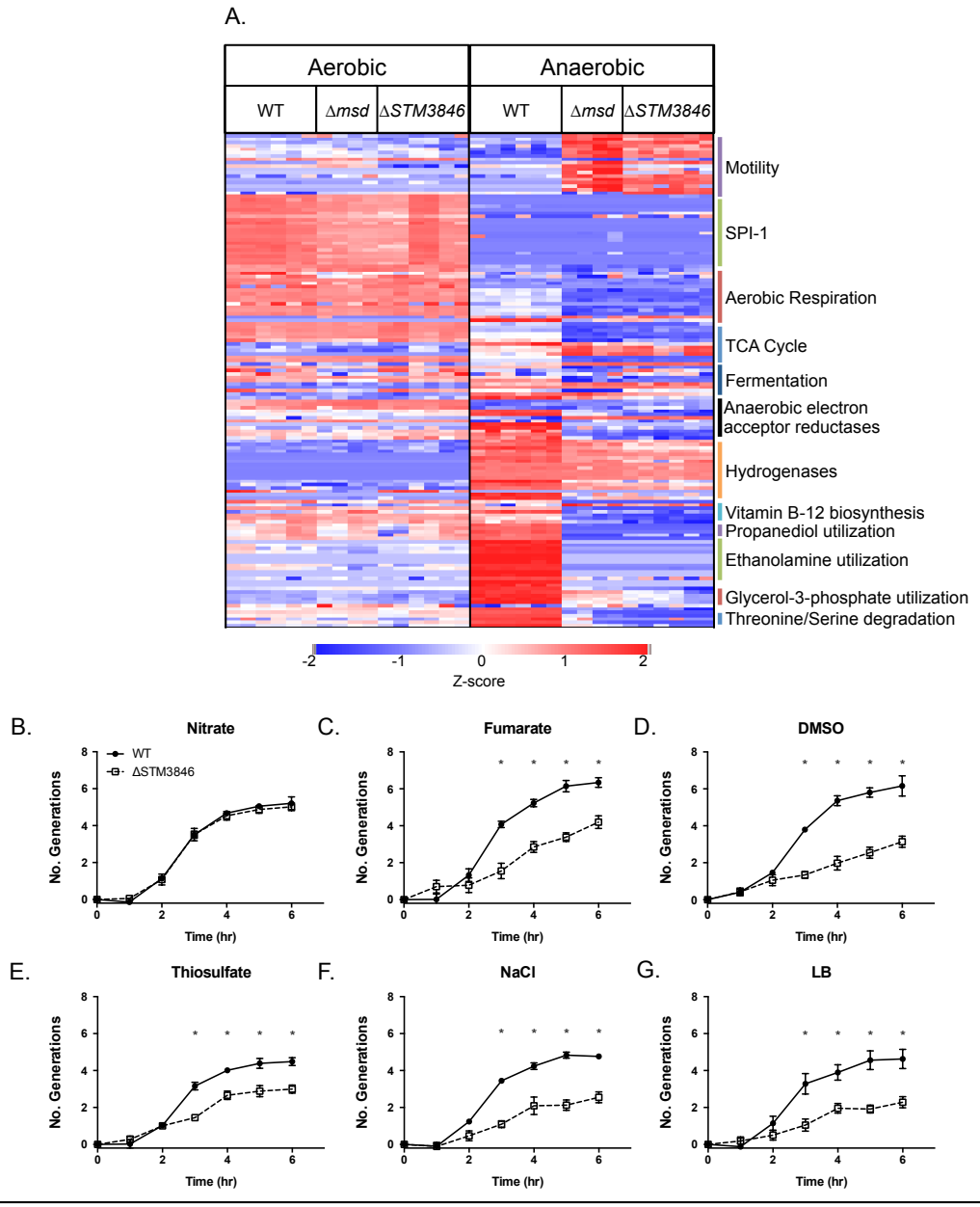


Figure 3.4: Proteins needed for anaerobic metabolism are dysregulated in mutants lacking msDNA. (A) Z-scores of 148 of 1505 total proteins identified from WT (n=3) and Δ*msd* (n=2) and Δ*STM3846* (n=3) mutant cultures grown in both aerobic and anaerobic conditions. Functions of selected proteins are listed to the right of the heat map. (B-G) Number of generations of anaerobically grown WT and Δ*STM3846* mutant in LB with added electron acceptor [B – nitrate (40 mM), C – fumarate (40 mM), D – DMSO (1% v/v), E – thiosulfate (40 mM) or control (F – NaCl (40 mM) or G – LB only)]. Each assay was performed on four separate occasions; each data point is mean +/- SEM. Statistical significance determined by t-test with * P<0.05.



In anaerobic conditions however, we identified 238 proteins that differed in abundance between the wild type and msDNA-deficient mutants. Forty-three percent of proteins with reduced abundance in the mutant were involved in amino acid and carbohydrate transport/metabolism and energy production/conversion (Figure 3.5A). Twenty-five percent of all proteins of altered abundance did not belong to a functional grouping (Figure 3.5A and 3.5B). The abundance of numerous proteins known to be important for anaerobic growth was severely reduced, including proteins for 1,2 propanediol utilization [91], ethanolamine utilization [90], anaerobic sn-glycerol-3-phosphate utilization [221], anaerobic vitamin B12 biosynthesis [222], and serine/threonine degradation [223].

Figure 3.5: Functional groupings of dysregulated proteins in msDNA mutants. (A) Proteins reduced in abundance and (B) proteins increased in abundance in msDNA mutants as determined by clusters of orthologous grouping [183].

A.

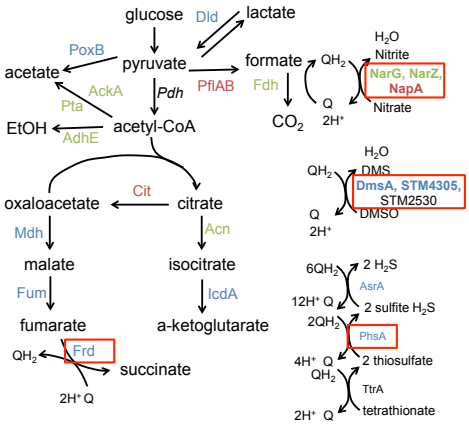
COG grouping	# proteins
amino acid transport and metabolism	32
energy production and conversion	27
carbohydrate transport and metabolism	14
cell wall/membrane/envelope biogenesis	9
coenzyme transport and metabolism	8
secondary metabolite transport, biosynthesis, catabolism	8
signal transduction	6
nucleotide transport and metabolism	5
transcription	5
inorganic ion transport and metabolism	4
post-translational modification, protein turnover, chaperone	3
intracellular trafficking, secretion, vesicular transport	2
cell cycle control, chromosome partitioning, cell division	1
cell motility	1
lipid transport and metabolism	1
general or unknown function	42

B.

COG grouping	# of proteins
cell motility	8
translation ribosomal structure, and biogenesis	8
energy production and conversion	5
inorganic ion transport and metabolism	5
amino acid transport and metabolism	4
cell wall/membrane/envelope biogenesis	4
signal transduction mechanisms	3
transcription	3
coenzyme, lipid, nucleotide transport and metabolism	3
carbohydrate transport and metabolism	2
post-translational modification, protein turnover, chaperone	2
replication, recombination, and repair	2
cell cycle control, cell division, chromosome partitioning	1
intracellular trafficking, secretion, vesicular transport	1
general or unknown function	18

Utilization of tetrathionate, nitrate, and fumarate as terminal electron acceptors is essential for the biology of facultative anaerobes (*Salmonella* and *E. coli*) in the intestine of mammals [88, 217, 218, 224, 225]. NTS benefit from the host inflammatory response by using the alternate electron acceptors tetrathionate, generated by the neutrophilic respiratory burst, and nitrate, generated by the epithelium in response to infection [88, 89, 225]. Numerous proteins involved in reduction of anaerobic electron acceptors [62] were altered in abundance between msDNA mutants and wild type bacteria during anaerobic growth (Figure 3.6). Proteins important for the reduction of thiosulfate (PhsAB)

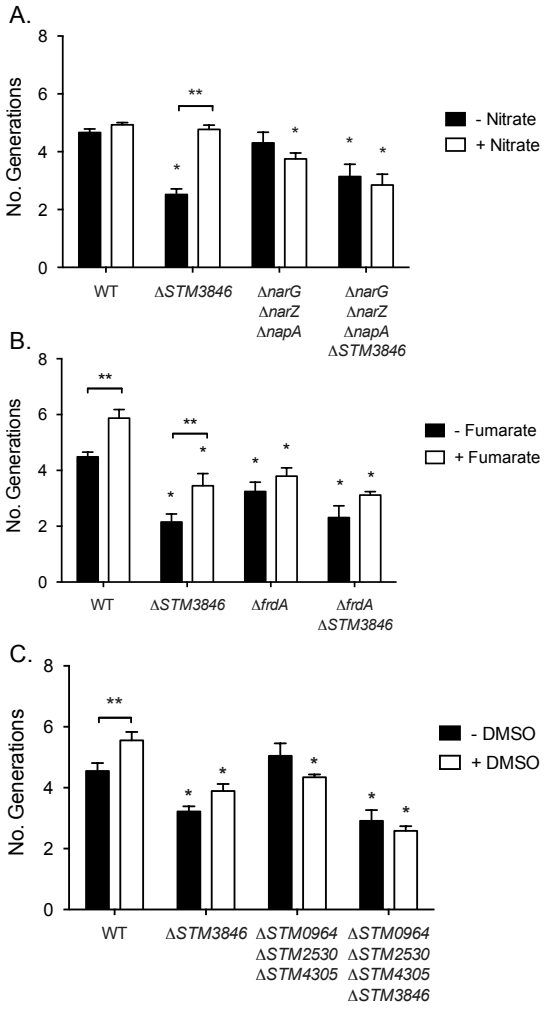
Figure 3.6: Anaerobic carbon metabolism is severely disrupted in msDNA-deficient mutants. Proteins necessary for a given reaction are listed beside the reaction. Proteins listed in blue are decreased in abundance, in red are increased in abundance, and in green are unchanged in abundance in the mutants. Proteins shown in black were not observed. Boxed proteins are those alternate anaerobic electron acceptors Figure adapted from [241].



and sulfide (AsrC) were of low abundance (Figure 3.4A). In addition, proteins necessary for reduction of DMSO (DmsA, STM4305.s) and fumarate (FrdA) were also of low abundance in msDNA mutants, although they did not meet our stringent criteria for statistical significance. Expression of genes necessary to utilize alternate electron acceptors is often induced by the presence of the electron acceptor [62] so the absence of a statistically significant reduction in some of these proteins is not surprising since these compounds were not present in the growth conditions. Interestingly, NapA, encoding the periplasmic nitrate reductase [62], was one of the proteins that was increased in abundance in msDNA deficient mutants compared to the WT and there was no change in the abundance of NarGH, one of the two other nitrate reductase complexes. These data are consistent with the growth defect of our mutants in anaerobic conditions, and suggest that in msDNA-deficient mutants there is severe dysregulation of proteins necessary for reduction of terminal electron acceptors needed during anaerobiosis.

We determined the effects of the addition of various terminal electron acceptors on growth of the *STM3846* mutant in anaerobic conditions. We found that the addition of fumarate, DMSO, or thiosulfate to the culture media during anaerobic growth did not restore the growth defects of strains lacking msDNA (Figures 3.4C-E and 3.7B-C). Thus, the reduced abundance of thiosulfate reductase, sulfide reductase, two DMSO reductases, and fumarate reductase noted in our proteomic analysis translates to an inability to use these

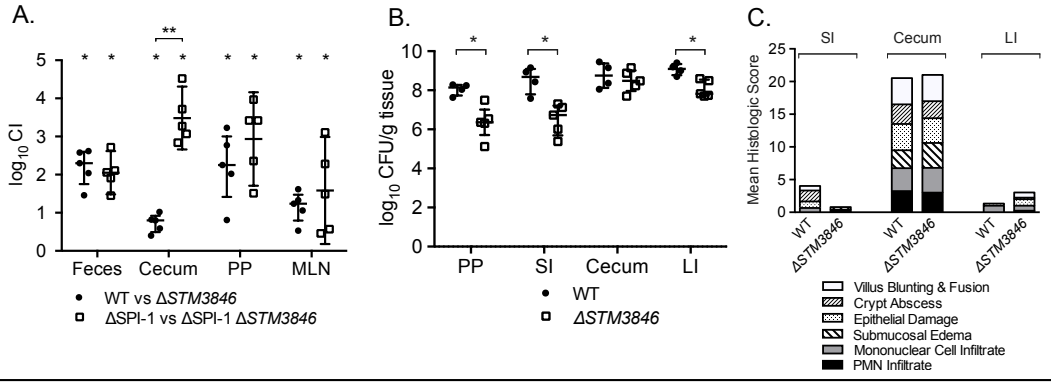
Figure 3.7: Nitrate, but not fumarate or DMSO, rescues the anaerobic growth defect of the Δ*STM3846* mutant to WT levels in LB. (A) Anaerobic growth of WT, Δ*STM3846* mutant, a mutant lacking all three nitrate reductases (Δ*narG* Δ*narZ* Δ*napA*), and Δ*narG* Δ*narZ* Δ*napA* Δ*STM3846* in the presence (black bars) or absence (white bars) of nitrate after a 4-hour incubation. (B) Anaerobic growth of WT, Δ*STM3846* mutant, a mutant lacking the fumarate reductase (Δ*frdA*), and Δ*frdA* Δ*STM3846* in the presence (black bars) or absence (white bars) of fumarate after a 4-hour incubation. (C). Anaerobic growth of WT, Δ*STM3846* mutant, a mutant lacking all three putative DMSO reductases (Δ*STM0964* Δ*STM2530* Δ*STM4305*), and Δ*STM0964* Δ*STM2530* Δ*STM4305* Δ*STM3846* mutants in the absence (black bars) or presence (white bars) of DMSO after a 4 hour incubation. Bars represent mean +/- SEM. * indicates significant difference between mutant and WT for that condition (P<0.05 by ANOVA). ** indicates significant difference between conditions (P<0.05 by t-test).



compounds as terminal electron acceptors to support anaerobic growth. We further determined that the addition of nitrate to culture medium rescued the anaerobic growth of the reverse transcriptase mutant (Figure 3.4B and 3.7A). These data are consistent with our proteomic data that show that mutants lacking msDNA have adequate NarGH, and an increased amount of NapA, allowing these strains to use nitrate as a terminal electron acceptor during anaerobic growth.

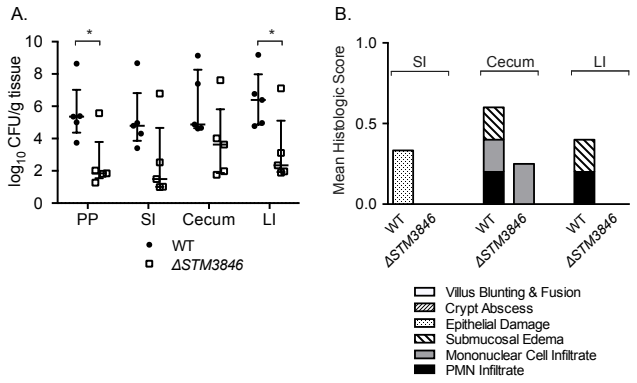
In the presence of an intact TTSS-1, NTS induce an inflammatory response that includes recruitment of luminal neutrophils and induction of inducible nitric oxide synthase by the epithelium [51, 88, 225] resulting in generation of tetrathionate and nitrate as available terminal electron acceptors in the inflamed intestine. To determine whether the colonization defects we observed were dependent on a functional TTSS-1 and host neutrophilic inflammatory response, we performed competitive infection experiments between the virulent WT and Δ*STM3846* mutant both in the presence and absence of SPI-1 (Figure 3.8A). We observed that a Δ*STM3846* mutant colonizes poorly in the intestine and associated organs. The modest colonization defect may be due to an inability to utilize carbon and amino acid sources within the inflamed intestine [90], or due to poor growth as compared with WT prior to the host inflammatory response. Interestingly, the colonization defect of the Δ*STM3846* mutant in the mouse cecum was exacerbated in the absence of a functional TTSS-1, suggesting that a robust inflammatory response partially

Figure 3.8: The STM3846 reverse transcriptase mediates intestinal colonization of *Salmonella* Typhimurium. (A) Competitive index (CI WT/mutant) of WT vs Δ*STM3846* (n=5 mice; closed circles) and ΔSPI-1 vs ΔSPI-1 Δ*STM3846* (n=5; open squares) 4 days post infection in the murine colitis model. (B) Organ colonization of mice infected with either WT (n=4; closed circles) or Δ*STM3846* mutant (n=5; open squares) in the murine colitis model. (C) Mean of histologic scores from indicated intestinal segments from mice in (B). Each point represents data from a single mouse with median and interquartile range indicated. Statistical significance determined as in figure 3.1. PP – Peyer's patches; SI – small intestine; LI – large intestine.



rescues mutants unable to produce msDNA (Figure 3.8A). Consistent with this finding, both the small and large intestines, which lack appreciable neutrophilic inflammation (Figure 3.8C), are poorly colonized with the Δ*STM3846* mutant in mice inoculated with this strain alone (Figures 3.8B and 3.8C). In murine models that do not develop a neutrophilic infiltrate in the intestine in response to infection (murine typhoid model), the Δ*STM3846* mutant also colonizes poorly after oral infection (Figures 3.9A and 3.9B). Our results suggest that *STM3846* is essential for STm to colonize the intestine, a defect that is partially rescued in the presence of a profound host inflammatory response.

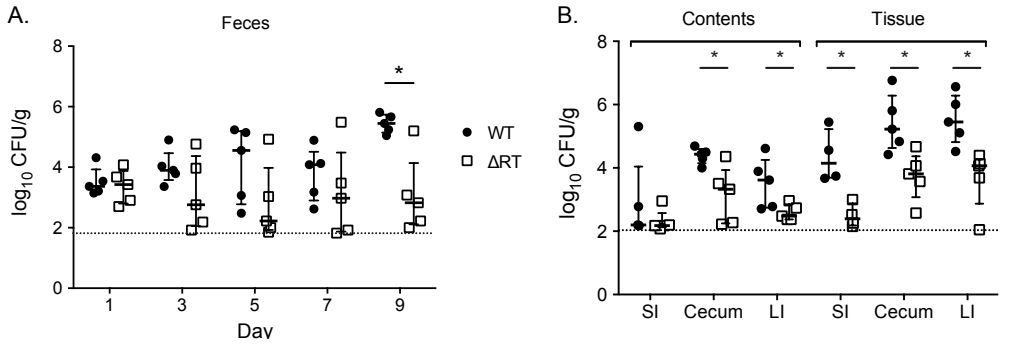
Figure 3.9: The Δ*STM3846* mutant colonizes the non-inflamed intestine poorly in the murine typhoid model. (A) Organ colonization of mice infected with either WT (closed circles; n=5) or Δ*STM3846* mutant (open squares; n=5). (B) Mean histologic score from mouse organs in (A). Each point represents a single mouse with median and interquartile range indicated. Statistical significance between WT and Δ*STM3846* mutant as determined by a t-test is indicated by * (P<0.05).



The msDNA of STm is similar in predicted secondary structure to msDNA of other enteric pathogens including enteropathogenic *E. coli* [112], a close phylogenetic relative of STm. Despite the fact that the pathology caused by these organisms is quite different, both colonize the intestine and cause diarrheal illness in susceptible hosts. We hypothesized that the RT of EPEC O127:H6, a serotype previously shown to produce msDNA [120], is also necessary for intestinal colonization. An EPEC mutant lacking the RT colonizes the intestine of mice poorly, both in the luminal contents and tissue adherent bacteria (Figure 3.10A and B). These data suggest that the importance of retron

reverse transcriptases during intestinal infection is not restricted to salmonellae, and thus are likely to be more broadly applicable to enteric pathogens.

Figure 3.10: Enteropathogenic *E. coli* is dependent on the retron reverse transcriptase to persist within the murine intestine. Fecal shedding (A) of WT (closed circles) and ΔRT mutant (open squares) throughout infection and (B) in intestinal contents (luminal bacteria) and washed tissue (tissue adherent bacteria) after 10 days competitive infection. Statistical significance was determined as for figure 3.1.



Discussion

We have used *S*. Typhimurium, a model enteric pathogen to show that msDNA produced by a retron reverse transcriptase directs colonization of the intestine through regulation of proteins necessary for central anaerobic metabolism. Recent evidence suggests that *Salmonella* induces a host inflammatory response to exploit alternate energy sources and electron

acceptors within the inflamed intestine [88, 90, 225]. We have shown that some of these processes in STm are altered in mutants unable to produce msDNA, along with many other processes with less clearly defined roles in pathogenesis. While the regulatory targets of msDNA may differ between species, the role of retron reverse transcriptases in intestinal colonization by enteric pathogens is likely ubiquitous, as we have shown similar phenotypes for two pathogens lacking a retron reverse transcriptase.

Decades of precedent indicate that inhibition of reverse transcriptases, where they are critical for biology of an organism (retroviruses), is a mainstay of effective therapy against these infections. Further studies elucidating the mechanism of msDNA function and its regulatory targets may lead to generation of novel therapeutics to target increasingly antibiotic-resistant bacteria.

CHAPTER IV

A *SALMONELLA* REGULATOR MODULATES GROWTH WITHIN THE MICROBIAL COMMUNITY OF THE INTESTINE

Overview

Salmonella Typhimurium has numerous mechanisms for colonization of the mammalian intestine. It is essential for *Salmonella* to compete with the host and resident microflora for nutrients in this niche. Phosphonates, molecules characterized by stable carbon-phosphorus bonds, are microbial-derived products that are produced by microorganisms to store phosphorus, and some have antimicrobial properties. *Salmonella* lacks the capability to produce these molecules but can use them, but role of phosphonate metabolism during enteric infection has not been explored. We previously showed that a transcriptional regulator encoded by *STM3602* is needed for colonization of the intestine of calves. Here we report that the necessity of for colonization of the murine intestine depends on the composition of the intestinal microflora. *STM3602* is needed for full activation of the type-3 secretion system-1 and adequate epithelial cell invasion. The Δ*STM3602* mutant grows poorly when phosphonoacetic acid (PA) is the sole phosphorus source, but this mutant is capable of utilizing 2-aminoethylphosphonate. Finally, we show that the enzyme required for PA breakdown, *phnA*, is not under the control of STM3602 suggesting an additional mechanism for utilization of PA in *S*. Typhimurium.

Together, these data suggest that *STM3602* has multiple regulatory targets that are necessary for survival within the complex microbial community in the intestine. Elucidation of the regulon of this gene is a fascinating area of future work and will likely illuminate important new pathways needed for colonization of the host.

Introduction

Non-typhoidal salmonellae (NTS) compete in a complex microbial community during infection of the host. Nutrient availability within the intestinal lumen varies along the length of the intestine and both the host and microorganisms vie for nutrients in this environment. To gain a foothold during infection, *Salmonella* Typhimurium (STm) exhibits numerous mechanisms to gain nutrients and survive in this niche. One notable strategy is the generation of a host inflammatory response with the products of the type 3-secretion system-1 (TTSS-1) to alter the composition of the luminal contents. Through the host inflammatory response, tetrathionate and nitrate are produced which STm uses as terminal electron acceptors [88, 225]. Host-derived sugars such as ethanolamine are also released and utilized as energy sources during inflammation [90].

Metals, such as iron, zinc, and manganese are highly sought after during infection (reviewed in [93]. Siderophores are produced by Enterobacteriaceae to scavenge iron. Salmonellae modify enterobactin to salmochelin [226] to

scavenge iron from host-derived lipocalin-2 in the inflamed intestine [227]. Zinc is sequestered by host-derived calprotectin during intestinal inflammation, a process which *Salmonella* overcomes with a specialized high-affinity zinc transporter to acquire this nutrient during infection [94]. While these are some essential mechanisms by which salmonellae acquire nutrients to replicate in the intestine, it is highly likely that these represent only a small fraction of the potential mechanisms.

Phosphonates are molecules with stable carbon-phosphorus bonds. Microorganisms produce these molecules to store phosphorus during periods of phosphate limitation [151]. In addition to a metabolic utility of these molecules, some of these compounds have potent antimicrobial activity [153, 154] and may be produced by microbes for interspecies competition. The carbon-phosphorus bond is produced by the action of the coupled enzymes phosphoenolpyruvate phosphomutase (Ppm) and phosphonopyruvate decarboxylase (Ppd) to generate phosphonoacetaldehyde, which is converted to 2-aminoethylphosphonate (2-AEP) by AEP transaminase [151, 154]. These are the first steps in the production of compounds containing the stable carbon-phosphorus bond. Although *Salmonella* lacks genes for the biosynthesis of phosphonates, it has the capability to utilize these compounds [160, 161].

We previously found that a mutant in ∆*STM3602* colonized ligated ileal loops in calves poorly [106]. In this model, *Salmonella* Typhimurium is inoculated into intestinal segments with undisturbed microflora and the host responds with

a profound neutrophilic inflammatory response [45, 46]. Thus, salmonellae must both compete with the naturally present microbiota and withstand the host inflammatory response to colonize in this model.

We report that *STM3602* is necessary for colonization of the intestine of mice regardless of the composition of the microflora. We have found that *STM3602* is needed for full activation of the TTSS-1 and adequate epithelial cell invasion. However, this gene is dispensible for TTSS-2 expression and intracellular replication. The Δ*STM3602* mutant is defective for growth with phosphonoacetic acid (PA) as a sole phosphorus source, but is capable of utilizing 2-AEP. Finally, we show that the known enzyme for PA breakdown, *phnA*, is not under the control of STM3602 suggesting an additional mechanism for utilization of PA. Our data suggest that *STM3602* has a diverse regulon including numerous genes needed for survival within the intestine.

Methods

Bacterial strains and plasmids

All bacterial strains are derivatives of ATCC 14028s. Unless stated otherwise, bacteria were grown in Luria-Bertani (LB) broth or LB agar supplemented with the following antibiotics as appropriate: kanamycin (50 mg/L), nalidixic acid (50 mg/L), carbenicillin (100 mg/L), streptomycin (100 mg/L) or chloramphenicol (20 mg/L). Mutants were constructed by a modification of the λ–red recombination technique and antibiotic cassettes removed as described

[97, 207]. Chromosomal transcriptional fusions to *lacZY* were constructed as described [228]. All mutations were moved into a clean genetic background by bacteriophage P22-mediated transduction [169].

Mouse infection

The Texas A&M University Institutional Animal Care and Use Committee approved all animal experiments. All mouse experiments were performed using 10-12 week old female C57BL/6J or CBA/J mice as indicated (Jackson Laboratories) as previously described [28].

For the acute murine colitis model, mice were administered 20 mg streptomycin in 75 µL sterile water by gavage. Twenty-four hours after treatment, mice were infected with approximately 10^8 CFU of an equivalent mixture of WT and mutant bacteria in 100 µL volume by gavage. Feces were collected 24 hours after infection. Mice were euthanized 96 hours post-infection and organs harvested, homogenized, serially diluted, and plated on LB agar with appropriate antibiotics for enumeration of CFU.

For the chronic murine colitis model, mice were administered 20 mg streptomycin in 75 µL water or 75 µL sterile water by gavage. Forty-eight hours after treatment, mice were infected as above. Feces were collected on the indicated days and mice euthanized 14 days post-infection. Competitive index

was determined by comparing the ratio of WT to mutant bacteria in the tissue to that of the inoculum.

Calf ligated ileal loop infection

Six 3-6 week old Angus-cross calves were used for ligated ileal loop experiments as previously described [106].

Invasion assays

Cell lines were purchased from American Type Culture Collection (ATCC) and used within 15 passages. HeLa cells (human cervical adenocarcinoma epithelial, ATCC CCL-2) were grown as recommended by ATCC. HeLa cells were seeded in 24-well plates at 5 x 10^4 cells/well approximately 24 hours prior to infection.

Late-log phase cultures were prepared by inoculating 10 ml LB broth with 0.3 ml overnight shaking culture. Flasks were grown at 37°C with agitation for 3 hours. Bacteria were collected by centrifugation at 8000 x g for 90 seconds, resuspended in an equal volume of Hanks' buffered saline solution (HBSS, Mediatech) and added directly to mammalian cells seeded in 24-well plates for 10 minutes. The multiplicity of infection was approximately 50:1 (bacteria:eukaryotic cell) for HeLa cells. Non-internalized bacteria were removed by aspiration, monolayers washed three times in HBSS and then incubated in

growth media until 30 minutes post-infection. Thereafter, gentamicin was added at 50 μg/ml from 30-90 minutes post-infection to kill extracellular bacteria and the media was replaced with media containing 10 μg/ml gentamicin from 90 minutes post infection. For enumeration of intracellular bacteria, monolayers were washed once in phosphate-buffered saline, solubilized in 0.2% sodium deoxycholate and serial dilutions were plated on LB agar.

β-galactosidase assays

For induction of SPI-1 expression, bacterial cells bearing plasmid constructs were grown overnight in LB with appropriate antibiotics. Overnight cultures were diluted 1:100 and incubated at 37˚C with agitation for 3 hours. SPI-2 inducing media was used as described (5mM KCl, 7.5mM $(NH_4)_2SO_4$, 0.5mM K_2SO_4, 8μM $MgCl_2$, 337μM KH_2PO_4, 80mM MES, 0.3% (v/v) glycerol, 0.1% (v/v) casamino acids, pH 5.8) [229]. Cells were grown overnight in SPI-2 inducing media then diluted 1:50 and incubated for an additional 24 hours for evaluation of SPI-2 expression. For evaluation of the expression of *phnA* in the presence of PA, overnight cultures were diluted 1:100 into LB with 40mM MOPS at pH 6.8 with the indicated phosphorus source. Cultures were incubated at 37˚C with agitation for 3 hours.

β-galactosidase activity was determined using standard methodology [230]. Briefly, bacterial cells were pelleted by centrifugation and resuspended in Z-buffer (60mM Na_2HPO_4, 40mM NaH_2PO_4, 10mM KCl, 1mM $MgSO_4$, 50mM b-

mercaptoethanol). The OD_{600} was taken of bacterial cells in Z-buffer. The cells were permeabilized with chloroform and 0.1% SDS prior to addition of substrate (*o*-nitrophenyl-β-D-galactoside; ONPG 4 mg/mL). The reactions were performed at 28˚C and were stopped with 1M Na_2CO_3 for determination of OD_{420} and OD_{550}. β-galactosidase activity (Miller units) was calculated using the following equation: 1000 x [OD_{420} – (1.75 x OD_{550})] / [time x volume x OD_{600}].

Phosphonate growth

Modifications were made to a phosphorus-limited minimal medium [231] to assess the ability of different bacterial strains to utilize different phosphorus sources. The final media composition (MMMM) was as follows: 20 mM NH_4Cl, 2.5 mM Na_2SO_4, 80 mM NaCl, 0.35 mM $CaCl_2$, 20 mM KCl, 40 mM MOPS, 1 mM $MgSO_4$, 0.01 mM $FeSO_4$, 0.2% glucose, pH 6.8. Phosphorus sources were Na_2HPO_4 (Pi, Sigma), 2-aminoethylphosphonate (2-AEP, Sigma), and phosphonoacetic acid (PA, Sigma) added at the concentrations indicated. Bacterial strains were grown overnight at 37˚C with agitation in MMMM with Na_2HPO_4 at the indicated concentration. Overnight cultures were diluted 1:100 into MMMM with indicated phosphorus source. Aliquots were removed, serially diluted, and plated to determine CFU.

Data analysis

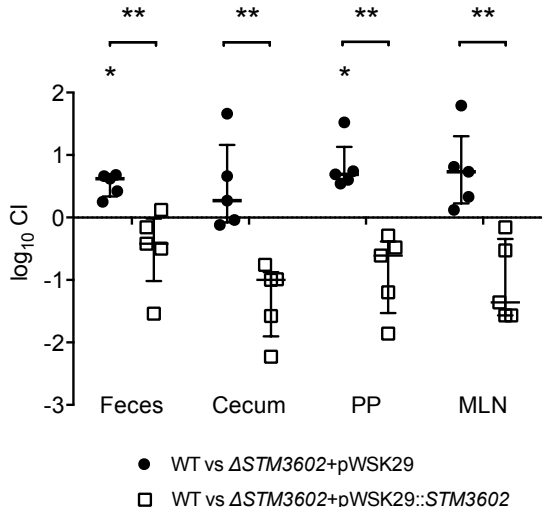
All data were log transformed prior to analysis. Statistical significance was determined using Student's t-test with significance set at $P < 0.05$.

Results

STM3602 in intestinal colonization

We have previously shown that *STM3602* is necessary for STm to colonize the intestine of calves [106]. We used the murine colitis model [28] to further dissect the function of this gene during infection. In the murine colitis model, mice are treated with high doses of streptomycin prior to infection with STm to alter the composition of microflora within the intestine, allowing colonization and development of a neutrophilic inflammatory response. We found that the Δ*STM3602* mutant has a fitness defect in feces by 24-hours post-infection and in Peyer's patches and mesenteric lymph nodes by 96-hours post infection (Figure 4.1). This defect is reversed by complementation *in trans*. Failure to observe a statistically significant defect of the Δ*STM3602* mutant in the murine cecum was likely due to large variation in competitive index between animals. Overall, these results are consistent with a requirement of *STM3602* for colonization of the intestine of mammals in the presence of a profound inflammatory response.

Figure 4.1: The Δ*STM3602* mutant poorly colonizes the murine Peyer's patches and mesenteric lymph nodes during acute colitis. Ten C57BL/6 mice were treated with streptomycin (20mg) then infected with ~10^8 CFU of an equivalent mixture of WT and Δ*STM3602* mutant bearing an empty plasmid (pWSK29) or complementing plasmid (pSTM3602) 24-hours after antibiotic treatment. Feces (F) were collected 24 hours after infection and mice were euthanized 24-hours post-infection for collection of Peyer's patches (PP), mesenteric lymph nodes (MLN), and cecum (C). Competitive index (CI) was determined by comparing the ratio of WT to mutant in the tissue to that of the inoculum. Each data point represents a single mouse with median and interquartile range indicated by horizontal bars. Significant difference in CI is indicated by an asterisk (*) and difference between groups is indicated by two asterisks (**) with $P < 0.05$.



STM3602 and SPI-1 regulation

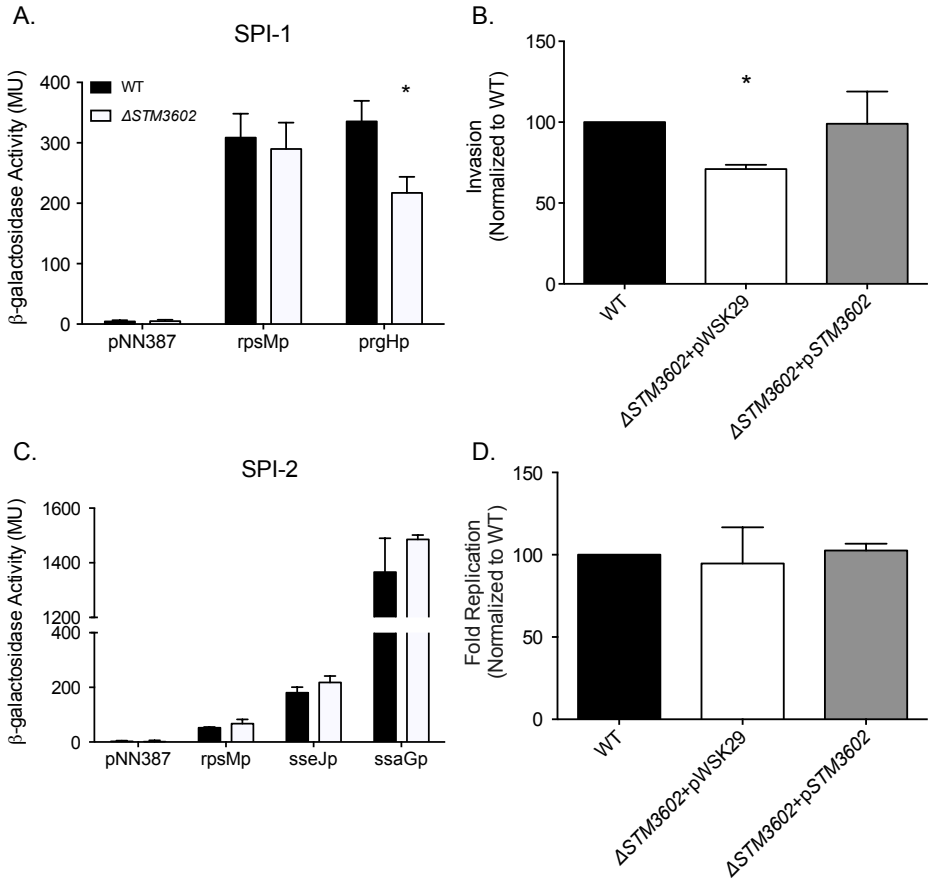
Salmonellae possess two type-3 secretion systems, encoded by *Salmonella* pathogenicity islands 1 and 2 (SPI-1 and SPI-2). The current dogma is that the TTSS-1 and its associated effectors are essential for invasion of non-phagocytic epithelial cells and penetration of the intestinal epithelium [51, 77]. The TTSS-2 is needed for maintenance of the *Salmonella*-containing vacuole

and intracellular replication [187]. We hypothesized that the intestinal colonization defect of Δ*STM3602* mutants could be due to a reduced ability of these mutants express the TTSS-1.

Using a plasmid containing *lacZY* under the control of a promoter of *prgH*, a TTSS-1 structural gene, we observed that the Δ*STM3602* mutant activates the *prgH* promoter less efficiently in SPI-1 inducing conditions than when the fusion is in the WT background (Figure 4.2A). Deletion of *STM3602* results in a similar reduction in invasion efficiency in cultured epithelial cells (Figure 4.2B). Conversely, the Δ*STM3602* mutant is capable of full activation of TTSS-2 apparatus (*ssaG*) and effector promoters (*sseJ*) (Figure 4.2C) as well as survival within cultured epithelial cells (Figure 4.2D).

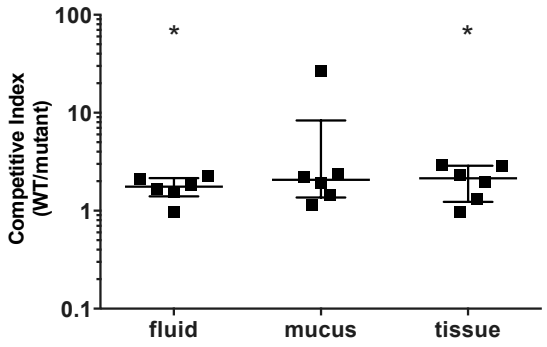
While these results are suggestive of a role of *STM3602* in the complex regulatory network of SPI-1, the invasion defect of strains lacking *STM3602* is small, and is unlikely to fully explain the phenotype that the Δ*STM3602* mutant displays during infection in murine and bovine models. The secretion of all 5 effectors s*ipAsopABDE2* is essential for full epithelial cell invasion and pathology in the calf model of infection [51, 77] while removal of a single effector causes only mild attenuation. In addition, we observed a statistically significant colonization defect of the Δ*STM3602* mutant in a ΔSPI-1 background in competition with a ΔSPI-1 mutant in calf ligated ileal loops (Figure 4.3). The median competitive index of the Δ*STM3602* mutant in the SPI-1 deletion background was 2.14 (1.2-2.9; interquartile range [IQR]), similar to that of

Figure 4.2: The Δ*STM3602* mutant has reduced invasion efficiency into cultured epithelial cells due to poor activation of SPI-1. (A) Activation a terminal SPI-1 promoter (*prgH*p-*lacZY*) in SPI-1 inducing conditions as determined by β-galactosidase activity. (B) Invasion efficiency of Δ*STM3602* and complemented Δ*STM3602* mutants into HeLa cell monolayers normalized to the efficiency of the WT at 1 h post-infection. (C) Activation of two SPI-2 terminal promoters (*sseJ*p-*lacZY* and *ssaG*p-*lacZY*) in SPI-2-inducing conditions as determined by β-galactosidase activity. (D) Fold-replication of Δ*STM3602* and complemented Δ*STM3602* mutants 7 h post-infection/1 h post-infection normalized to WT fold-replication. Bars represent the mean +/- SD. Assays were performed on three separate occasions. * P<0.05.



published data for the Δ*STM3602* mutant in the WT background (median 1.7, 1.3-3.0 IQR) [106]. These data suggest that *STM3602* participates in additional pathways that result in the reduced fitness of the Δ*STM3602* mutant during infection.

Figure 4.3: The colonization defect of the Δ*STM3602* mutant persists in the absence of an intact TTSS-1. Ligated ileal loops from 6 calves were inoculated with an equivalent mixture of ΔSPI-1 mutant and Δ*STM3602* ΔSPI-1 double mutant. Loops were harvested 12 hours after infection. Each data point represents a singe calf. Median and interquartile range of competitive index (WT/mutant) indicated with horizontal bars. Analyses for statistical significance determined as in figure 4.1.

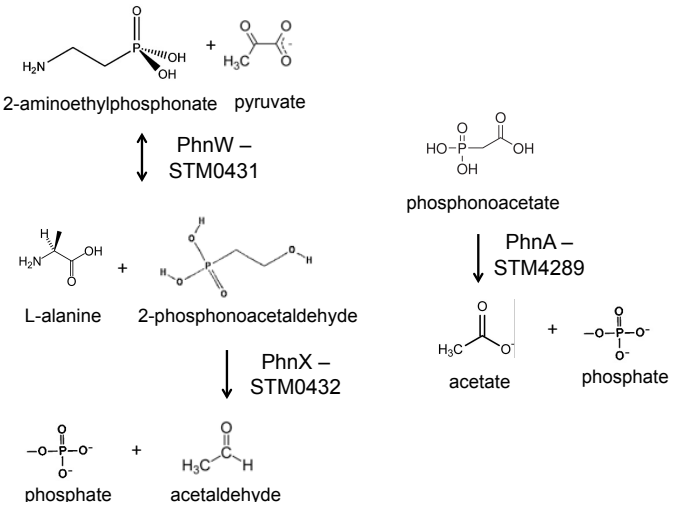


Growth in phosphonoacetate as sole phosphorus source

Although *Salmonella* lacks genes for the biosynthesis of phosphonates, it has the capability to utilize these compounds (Figure 4.4 and reference [160, 161]). *STM3602* belongs to the GntR family of transcriptional regulators and

shares a conserved domain with *phnF*, a regulator of the phosphonate utilization operon in *E. coli* [232]. However the putative mechanism of phosphonate degradation differs between *E. coli* and *Salmonella*. *E. coli* degrades phosphonates using the C-P lyase system encoded within a 14 gene operon (*phnCDEFGHIJKLMNO*) [233]. *Salmonella* has two loci annotated for

Figure 4.4: *Salmonella* enzymes with an annotated role in phosphonate metabolism. See text for description. Not included is PhnO (STM4287), aminoalkylphosphonic acid N-acetyltransferase.

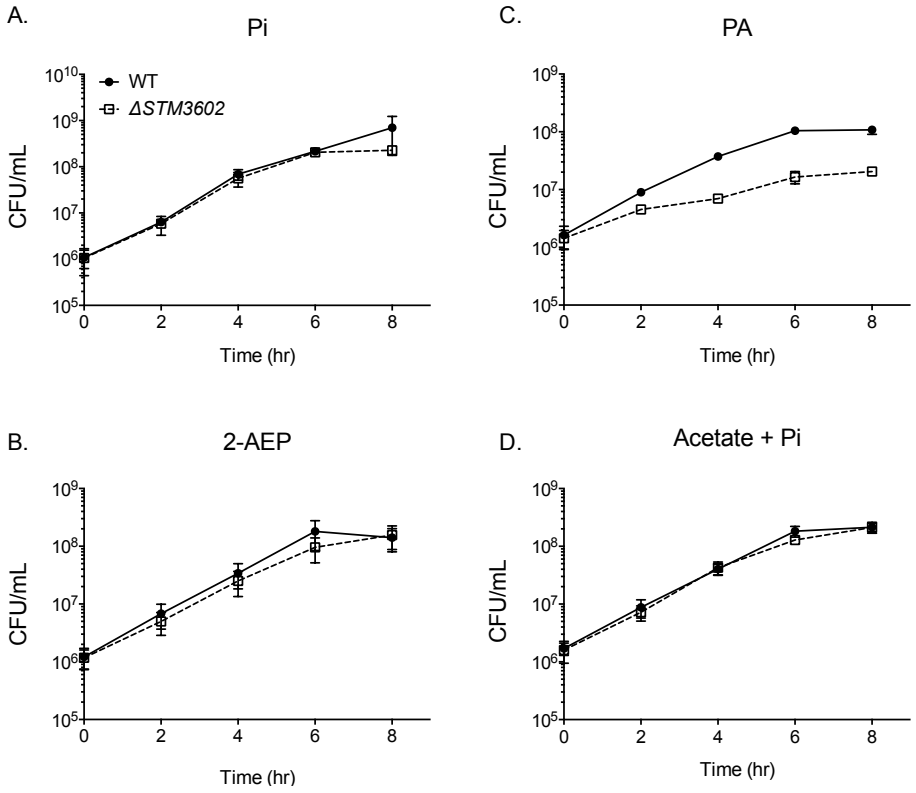


degradation of phosphonates via the phosphonatase pathway, *phnABO* and *phnVUTRWX* [161]. There are four genes annotated for phosphonate degradation (Figure 4.4), *phnW* (*STM0431*; 2-AEP-pyruvate aminotransferase),

phnX (*STM0432*; phosphonatase), *phnA* (*STM4289*; phosphonoacetate hydrolase), and *phnO* (STM4287; aminoalkylphosphonic acid N-acetyltransferase) [160, 161]. The *phnVUTRWX* locus is under the control of the *pho* regulon and is activated under conditions of phosphate deprivation [161].

Based on the shared conserved domain with PhnF, we hypothesized that STM3602 regulates one or both of these phosphonate utilization loci. We chose to characterize the survival and growth of the Δ*STM3602* mutant in media containing either 2-AEP or PA as the sole phosphorus source. When bacterial strains were grown in the presence of 5mM PA, the Δ*STM3602* mutant replicated poorly (Figure 4.5C). However, this mutant has normal growth characteristics in the presence of either 5mM 2-AEP or Pi (Figure 4.5A-B) as sole phosphorus sources. PA is degraded to acetate and inorganic phosphate (Figure 4.4). One possible explanation for the phenotype of the Δ*STM3602* mutant in PA-containing medium is an inability to properly utilize or excrete acetate. However, the Δ*STM3602* mutant exhibits similar growth kinetics to WT in the presence of both 5mM acetate and 5mM Pi (Figure 4.5D) suggesting a mechanism specific to metabolism of PA. These data suggest a role for *STM3602* in phosphonoacetate utilization.

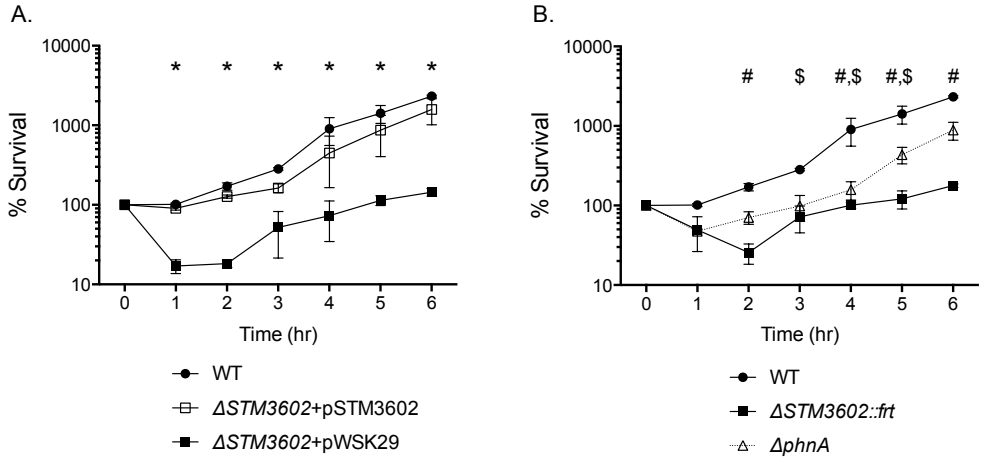
Figure 4.5: *STM3602* is required for adequate growth with phosphonoacetic acid as a sole phosphorus source. (A-D) Growth curve of WT and Δ*STM3602* in 5mM Pi (A), 2-AEP (B), PA (C) and 5mM sodium acetate with 5mM Pi (D). Bacteria were grown overnight in a minimal medium with 5mM Pi then diluted 1:100 into media with the indicated phosphorus or carbon additions. CFU were determined every 2 hours.



Next, we determined the growth kinetics of the Δ*STM3602* mutant in media with 5mM PA after phosphorus deprivation. When bacteria were grown in phosphorus-limiting conditions (0.5mM Pi) and transferred to media containing 10 times more phosphorus in the form of PA, the Δ*STM3602* mutant initially lost viability (Figure 4.6A-B) before recovering to the starting CFU. This defect was

reversed in the complemented strain (Figure 4.6A). A mutant deleted for *phnA*, a gene that encodes the phosphonoacetate hydrolase, had a significant growth defect in these conditions compared to the WT (Figure 4.6B). Interestingly, the growth of the Δ*phnA* mutant was significantly improved compared with the growth of the Δ*STM3602* mutant. These results suggest that both *STM3602* and *phnA* are needed for growth in the presence of PA. However, it is possible that they do not participate in the same pathway to metabolize this compound.

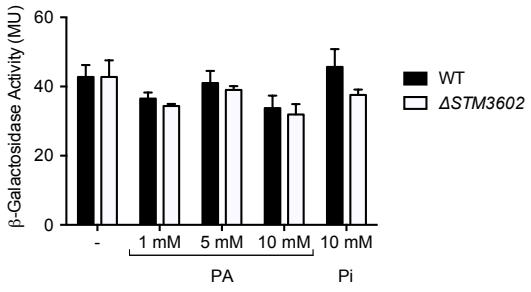
Figure 4.6: The growth defect of the Δ*STM3602* mutant with PA as a sole phosphorus source is reversed by complementation *in trans* and differs from a mutant in Δ*phnA*. Bacterial strains were grown overnight in 0.5 mM Pi and diluted 1:100 into media with 5 mM PA. CFU were determined hourly. (A) Growth of WT, Δ*STM3602* bearing empty vector and complemented Δ*STM3602* mutant. (B) Growth of WT, Δ*STM3602* and Δ*phnA*. CFU data were log transformed and statistical significance determined by ANOVA. (*) indicates significant difference in CFU between Δ*STM3602* + pWSK29 and WT; ($) indicates significant difference between Δ*phnA* and WT; (#) indicates significant difference between Δ*STM3602* and Δ*phnA* with $P < 0.05$.



Expression of phnA

The Δ*STM3602* mutant grows poorly in the presence of PA. We hypothesized that *STM3602* regulates the *phnABO* operon because *phnA* and *STM3602* mutants both have growth defects when grown in PA as a sole phosphorus source. In order to test this hypothesis, we generated a mutant strain bearing allelic replacement of *phnA* (with the first 10 amino acids intact) with *lacZY* to monitor transcription from the *phnA* promoter. Using this construct, we found that the expression of *phnA* in rich media was not affected by the addition of PA at varying concentrations (Figure 4.7). In addition, we observed no effect of a deletion of *STM3602* on the expression of *phnA* in these conditions during log phase growth (Figure 4.7), a time at which *STM3602* is expressed [234]. These data suggest that the expression of *phnA* is not dependent on the presence of PA or *STM3602* in rich medium.

Figure 4.7: The expression of *phnA* is not affected by deletion of *STM3602*. The expression of *phnA::lacZY* was determined from log phase cultures in rich medium by measuring β-galactosidase activity in both the WT (black bars) and Δ*STM3602* mutant (white bars) backgrounds. Bars represent the mean +/- SEM of 3 independent experiments.

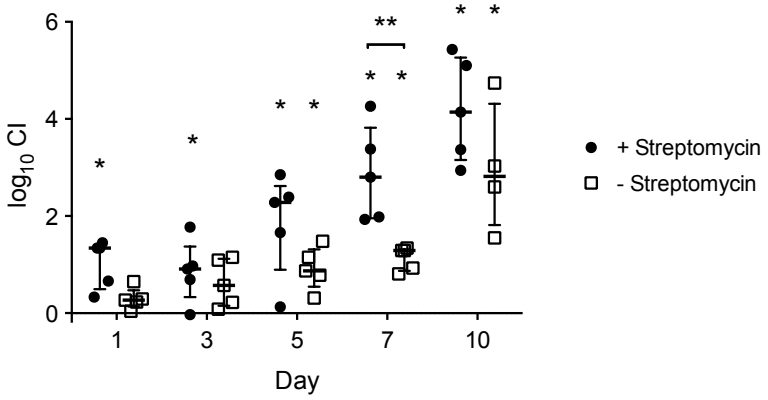


Impact of microbiota on ∆STM3602 intestinal colonization

The first phosphonate to be discovered, 2-AEP, was identified in both the ruminal and duodenal contents of sheep [156]. This compound was associated with both ruminal protozoal and bacterial populations and was less abundant in defaunated animals compared with those with a normal microbial composition. Phosphonoacetic acid is one product of 2-AEP metabolism mediated by the enzyme PhnY. Although salmonellae lack a gene with this annotated function, it is likely that members of the microflora possess this gene to produce PA [151, 159] as this gene has been found in Cyanobacteria, Proteobacteria and Firmicutes, phyla known to inhabit the murine intestine [235].

We hypothesized that the colonization defect of the ∆*STM3602* mutant would correlate with the microbial composition of the host. We used the chronic carriage mouse model to determine the kinetics of the previously observed colonization defect. In competitive infection with the WT in the presence of streptomycin (altered microbiota), the ∆*STM3602* mutant failed to colonize the intestine on day 1 whereas the colonization defect was only apparent in conventional mice beginning at day 5 (Figure 4.8). These results suggest that the colonization defect we observed occurs earlier in animals with disrupted microbiota than with intact microbiota.

Figure 4.8: The Δ*STM3602* mutant fails to colonize the intestine of mice with an altered microflora and fails to persist in the intestine of mice with an intact microflora. Ten CBA/J mice were treated with streptomycin (20mg; closed circles) or an equivalent volume of sterile water (open squares) and infected 48 hours later with ~10^8 CFU of an equivalent mixture of WT and Δ*STM3602* mutant by gavage. Feces were collected on the indicated days. Significant difference in CI (WT/mutant) is indicated by an asterisk (*) and difference between groups is indicated by two asterisks (**) with $P < 0.05$. Analyses for statistical significance determined as in figure 4.1.



Discussion

We report that the putative GntR family regulator STM3602 is necessary for colonization of the murine intestine, similar to published results of a colonization defect in the bovine intestine [106]. Two possible mechanisms for the observed colonization defect are a role for STM3602 in the activation of the TTSS-1 and in metabolism of the microbial-derived product, phosphonoacetic acid. Our data are consistent with a global regulatory role of STM3602 in modulating virulence and metabolism in the infected animal host.

2-AEP was the first phosphonate discovered from sheep rumen [155]. This compound is found in low amounts in feed and is associated with bacterial and protozoal populations within the intestine [156]. 2-AEP is also found in mammalian tissues [157, 236] likely from assimilation from microbial and feed sources because mammals lack the enzymes to produce such molecules [154]. Numerous marine bacterial phyla contain genes for the biosynthesis and degradation of phosphonates [151]. This finding is not restricted to the marine biosphere. The leading bacterial phyla containing phosphonate biosynthetic genes from mammalian and bird microbiome metagenomes are Firmicutes, Proteobacteria, and Bacteroidetes [152]. These phyla are abundant in the murine cecum, although the relative abundance of these phyla is substantially altered following streptomycin treatment [235].

STm lacks the enzymes for synthesis of phosphonates, but possesses four enzymes annotated for the metabolism of these molecules. We have shown that a mutant in Δ*STM3602* has both a colonization defect in the intestine of mammals and fails to utilize phosphonoacetic acid as a sole phosphorus source. The relationship between these phenotypes remains unclear. We found that the Δ*STM3602* mutant fails to colonize the intestine of mice, regardless of whether mice have intact or altered intestinal microbiota. In addition to the difference in microbial composition, these two mouse models differ in the host response. Mice with altered microbiota infected with STm develop neutrophilic inflammation [38], whereas those with intact microbiota do not develop a neutrophilic inflammatory

response [25]. Thus, in addition to alterations in microflora composition, there is substantial difference in the host response that may affect the fitness of the mutant. It is also possible that the host acts as a source of phosphonates, having absorbed them from the microflora and incorporated them into phosphonolipids [157, 236]. Further studies evaluating the fitness of mutants unable to utilize phosphonoacetic acid and other phosphonates during intestinal inflammation in combination with evaluation of the PA content of intestinal fluid and tissue in conventional and germ-free mice will be very informative.

The locus needed for growth on 2-AEP (*phnVUTSRWX*) is under the regulatory control of the Pho regulon [161] but no studies have evaluated the regulation of the *phnABO* operon. Our data suggest that *phnA* is expressed at low levels in rich medium and that the expression of *phnA* is not induced in the presence of PA. Our data also suggest that the expression of *phnA* is not affected by deletion of STM3602. We measured *phnA* expression during log phase growth, a time when *STM3602* is expressed [234]. The phosphorus content of LB broth is undefined, and it is possible that the expression profile of *phnA* would differ in the defined media in the presence of different phosphorus sources. The phenotypes of the Δ*STM3602* mutant have subtle differences depending on starting culture conditions (Figures 4.5 and 4.6) so we can not rule out that different culture conditions would affect the expression of either *STM3602* or the involvement of *STM3602* in *phnA* expression.

GntR family regulators have characteristic N-terminal DNA-binding domains and variable C-terminal small ligand binding domains [201, 237]. The crystal structure of *phnF* from *E. coli* has been solved and a small molecule ligand has been modeled into a binding site [237]. Despite this advance, we are not aware of any experimental data to confirm the identity of the small molecule ligand or the regulon of this gene. STM3602 shares a conserved domain with PhnF, but has only 23.4% amino acid identity. Despite the close phylogenetic relationship of *E. coli* to STm, the mechanisms of phosphonate degradation differ between these organisms. *E. coli* uses the C-P lyase pathway, a relatively non-specific mechanism whereas STm uses the phosphonatase pathway [161, 232, 233, 238]. These observations suggest that the utilization of phosphonates is important for the biology of these similar organisms. Whether STM3602 and PhnF share regulatory targets remains unknown.

NTS use the TTSS-1to invade normally non-phagocytic intestinal epithelial cells and induce a strong neutrophilic inflammatory response [51, 77, 173]. The great importance of this virulence island is illustrated by the fact that salmonellae lacking a functional TTSS-1 are avirulent in calf, pig, and mouse models of infection [28, 47, 51, 173]. The regulatory network controlling the expression of the TTSS-1 is intricate. Transcriptional regulation is carefully controlled by regulatory proteins encoded both within SPI-1 and located elsewhere on the chromosome [186]. The master regulator of the TTSS-1, *hilA*, is encoded within SPI-1 and integrates regulatory input from numerous sources

to activate operons necessary for the production of protein components of the TTSS-1 and its associated effectors (reviewed in [239]).

We have shown a minor role for *STM3602* in the regulation of SPI-1 and invasion of tissue cultured epithelial cells. However, competitive infection of the double mutant in Δ*STM3602* Δ*SP1-1* vs Δ*SPI-1* exhibited a colonization defect in ligated ileal loops in calves (Figure 4.3), suggesting that inadequate activation of SPI-1 does not fully explain the observed phenotype. However, the role of *STM3602* in regulation of SPI-1 remains an interesting area of further study to contribute to the breadth of knowledge on this essential virulence mechanism.

In the study we report here, we have confirmed that STM3602 is needed for colonization of the mammalian intestine. We report that a deletion mutant lacking *STM3602* grows poorly with PA as a sole phosphorus source and that STM3602 has no effect on the expression of *phnA*, the enzyme required for utilization of this compound. Finally, we show that *STM3602* plays a minor role in the regulation of the TTSS-1.

Phosphonates are compounds that are produced by microorganisms and have been identified in the intestine of mammals. Further studies evaluating the role of *STM3602* in co-culture with microorganisms known to produce diverse phosphonates will elucidate the full complement of compounds to which this regulator responds. In addition, definition of the regulon of STM3602 may elucidate novel mechanisms for phosphonate transport or utilization in the large complement of genes of unknown function scattered within the genome of STm.

CHAPTER V

CONCLUSIONS AND FUTURE DIRECTIONS

The first genome sequences of *Salmonella enterica* were published in 2001, one of the host-generalist serotype Typhimurium [129], and the other of the human host-specific serotype Typhi [240]. The availability of these genome sequences ushered the study of *Salmonella* into the genomic era. The availability of two genome sequences from organisms with different host specificity and disease presentation allowed the development of hypotheses as to the similarities and differences between these two serotypes, an area of ongoing research. It is clear from comparison of genome sequences of the serotypes causing enteric and systemic disease that there are specific genetic requirements for survival in these niches [241]. Further work comparing the host-specific systemic serotypes may define gene signatures important for host-specific colonization. These types of analyses provide framework to experimentally test hypotheses regarding host- and niche-specificity with the ultimate goal of development of new therapeutics to combat this pathogen.

Approximately 25% of the genome of STm contains genes that are absent in close phylogenetic relatives. It has been hypothesized that these genes are necessary in the lifecycle of *Salmonella* in the environment and in the host, and some may be necessary for growth and virulence STm during infection

[97]. To test this hypothesis, a library of ~1000 mutants in *Salmonella*-specific genes was constructed and used to for systemic infection of *Salmonella*-sensitive mice. This carefully designed genetic screen identified 52 genes predicted to be important for systemic infection in mice, not including those in known virulence determinants. In the work described here, the same library was used to test the hypothesis that STm uses specific genes for early colonization of the intestine of the calf, a natural host of *Salmonella* [106]. In this screen a total of 54 genes of predicted necessity during early enteric infection were identified. Of these, 31 were in genes not previously reported to be essential for enteric infection, and 21 not previously predicted to be needed for this purpose. Not surprisingly, only 7 genes outside of known virulence pathways were predicted to be necessary for both enteric and systemic infection.

From direct comparison of the results of these two genetic screens that used the same starting library and same methodology for evaluating mutant abundance in the pool, we have gained insight into the genes necessary for *Salmonella* to cause enteric and systemic infection. Similar work has been done by another group using highly complex transposon-insertion libraries to identify host-specific colonization genes [100, 105]. However, these studies lack experimental verification in the target animal species, making it difficult to determine the relative importance of each predicted gene as a host-specific factor. Unfortunately, the extreme differences between calf ligated ileal loops and mouse systemic infection preclude the assignment of each of these genes

into host- or niche-specific colonization requirements. Further work is needed to test the hypothesis that the genes predicted to be niche-specific by these screens are, in fact, niche-specific and do not represent host-specific colonization factors. The candidate genes needed for colonization of both mammalian hosts warrant further exploration to determine their function as they likely represent good targets for development of therapeutic interventions that may be extrapolated to other mammalian hosts.

Surprisingly, one of the genes needed during both bovine and murine infection encodes a putative reverse transcriptase. This gene was of immediate interest to us, because reverse transcriptases are known to be essential for the pathogenesis of retroviruses, but had not previously been described in the pathogenesis of bacteria. The STm reverse transcriptase is encoded on a horizontally-acquired operon called a retron that makes a covalently linked RNA-DNA hybrid molecule. This molecule, multicopy single-stranded DNA (msDNA), has been studied for 30 years, but no known phenotypes have been associated with mutants unable to produce the molecule. Using knowledge of the biology of the host gastrointestinal tract, we identified the first *in vitro* phenotype for a mutant deficient in msDNA: inadequate growth in anaerobic conditions. This phenotype allowed us to determine that msDNA is needed for adequate production of over 230 proteins, only during anaerobiosis. This study is an example of functional identification of a gene product in the context of infection of a host, using the host biology as a guide to discovery and highlights the

importance of knowledge of host physiology for the study of bacterial pathogenesis.

I hypothesize that msDNA, a covalently linked RNA-DNA hybrid molecule, represents a new class of regulatory molecules. It is unlikely that msDNA directly alters the abundance of more than 230 proteins. A more likely explanation is that it exerts its effects by altering a global regulatory process. This molecule may bind directly to other nucleic acids or nucleic acid-binding proteins to exert its regulatory function. It is possible that this molecule can regulate transcription by directly binding DNA in a manner that may or may not depend on the primary nucleotide sequence to alter the binding of RNA polymerase or other regulatory molecules such as transcriptional regulators or small RNAs. Another possibility is that this molecule may bind to mRNA transcripts creating RNA-RNA or RNA-DNA duplexes that may alter the kinetics of mRNA degradation. It may also have a direct effect on translation by binding to rRNA preventing the ribosome from forming normally. Finally, it may have an effect on either transcription or translation through interaction with a nucleic acid-binding protein and altering the function of that protein.

The data suggest that msDNA is needed only during growth in the absence of oxygen, as deletion of this molecule has no obvious consequences during growth under standard aerobic laboratory conditions. msDNA is produced in large amounts during exponential growth in both aerobic and anaerobic conditions, but has no apparent role in aerobic growth. This suggests that the

molecular target of msDNA is not present under standard laboratory conditions, but only during anaerobiosis. The role of oxygen in the mechanism of msDNA function is a fascinating area for further study.

There are numerous potential functional portions of msDNA. It is possible that either the RNA or DNA encode the functional region. Additionally, the primary sequence or secondary or tertiary structures may relate to the function of this molecule. Additional experiments are needed to (i) determine whether the RNA or DNA portion of the molecule contain the functional region and (ii) identify whether function depends on primary sequence or secondary structure. These experiments are essential to elucidate the mechanism of msDNA function.

Retrons have been identified in numerous other pathogenic bacteria, opening the possibility that inhibition of the reverse transcriptase or of the function of msDNA may become a new antimicrobial strategy to combat bacterial pathogens in an era of increasing antibiotic resistance [21]. This pathway for antimicrobial discovery is particularly attractive because it can use the framework set by decades of research into the reverse transcriptase of HIV-1 and use the hundreds of small molecule libraries that have already been generated to inhibit this enzyme. Future mechanistic work on the retrons of STm, Typhi, and other enteric pathogens (EPEC, *Shigella*, *Vibrio*, etc.) has the potential to generate broad-spectrum therapeutics, some of which may have already been used in safety trials in people in the context of retroviral inhibition.

I also examined the role of *STM3602*, encoding a putative transcriptional regulator in enteric colonization. There are many classes of transcriptional regulators, some affect gene expression of neighboring operons while others have *trans* regulatory properties, some respond to the presence or absence of a metabolite while others are activated in a hierarchical fashion. This diversity makes transcriptional regulators a challenging class of proteins to study. *STM3602* shares a conserved domain with a regulator of phosphonate metabolism. Though experiments described in this proposal, we have identified a role for this regulator in metabolism/utilization of phosphonoacetic acid. The importance of this finding during infection of a host remains to be determined.

Many questions have arisen from this work. The only phosphonate to be identified in the mammalian intestine is 2-AEP. However, to my knowledge no studies have surveyed mammalian intestinal contents to determine the full complement of these molecules in this niche. Published data suggest that organisms that produce these molecules are in the intestine. I hypothesize that there are numerous phosphonate species in intestinal contents, produced by microorganisms for their antimicrobial effects as well as to store phosphorus, an element that may be limited in certain portions of the intestine. Phosphonate-containing molecules could represent a mechanism of interspecies competition in this niche. Second, the complement of phosphonates incorporated into host cells is also unknown. It is possible that the host incorporates these molecules of microbial origin secreted directly from intestinal microbes or indirectly from feed

sources. These molecules in the form of phosphonolipids could increase in availability during epithelial damage as a result of *Salmonella* infection. Finally, it remains to be determined whether compounds containing these molecules or organisms producing these molecules could have therapeutic roles to clear *Salmonella* from the intestinal tract of an infected animal. Numerous phosphonate-containing compounds are in clinical use as antimicrobials (and have other functions) and much work has been done to elucidate the mechanism of both biosynthesis and chemical synthesis of molecules containing a carbon-phosphorus bond.

The role of phosphonates in the pathogenesis of enteric salmonellosis merits further work. I hypothesize that there is a small contribution of feed material to the overall phosphonate content of the host but that the host colonized with phosphonate-producing microorganisms will have a greater phosphonate content of both ingesta and host tissues. Germ-free mice are a valuable resource for these studies. First, it is necessary to determine the complement and quantity of phosphonates in ingesta and incorporated into tissues of germ free animals. This will determine the relative contribution of feed matter to host phosphonate content. Subsequent studies to evaluate the contribution of bacteria to the phosphonate content of both ingesta and host tissues can be performed through recolonizing littermates with single or multiple bacterial species. I believe it is possible to control the phosphonate content of intestinal contents and tissues through feeding these compounds directly or

through colonization of germ-free mice with phosphonate-producing microorganisms. These studies are a necessary start to identify the role of these compounds in the pathogenesis of *Salmonella* as well as the role of *STM3602* in the colonization of the host.

The work presented here is the starting point for the study of new strategies used by *Salmonella* to colonize the host. *Salmonella* is one of the most heavily studied pathogens because it is genetically tractable, has many metabolic similarities to the model organism *E. coli*, and has great importance for animal and human health. I have shown that interrogation of the genome using carefully chosen animal models of infection will identify new and interesting genes needed for colonization of the host in this heavily studied organism. This type of study is critical because the number of cases of enteric salmonellosis has not changed despite an overall decrease in the number of cases of all bacterial foodborne disease [95]. Additionally, there is an emergence of invasive NTS that has the potential to cause life-threatening bacteremia [22]. Finally, the number of NTS that are resistant to available antibiotics is increasing but there is not a corresponding increase in the development of new antibiotics [21]. Therefore, further work on pathogenesis using relevant and carefully chosen animal models of disease is critical to understand the biology of this organism within a host. Each of the genes described in this work represent a new target for a therapeutic intervention or a candidate modified live vaccine strain. By studying pioneer genes outside the known virulence pathways, there

is the opportunity for tangible advances in the field of *Salmonella* pathogenesis with the ultimate goal of reduction in the morbidity and mortality caused by this highly successful pathogen.

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