THE REGULATION OF *SALMONELLA* TYPHI VI CAPSULAR ANTIGEN EXPRESSION IN INTESTINAL MODEL EPITHELIA AND THE BOVINE LIGATED-ILEAL LOOP MODEL

A Dissertation

by

QUYNH TIEN-NGOC TRAN

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

DOCTOR OF PHILOSOPHY

August 2009

Major Subject: Veterinary Microbiology

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Approved by:

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ABSTRACT

The Regulation of *Salmonella* Typhi Vi Capsular Antigen Expression in Intestinal Model Epithelia and the Bovine Ligated-Ileal Loop Model. (August 2009)

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Salmonella enterica serovar Typhi, a major public health concern in developing countries, continues to be a priority for the World Health Organization. *S.* Typhi possesses a *viaB* locus responsible for the biosynthesis of the Vi-capsular antigen, a significant virulence factor at the focus of developing improved prophylaxis for typhoid fever. Tissue culture experiments have demonstrated that *S.* Typhi wild-type capsule-expressing strain elicits less chemokine secretion than a *viaB* mutant. Calf experiments using the *viaB* mutant resulted in an increase inflammatory response. Osmolarity is one of the control signals that affect the biosynthesis of the Vi antigen. Under high osmolarity growth conditions of 300 mM and greater, Vi production is suppressed and *S.* Typhi is highly invasive. Studies reveal that the *viaB* mutant displays increased invasion towards intestinal epithelial cells. Our first objective was to implement direct and indirect methods to localize and detect Vi expression within intestinal epithelial cells and bovine Peyer's patch. The second objective was to compare the invasiveness between a

viaB mutant, an *ompR* mutant, and *S.* Typhi grown under hyperosmolarity. We also measured the effects of these strains in eliciting inflammation in the calf model.

We report that *tviB* was significantly up regulated intracellularly within T84 polarized cells. In the calf experiments, *tviB* was expressed at levels significantly higher in calf tissue following invasion compared to inoculum grown under Vi-suppressing conditions. Together, these results support the idea that the Vi capsular antigen is expressed after invasion of intestinal epithelial cells *in vivo.*

We found that *S.* Typhi grown under high osmolarity, the *viaB* mutant, and the *ompR* mutant had increased invasion in polarized T84 cells and bovine ileal tissue. Fluid accumulation among Vi-deficient and Vi-suppressed strains was similar. The histopathology of the inflammatory lesions of the small intestine produced by the Vi-deficient and suppressed strains was quite comparable. Our data supports the notion that Vi-suppressed and Vi mutants of *S.* Typhi exhibit similar levels of increased invasion and inflammation, perhaps mechanistically through the inactivation of the Vi antigen.

DEDICATION

To all who believe dreams do come true

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Thank you Pippi for always being so happy! It has been a long journey, but I'm so lucky you've been on it with me this entire time!

NOMENCLATURE

RT-PCR	Real Time-Polymerase Chain Reaction
DMEM	Dulbecco's Modified Eagle Medium
LBH	300 mM Sodium Chloride Containing Luria Bertani Broth
SOB	10 mM Sodium Chloride Containing Luria Bertani Broth
LB	Luria Bertani Media
egfp	Encoding Green Fluorescent Protein
TLR	Toll Like Receptor
PAMP	Pathogen Associated Molecular Pattern
MHC	Major Histocompatibility Complex
T3SS	Type III Secretion System
SPI-1	*Salmonella* Pathogencity Island 1
SPI-2	*Salmonella* Pathogencity Island 2
SPI-7	*Salmonella* Pathogencity Island 7
LPS	Lipopolysacharride

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CHAPTER I

INTRODUCTION

BACKGROUND

The organism. *Salmonella* Typhi, the etiological agent of typhoid fever, is responsible for 16 million cases of human disease and 600,000 deaths worldwide each year (68). This disease is endemic to many developing countries including regions of Africa, South America, and Asia. Children living in endemic areas, travelers to affected locations, and microbiological laboratory workers are among those at particularly high risk for contracting typhoid fever. This gram-negative enteric bacillus belongs to the family *Enterobacteriacea,* and it is a motile, facultative anaerobic intracellular pathogen. Epidemic recurrences of typhoid fever remain among the most costly human infections in terms of both morbidity and mortality (107, 74).

Transmission. *S.* Typhi is transmitted via contaminated water and food and is strictly host adapted to humans. Due to the recent emergence of multidrug-resistant strains, the treatment of *S.* Typhi infection has proven to be difficult and poses a serious threat to future treatment options (6, 75, 86, 106). Infected patients present with a history of prolonged fever, headache, abdominal discomfort, and general lethargy.

This dissertation follows the style of Infection and Immunity.

Around 10% of these develop severe or complicated disease and without necessary treatment 5-30% of typhoid fever patients may die.

Pathogenesis: typhoidal versus non-typhoidal salmonellosis. Experiments using the calf model and cultured human cell lines have elucidated some of the mechanisms by which *S.* Typhimurium causes the massive neutrophil influx infiltrate in the intestine, which is a pathological hallmark of human non-typhoidal enteritis. The invasion-associated Type III secretion system (T3SS-1), encoded by Salmonella pathogenicity island 1 (SPI-1), allows *S.* Typhimurium to invade intestinal epithelial cells *in vitro* (29, 84). The mechanisms in which *S.* Typhimurium and *S.* Typhi use for invasion (26) and for intracellular trafficking are quite similar. Once *S.* Typhimurium invades, like *S.* Typhi, it induces membrane ruffling and cytoskeletal rearrangements upon contact with HeLa cell surfaces. Ruffling induced by *S*. Typhi and *S.* Typhimurium is then accompanied by macropinocytosis and aggregation of cell surface class I MHC (26). Once *S.* Typhimurium crosses the intestinal epithelial barrier, the innate immune system is able to recognize pathogen-associated molecular patterns (PAMPS) by toll-like receptors (TLRs) expressed by intestinal epithelial cells. TLR5, which recognizes flagella, appears to be preferentially expressed at the basolateral pole of intestinal epithelial cells *in vitro* (31) and *in vivo* (12). The TLR stimulation leads to the production of CXC chemokines (e.g. IL-8), which consequently leads to the recruitment of a massive neutrophil influx characteristic of gastroenteritis in humans. One mechanism by which neutrophils may contribute to diarrhea is related to neutrophil

de-granulation and the generation of tissue injury, resulting in damage to the epithelial barrier and leakage of extravascular fluids, allowing liquid to flow from blood into the intestinal lumen (111). Both *S.* Typhi and *S.* Typhimurium secrete known effectors, SipA, SopB, SopD, SopE, that contribute to invasion and inflammation in the host; however, SopE2 and SopA are pseudogenes in *S.* Typhi. In addition, it has been found that the alteration of these effectors do not contribute to the strict host adaptation of *S.* Typhi (83).

S. Typhi possesses a single, monophasic flagellum (*fliC*) that has the same antigenic specificity whereas *S.* Typhimurium produces a biphasic (*fliCfljB*) flagella that has two different H antigenic specificities. The role of flagella is believed to aid in motility and is generally associated with the extracellular life of the pathogen. However, continuous research is revealing the significance of novel roles of flagella during salmonellosis.

S. Typhi is highly host adapted to humans and higher nonhuman primates. Thus, there is a lack of animal models suitable to study the molecular mechanisms of this pathogen as well as its host-pathogen interactions. The *S.* Typhimurium murine model of infection is commonly used to study the pathogenic mechanisms of typhoid fever. *S.* Typhimurium is a zoonotic pathogen that can cause enteritis in a broad host range. Upon comparison of the clinical syndromes of typhoidal with those of non-typhoidal salmonellosis (caused mainly by *S.* Typhimurium and other *Salmonella enterica* serovars), significant differences in their pathology and host agent interactions are exposed. First, *S.* Typhi causes a systemic infection characterized by bacterial

colonization of the liver, spleen, bone marrow, mesenteric lymph nodes and Peyer's patches. The propagation of *S.* Typhi at systemic locations is believed to occur within macrophages and evidence provided by *in vitro* studies demonstrate that the organism survives well in human monocyte derived macrophages and other macrophage cell lines (21, 24, 42, 45, 46, 90). SPI-2 has been shown to be important for the survival of *S.* Typhimurium and *S.* Typhi in macrophages. In contrast, *S.* Typhimurium infection in human causes a localized gastroenteritis, while bacteremia only occurs in a small percentage of patients (approximately 1%). Typhoid fever also has a longer incubation period (median of 5-9 days) and longer duration of symptoms (fever lasting for approximately 3 weeks) when compared to humans infected with non-typhoidal salmonellosis. The incubation period of gastroenteritis caused by *S.* Typhimurium is short (12-72 h) and is followed by a short course of disease (< 10 days), suggesting that the infection is successfully cleared by the host's immune response.

Another major difference between typhoid fever and *S.* Typhimurium-induced diarrhea in human is the type of inflammatory response elicited in the intestine. The intestinal pathology observed during *S.* Typhimurium infection in humans is dominated by a massive neutrophil influx in the terminal ileum and proximal colon. Analysis of intestinal biopsies from patients infected with *S.* Typhimurium reveals an infiltrate primarily composed of neutrophils, although mononuclear cells are also present (19, 66). Similarly, neutrophils are the predominant cell type (75% fecal leukocytes) in stools from patients diagnosed with non-typhoidal salmonellosis (36). In contrast, typhoid fever is not a typical diarrheal disease and biopsies from typhoid fever patients or from

volunteers infected with *S.* Typhi reveal an infiltrate dominated by mononuclear cells (53). Though *S.* Typhimurium and *S.* Typhi share similar mechanisms in invading the intestinal epithelium, penetration of the intestinal mucosa by *S.* Typhi does not induce IL-8 production from intestinal epithelial cells nor does it trigger the same massive neutrophil infiltration seen with *S.* Typhimurium. *S.* Typhi infection does however cause the production of IL-6 production due to unclear mechanisms (107). In addition, diarrhea is not a significant sequelae to human infection with *S.* Typhi, in which diarrhea only develops in 1% of typhoid fever patients and only after the onset of fever (44). Differences in disease manifestations in humans between *S.* Typhi and *S.* Typhimurium indicate that *S*. Typhi possesses distinct virulence factors important in its pathogenesis.

Animal models of infection. Our knowledge of the pathogenesis of *S.* Typhi is limited due to the fact that it only infects humans, resulting in the lack of *in vivo* models to study host-pathogen interactions. The implementation of a variety of cell culture lines have elucidated some of the molecular mechanisms in which *S.* Typhi causes disease; however *in vivo* studies are still warranted in order to validate *in vitro* findings and to promote further understanding of typhoid fever pathogenesis in the host. Much of our information comes from observations in clinical settings, mainly conducted during the first half of the 20th century, and in the course of experimental challenge studies in healthy adult volunteers carried out in the 1950s and 1960s (44, 60). Two animal models have contributed significantly to our understanding of the human pathogenesis of

typhoid infection: oral challenge of chimpanzees with *S.* Typhi (22, 28) and oral or systemic challenge of mice with *S*. Typhimurium (13, 15).

Initial studies conducted at the Walter Reed Army Institute showed that orally inoculated chimpanzees with *S.* Typhi develop a clinical illness that mimicked human typhoid fever (22, 28). The animals experienced a bacteremia, produced fever for 10 days, and excreted bacteria in their stools. Differences in clinical typhoid infections between chimpanzees and humans included a shorter incubation period followed by a milder clinical course of disease in the animal model. The systemic spread of the disease progressed from the primary site of infection, the intestinal lymphoid tissue and metastasized to the general lymphatic system and subsequently a circulating bacteremia. *S.* Typhi was found in the liver and spleen, in addition to the bile and gallbladder. Pathological examination of lesions present in the intestinal mucosa and lymphoid tissue, spleen and liver of infected animals resembled those seen in humans. Chimpanzees also had serum antibody and responses to *S.* Typhi LPS and flagella.

The use of higher non-human primates in research has declined in popularity overtime due to the expense and public concerns. Therefore, most studies have relied on a murine model of human typhoid that uses *S.* Typhimurium, which causes a typhoid-like illness in mice (90). As a consequence, most of what is known about the pathogenicity of *S.* Typhi has been extrapolated from *S.* Typhimurium infections in mice. A significant limitation to using this murine model is that *S.* Typhimurium does not cause typhoid fever in humans, but instead causes a localized purulent gastroenteritis resulting in diarrhea.

Mice infected with *S.* Typhimurium exhibit systemic disease resembling human typhoid fever. Therefore, the pathogenesis of typhoid fever has been commonly studied using *S.* Typhimurium infection in mice as an animal model. The intestinal pathology caused by *S.* Typhimurium in mice is similar to that observed in human typhoid fever patients and is characterized by edema and mononuclear cell infiltrates of villi which become shortened in height (91). Capillary thrombosis, hemorrhage, and ulcerations may be present in the ileum at areas of Peyer's patches while the epithelium in other areas remains largely intact (7, 53, 90, 94). In susceptible mice, rapid bacterial migration to the liver and other internal organs causes death within 6-10 days following *S.* Typhimurium infection (48).

More recently, studies have focused on the calf as a model for *S.* Typhimurium-induced enterocolitis (89, 98, 99, 110, 112). Calves develop diarrhea within 12-24 h post-ingestion of *S.* Typhimurium (98). The infection typically remains localized to the intestine and mesenteric lymph nodes (98, 109). Calves develop a necrotizing fibrinopurulent enterocolitis characterized by a severe, diffuse infiltration of polymorphonuclear leukocytes (22). In contrast, mice infected with *S.* Typhimurium develop diffuse moderate mononuclear enteritis in the small intestine characterized by a predominantly mononuclear leukocyte infiltrate with no association with diarrhea (91). The clinical and pathological features of *S.* Typhimurium infection in calves parallel the disease in man and provide a natural human model of disease.

In addition, the use of the calf ligated-ileal loop model has been used to study the pathogenesis of *S.* Typhi (82, 83, 91). Moreover, the role of the Vi capsular antigen, an

important virulence factor present in *S.* Typhi but absent in *S.* Typhimurium, has been shown to reduce inflammation in the calf model and research findings illustrate that the model is suitable for studying the effects of Vi antigen expression *in vivo* (82). Thus, the calf model is a good animal model that allows us to perform comparative studies investigating *S.* Typhi virulence factors that are absent from *S.* Typhimurium and those that may be responsible for allowing *S.* Typhi to cause typhoid fever in humans.

Current prophylaxis and treatment. The Vi capsular polysaccharide of *S.* Typhi is an important virulence factor and protective antigen during its pathogenesis. The Vi antigen is believed to block toll-like receptor recognition of pathogen associated molecular patterns and allows *S.* Typhi to go systemic by resisting complement-mediated phagocytosis. Immunization with a capsule-containing vaccine has been shown to confer protective antibody responses against typhoid fever and is generally considered efficacious. Thus, the Vi capsule is and continues to be a significant focus for the development of improved typhoid fever vaccines.

Inactivated whole-cell parenteral vaccines have been used previously to prevent typhoid fever infections, but the adverse associated reactions have made it unpopular as a public health vaccine. As a result, two vaccines have been developed in the past fifteen years and licensed for protection against *S.* Typhi. The first one is a parental vaccine based on the isolation of a purified Vi capsule polysaccharide from the blood of an infected patient. The second one is a live attenuated oral vaccine derivative of *S.* Typhi Ty21a strain.

Immunization with the capsular polysaccharide vaccine (Typhim Vi) has been shown to be effective in its ability to stimulate protective antibody responses against typhoid fever (40). Conversely, inoculation with the vaccine produces some adverse side effects. For example, 17% of adult recipients and 86.7% of children recipients experience non-severe and transient local pain at the injection site (40). The vaccine is given subcutaneously at a single dose of 25 μg to persons over 2 years of age, confers protection within 7-10 days post-inoculation, and requires 3-year boosters (57, 68).

The second licensed typhoid vaccine that is currently available is the *S.* Typhi strain Ty21a live, attenuated oral vaccine (Vivotif). Ty21a is characterized by a *galE* mutation and its inability to produce the Vi antigen (68). This mutation is not solely responsible for the attenuation of the Ty21a strain since a defined *galE* and Vi negative mutant still maintained some virulence when tested in human volunteers (43). This suggests that the mutations that attenuate Ty21a remain unclear. Hypothetically, a problem that may arise with the use of Ty21a is the ability to revert back to a virulence form, although no known cases have been reported (67).

Similar to the Vi capsular antigen vaccine, Ty21a has been proven to be effective and safe in several vaccine trials (8, 16, 57-59, 105). Ty21 elicits a moderate immunogenic response and requires at least 3-4 doses initially (8, 57-59, 72). Thus, the vaccine is usually given at the beginning as three to four bacteria-containing capsules on alternate days and requires boosters every five years (68), although a liquid formulation of the Ty21a vaccine has been shown to improve the protectiveness (57, 59) .

New generations of typhoid vaccines are being studied and developed to replace the current ones due to their drawbacks. One weakness associated with the use of the parenteral vaccine is the greater number of side effects that occur; though the oral vaccine may have fewer side effects, it may confer a reduced immunogenicity. The Ty21a strain vaccine offered less protective immunity with the simultaneous administration of antimalarial drugs (52). However, both vaccines are equally effective and offer 65% to 75% protection against the disease. Nevertheless, some of the innovative vaccines include a parenteral Vi polysaccharide-protein conjugate that is expected to produce higher antibody titers following initial and booster immunizations than the Vi vaccine (68) and a number of genetically defined attenuated strains of *S.* Typhi. Shortfalls in both vaccines and a greater understanding of the genetics of *S.* Typhi virulence and recombinant DNA technology promotes the search for a more efficacious and better defined vaccine.

The Vi (virulence) capsular antigen. Whole genome sequencing has revealed that *S.* Typhi genome contains 601 genes on 82 genetic islands that are absent from *S.* Typhimurium genome (76). The largest of these islands termed SPI-7, contains 134 kb of *S.* Typhi-specific DNA and carries biosynthesis genes (*viaB* locus) for the production of the Vi capsular antigen, a linear polymer of α-1,4 2-deoxy-2-N-acetylgalacturonic acid variably O-acetylated at the C3 position (38, 41) and the genes encoding Type IVB pilus, a structure involved in the invasion of intestinal epithelial cells (113). The Vi-antigen is expressed *in vitro* in macrophages (17, 24) and during human infection, as

indicated by the infection of anti-Vi antibodies in patient serum (Widal test) and the fact that with vaccination with Vi antigen confers protection against typhoid fever (49, 50, 62, 85). Some *S.* Typhi isolates were reported to lack the ability to express the Vi antigen as determined by slide agglutination (3, 87). However, a prospective analysis of clinical *S.* Typhi isolates for the presence of *viaB* by polymerase chain reaction (PCR) and for Vi expression by a more sensitive technique (i.e. immunofluorescence) found that *S.* Typhi isolates that are Vi-negative by slide agglutination are in fact Vi-positive and carry the *viaB r*egion (106). Although the Vi antigen appears to be present in fresh clinical isolates, SPI-7 can be lost by deletion upon passage or storage of *S.* Typhi in the laboratory (10, 73). Most importantly, the role of the Vi antigen during host-pathogen interactions is still poorly understood.

Recent *in vitro* observations have revealed that the expression of the Vi capsular antigen is regulated by osmolarity. Under conditions of high osmolarity, as expected in the intestinal lumen, the Vi antigen is turned off (78), while flagella and the T3SS-1 genes are expressed (2). When *S.* Typhi wild type is cultured in 300 mM NaCl containing Luria-Bertani broth (LBH), the expression of the Vi antigen is suppressed, but the secretion of invasion proteins (SipB, SipC, SipA) is increased (114).

Under the same conditions, *S.* Typhi wild type is highly invasive and destructive towards epithelial and M cells of rat's Peyer's patches (114). These data suggest that the Vi antigen may not be expressed in the intestinal lumen and possibly allow *S.* Typhi to have a more invasive phenotype as it penetrates the intestinal epithelium. In addition, the mechanisms by which Vi expression and invasion are controlled appear to act in

opposition to one another. Infection of polarized human epithelial cells (T84 cells) and human macrophage-like cells (THP-1) with a capsulated *S.* Typhi significantly reduces the amount of IL-8 production compared to a non-capsulated mutant (80). Furthermore, these findings support the notion that the Vi antigen may be expressed after invading cells and inhibit PAMP recognition by TLRs and resulting in no IL-8 production.

The two component positive regulatory systems, RcsBC and OmpR EnvZ, in addition to the promoter of the *viaB* region, located upstream of *tviA*, have been identified as contributors to Vi expression and are modulated by osmolarity. Under low osmolarity conditions, the production of Sip proteins, flagellin, and Vi antigen is differentially modulated by the RcsB-RcsC regulatory system. The transcription of *iagA*, *invF,* and *sipB* genes is negatively controlled by the RcsB regulator (2). The TviA protein is not essential for Vi synthesis, but it does function as a positive regulator in co-transcribing the *tviA* and *tviB* genes (37, 104). When the *tviA* gene is disrupted, the expression of the Vi antigen is strongly decreased. In addition, the TviA protein may act in concert with the RcsB protein at the *tviA* promoter to activate transcription of the genes involved in the Vi synthesis (103). Hence, the regulations of Vi expression under the control of the promoter of *tviA* and its co-regulatory functions with *rcsB* have not been clearly elucidated. *S.* Typhi strains harboring *ompR* deletions no longer agglutinated with Vi anti-serum (78), indicating that somehow the *ompR-envZ* system is involved in the Vi biosynthesis. Recently, other components of the *S. Typhi* genome have been implicated for involvement with Vi expression. The type IVB pillus has been shown to assist in the invasion of intestinal epithelial cells (113) and evidence suggests

that the pilus production and Vi synthesis may be regulated under similar promoters and that Vi synthesis precedes pilus production (54). The *rpoS* gene, which is a master regulator in stress response and required for survival under extreme conditions in *S.* Typhimurium, has been implicated as another regulator of Vi synthesis of *S.* Typhi (88).

Polysaccharide capsules are found on the surface of a wide range of gram-negative bacteria. Capsules have a significant role in determining access of certain molecules to the cell membrane, mediating adherence to surfaces, and increasing tolerance of desiccation. Furthermore, capsules of many pathogenic bacteria impair phagocytosis (63) and reduce the action of complement-mediated killing. Consistent with this belief, the Vi-antigen was shown to impede uptake of *S.* Typhi by human neutrophils (64). Thus, the Vi capsular antigen is likely to be major virulence determinant of *S.* Typhi.

Osmolarity has been shown to be one of the signals that control Vi capsule expression in *S.* Typhi *in vitro* (2, 78, 114). *S.* Typhi grown under hyperosmotic conditions in LBH medium suppresses Vi expression where as growing *S.* Typhi under hyposmotic conditions in SOB medium upregulates Vi expression. This research finding corresponds to the different stages of *S.* Typhi pathogenesis as it encounters different osmolarities *in vivo*. In the gut lumen, hyperosmotic conditions exist due the enzymatic digestion of macromolecular foodstuffs in addition the presence of sodium, chloride, and potassium ions in the intestinal fluid. In tissue and blood, the conditions are hypososmotic compared to the intestinal lumen as a result of homeostatic mechanisms. The Vi may be downregulated in the gut, facilitating interactions of *S.*

Typhi with epithelial cells, while it is upregulated in blood, where it is known that Vi is important for this stage of infection and in tissue, where it may inhibit TLR recognition. Therefore, the central hypothesis of this study is during *S.* Typhi invasion of intestinal epithelial cells, the Vi antigen is downregulated, which enhances the secretion of the SPI-1 (T3SS) effector proteins and promotes a more invasive phenotype, possibly allowing invasion into deeper tissues. Following invasion, the Vi is upregulated, blocking innate immune recognition of PAMPs. This theory has been formulated on previous studies that expression of the Vi antigen causes decreased levels of pro-inflammatory cytokine production in human epithelial cells (80) and in the calf ligated ileal-loop model (82).

The second aspect of this study involves the *ompR* regulator and unraveling its role in the expression of the Vi antigen and activation of invasion-associated genes. OmpR-Envz, the two-component regulatory system, has been shown to be a positive regulator of Vi expression (78), in which the construction of a *S.* Typhi *ompR* mutant no longer produced the Vi polysaccharide. Prior studies have shown that a *viaB* mutant and *S.* Typhi grown under Vi-suppressed secreted increased levels of invasion proteins and demonstrated increased invasion (71, 80, 114). Accordingly, we believe that the *ompR* mutant possesses a hyperinvasive phenotype through the inactivation of Vi expression. This would further support the idea that the mechanisms by which Vi expression and invasion are controlled appear to act in opposition to one another. However, currently there are no convincing reports of a S. Typhi *ompR* mutant demonstrating whether or not there is an increase invasion associated with this Vi-deficient strain. As a result, we

wanted to test our *ompR* mutant in the T84 polarized cell experiments and the calf ligated loop-model and study invasion.

In the first set of experiments, we tested the hypothesis that the Vi antigen will be upregulated and expressed following invasion of intestinal epithelial cells by infecting T84 polarized cells and inoculating calf ligated-ileal loops with *S.* Typhi and demonstrating Vi expression. We first constructed a *gfp* fusion to the promoter of the *viaB* region in *S.* Typhi and determined the fluorescence population between intracellular and extracellular bacteria in T84 experiments by using flow cytometry. We performed real-time PCR experiments to analyze Vi capsule expression and invasion genes in T84 polarized and the calf ligated-ileal loops. Ultimately, we directly detected the presence of the Vi capsule in calf ileal tissue with fluorescence immunocytohistochemistry.

In the second set of experiments, we tested and compared the *ompR* mutant, *viaB* mutant, *S.* Typhi grown under Vi-suppressing conditions, and *S.* Typhi grown under Vi-expressing conditions. We first analyzed the protein secretion profile from the supernatant of each strain. We conducted RT-PCR analysis to study the expression of invasion genes associated with each strain. We infected T84 polarized cells and inoculated calf ligated-ileal loops to perform invasion analysis. Lastly, slides from the calf ileal tissue were read and scored according to a scale for inflammatory changes.

Vi antigen expression has not been studied adequately in different cells lines nor has it been examined extensively in animal models. In addition, the mechanism in which the *ompR* regulon mediates Vi expression and affects invasion remains unclear.

Our experiments elucidated how *S.* Typhi may have adapted to the natural host environment and exploited the host environmental niche in order to promote disease in humans. Our results revealed some of the molecular mechanisms of the Vi capsular antigen expression and support its role in allowing *S.* Typhi to evade the host innate immunity.

CHAPTER II

VI ANTIGEN EXPRESSION IN CULTURED INTESTINAL EPITHELIAL CELLS AND BOVINE PEYER'S PATCH

INTRODUCTION

Typhoid fever is an acute, systemic infection of the reticuloendothelial system caused by *Salmonella enterica* serotype Typhi, that is annually responsible for an estimated 16 million illnesses and 600,000 deaths worldwide (68). Our knowledge of the pathogenesis of *S.* Typhi is limited because it only infects humans and higher non-human primates (28), resulting in the absence of *in vivo* models to study host-pathogen interactions. Many studies have relied on a murine model of human typhoid that uses *S. enterica* serovar Typhimurium, which causes a typhoid-like illness in mice. As a consequence, most of what is known about the pathogenicity of *S.* Typhi has been extrapolated from *S.* Typhimurium infections in mice. A significant limitation to using the murine model is that *S.* Typhimurium does not cause typhoid fever in humans, but instead causes a localized gastroenteritis resulting in diarrhea.

Whole genome sequencing has revealed that *S.* Typhi genome contains 601 genes on 82 genetic islands that are absent from the *S.* Typhimurium genome (76). The largest of these islands termed SPI-7, contains 134 kb of *S.* Typhi-specific DNA and carries biosynthesis genes (*viaB* locus) for the production of the Vi capsular antigen, a linear polymer of α-1,4 2-deoxy-2-N-acetylgalacturonic acid variably O-acetylated at the C3 position (38, 41). The Vi capsular antigen is a significant virulence factor for

typhoid fever as strains positive for Vi production have higher rates of infection (43, 44), and it continues to be the focus for improvement in current treatment and prophylaxis for this disease.

The two component positive regulatory systems, RcsBC and OmpR EnvZ, in addition to the promoter of the *viaB* region, located upstream of *tviA*, have been identified as contributors to Vi expression and are modulated by osmolarity. Under low osmolarity conditions, the production of Sip proteins, flagellin, and Vi antigen is differentially modulated by the RcsB-RcsC regulatory system. The transcription of *iagA*, *invF,* and *sipB* genes is negatively controlled by the RcsB regulator (2). The TviA protein is not essential for Vi synthesis, but it does function as a positive regulator in co-transcribing the *tviA* and *tviB* genes (37, 104). In addition, the TviA protein may act in concert with the RcsB protein at the *tviA* promoter to activate transcription of the genes involved in the Vi synthesis (103). *S.* Typhi strains harboring *ompR* deletions no longer agglutinate with Vi anti-serum (78). The *rpoS* gene, which is a master regulator in the stress response and required for survival under extreme conditions in *S.* Typhimurium, has been implicated as another regulator of Vi synthesis of *S.* Typhi (88). Indeed, extensive work has been attempted to characterize the regulation of the Vi expression *in vitro*; however, studies have shown that the gene regulation in infected hosts can markedly differ from what has been expected based on *in vitro* and cell culture work (5, 55, 56), stressing the need for *in vivo* studies to understand *Salmonella* virulence gene regulation fully.

As *S.* Typhi encounters different osmolarity environments during its pathogenesis, it may in fact possess adaptive responses to these changes (79). In the intestinal lumen, the osmolarity is high, with values considered to be equivalent to 300mM NaCl and greater (32, 70, 97). Once *S.* Typhi invades the intestinal barrier, it encounters a lower osmolarity condition equivalent to ~150mM (69), which has been reported to be the osmolarity of blood and plasma. It is thought that the hyperosmotic conditions within the intestinal lumen may promote a hyperinvasive phenotype while suppressing Vi expression and then following invasion, the Vi is activated and expressed whereas the T3SS-1 is down-regulated (81).

In contrast, other studies have shown that the capsule may inhibit bacterial adhesion and invasion of the intestinal epithelium (2, 71), suggesting that the Vi may be produced in the intestinal lumen. Experiments have also demonstrated that natural occurring typhoid fever infections as well as live *S.* Typhi vaccines provoke a poor host protective immune response even though the Vi is a good antigen (96), which may be due to the inactivation of Vi antigen expression once the pathogen invades the intestinal epithelium and resides in macrophages. This concept has been proposed to be a possible explanation for the reduced Vi antibody responses as decreased amounts of antigen may be presented to the host's immune system for processing (78). However, these assumptions are not supported by the current data on Vi capsule expression. Nevertheless, the location and the regulation of Vi capsule expression in the host remain unclear. The purpose of this study was to investigate the expression of the Vi antigen *in*

vitro using intestinal model epithelia (polarized T84 cells) and *in vivo* in the bovine ligated-ileal loop model of *Salmonella* infection.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *S.* Typhi strain Ty2 was obtained from the American Type Tissue Culture Collection (ATCC 19430). Strains were cultured aerobically in Luria-Bertani broth containing 300mM NaCl, (LBH) for optimal Vi suppression supplemented with the following antibiotics carbenicillin, 100 mg/L and/or kanamycin 100 mg/L, as appropriate. For T84 cell infection experiments, each strain was grown overnight at 37ºC shaking in LBH broth with appropriate antibiotics. The next day 1:1000 dilution of overnight culture was made and bacteria were grown until OD_{600}= 1.0-1.5 (late log phase) for optimal Vi suppression without antibiotics. Bacteria were added at a concentration of 10^7 cfu/well. For bovine ligated ileal loops, each strain was grown overnight at 37ºC shaking in 4 ml of LBH broth with appropriate antibiotics. A volume of 0.04 ml of overnight culture was used for inoculation of 4 ml of LBH broth without antibiotics, and bacteria were grown until OD_{600}= 1.0-1.5 (late log phase) for optimal Vi suppression, and the culture was used as the inoculum. Strains were cultured in SOB (Vi-inducing conditions) medium where indicated as controls. Strains and plasmids used in this study are listed in Table 1.

Table 1. Strains and plasmids

Strain or plasmid	Description	Reference and/or source
Strains		
S. Typhi		
Ty2	*Salmonella Typhi* wild type	ATCC 19430
QT74	Ty2Δ*ompR*::Kan^R	This study
QT81	Ty2::pBluescript::*egfp*::*viaB* promoter region	This study
QT113	Ty2Δ*ompR* carrying pBluescript SK+::*ompR*::Kan^{R}Carb^R	This study
Plasmids		
pcR 2.1	Cloning vector	Invitrogen
pBluescript SK+	Cloning vector	Stratagene
pEGFP	Green Fluorescent Protein (GFP) mut1variant expressing vector	Clonetech
pQT13	pGP704::*ompR*FR1	This study
pQT17	pGP704::*ompR*FR1::ompRFR2	This study
pQT19	pGP704::*ompR*FR1::*ompR*FR2::Kan^R	This study
pQT27	pEGFP::*viaB* promoter	This study
pQT28	pBluescript SK+::egfp::*viaB* promoter	This study
pQT50	pBluescript KS:: *ompR*	This study

Construction of a non-polar deletion of *ompR* in *S.* Typhi and the complementation strain. A region 1.2 kb upstream of *ompR* was amplified by PCR using FW (5'-GACGGTTCGTGTTCCAGAGCAG-3') and RV (5'-CTCTTGCATTGTCTGTACTCC-3') and a 1.0 kb region downstream of *ompR* was PCR amplified using FW (5'-CGCCGTATGGTGGAAGAAG-3') and RV (5'-ACCTGGATGCTGCCTGCCTG-3'). Both downstream and upstream flanking regions were cloned into pCR 2.1 (Invitrogen). Subsequently, the upstream fragment was subcloned into the BglII/SalI site of pgP704, giving rise to pQT13. The downstream fragment was cloned into pQT13 using the XbaI/SmaI site, giving rise to pQT17. The kanamycin cassette (1.5 kb) was excised from pKIXX and inserted in the SalI site in pQT17 to give rise to pQT19. The entire construct was confirmed via nucleotide sequencing and restriction enzyme digestion (Figure 1). Using the suicide plasmid pQT19, alleles were introduced into *S.* Typhi with standard allelic methodologies. Colonies were screened for kanamycin resistance and loss of carbenicillin resistance. A general schematic for the generation of QT74 is depicted in Figure 2.

A single colony, demonstrating loss of carbenicillin resistance and gain of kanamycin resistance, was further screened for the *ompR* deletion. Loss of the *ompR* gene was confirmed by Southern blotting with an *ompR*-specific probe, PCR analysis, and the inability to agglutinate with Vi antisera (Difco). The strain was designated QT74.

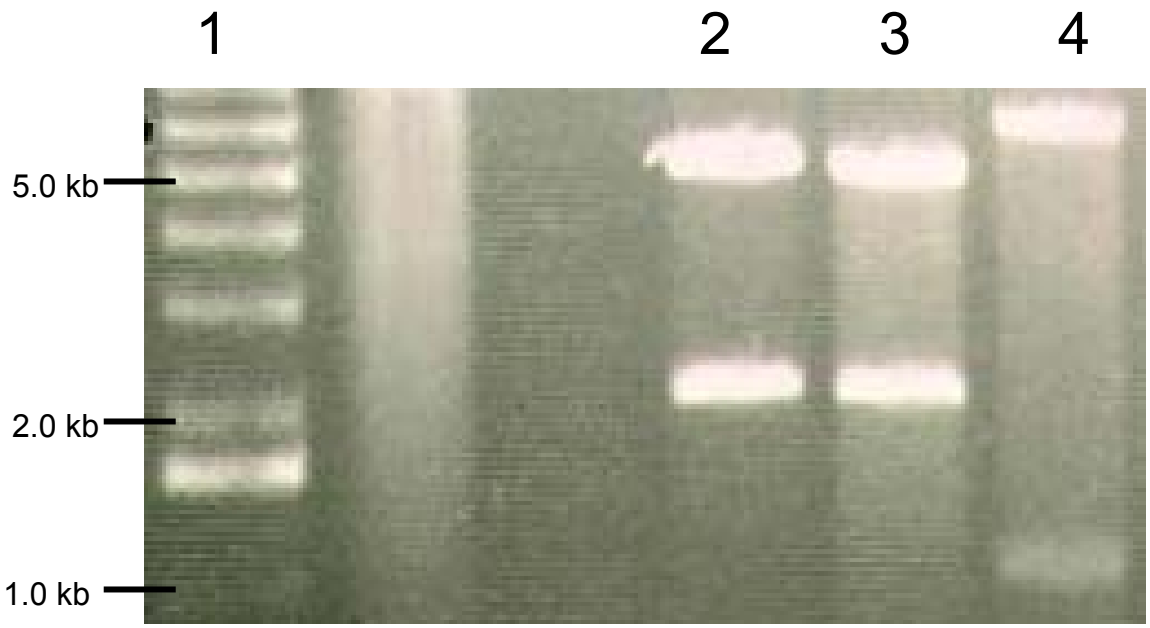

Figure 1. Restriction enzyme digestion of pQT19 with SmaI. Two possible orientations: 1st band~6.1 kb and 2nd band~1.15 kb (Lane 4) or 1st band~1.95 kb and 2nd band~5.25 kb (Lane 2 and 3). All colonies were correct for pQT19.

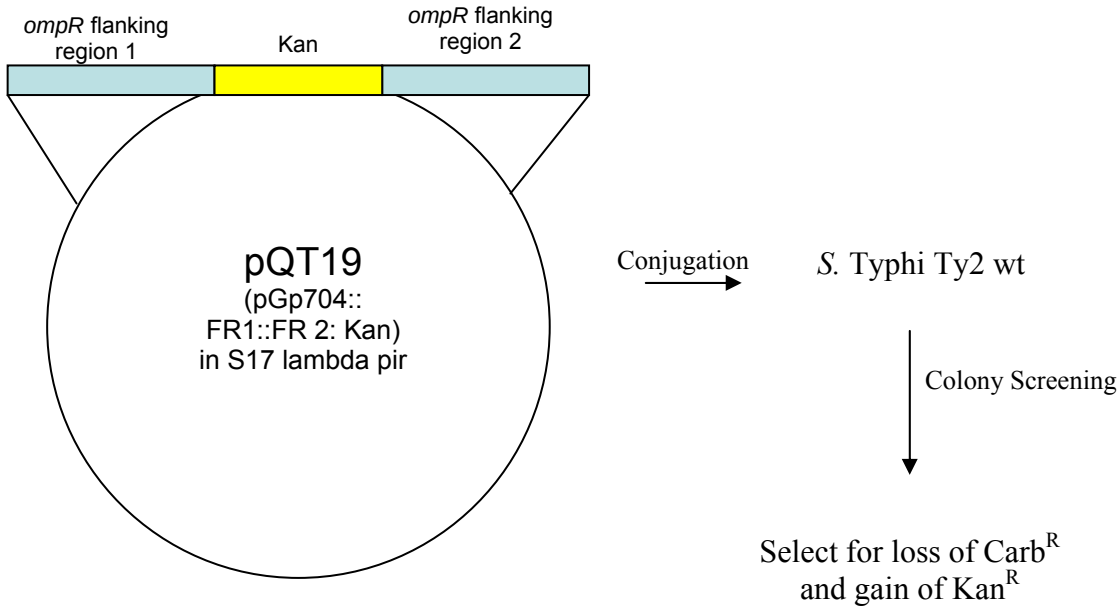

Figure 2. General schematic illustrating the generation of QT74 (*S.* Typhi Ty2 *ompR* mutant strain) from allelic exchange methodologies. *E.* coli (S17 lambda pir) harboring the plasmid construct (pQT118) and *S.* Typhi Ty2 wild type strain were grown over night shaking at 37 degrees C in appropriate liquid medium. Following day, conjugation experiments involving both strains were performed on LBH plates. Colonies were subsequently selected for loss of carbenicillin resistance and gain of kanamycin resistance.

To generate the complementation strain, *ompR* was amplified using FW(5'-TGCCAGCCATCAGCGGGGGCTT-3') and RV (5'-GCCCTGATGAATCTCGGTCAG-3') primers. The PCR fragment was cloned into pBluescript KS+ using EcoRV/EcoR1, creating pQT50. The plasmid was then electroporated into QT74, giving rise to QT113. The ability of QT113 to agglutinate with Vi antisera was restored, indicating the *ompR* gene was complemented successfully.

Construction of an *egfp* reporter system encoding the promoter of the *viaB* locus of *S*. Typhi. For construction of a Ty2 strain carrying a high copy plasmid encoding the *viaB* region promoter fused to a mutant variant of *gfp* (i.e. *egfp*), the promoter of the *viaB* locus was amplified by PCR using the primers FW (5'-tataccatgggaagtctccttatgctgaaa-3') and RV (5'-tatagtcgacgcagtcacgcaccatc-3') flanked with restrictions sites SalI and NcoI, respectively. The resulting 600 bp PCR fragment was cloned into pCR2.1 (Invitrogen), and the fragment was confirmed by nucleotide sequencing and restriction enzyme digestion (Figure 3). The fragment was subcloned into pEGFP, using the Nco I and Sal I multiple cloning site, giving rise to plasmid pQT27. The promoter of the *viaB* region and the *egfp* fragments were excised using SalI and EcoRI, and subsequently subcloned into pBluescript SK+ (Strategene), giving rise to plasmid pQT28. A general schematic demonstrating the construction of pQT28 is illustrated in Figure 4. The plasmid was electroporated into Ty2 to produce strain QT81. To confirm that Vi expression is modulated by osmolarity *in vitro*, QT81 was grown in culture containing different salt concentrations and investigated with flow cytometry.

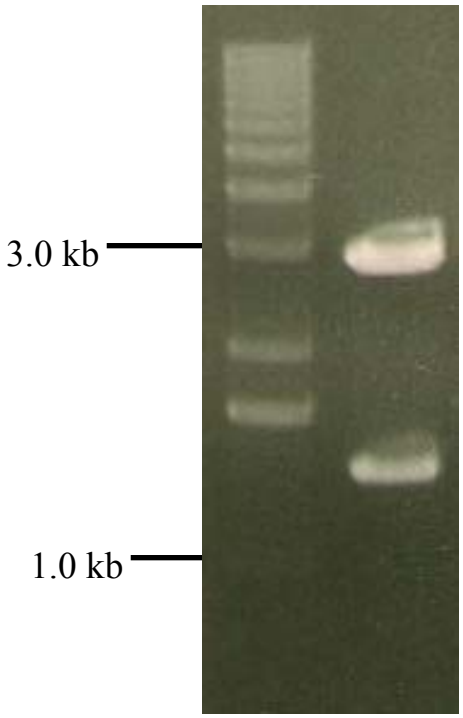

Figure 3. Restriction enzyme digestion of pQT28 with SalI and EcoR1. Band 1~1.36 kb (insert) and Band 2~ 3.0 kb (vector). The plasmid was subsequently electroporated into Ty2.

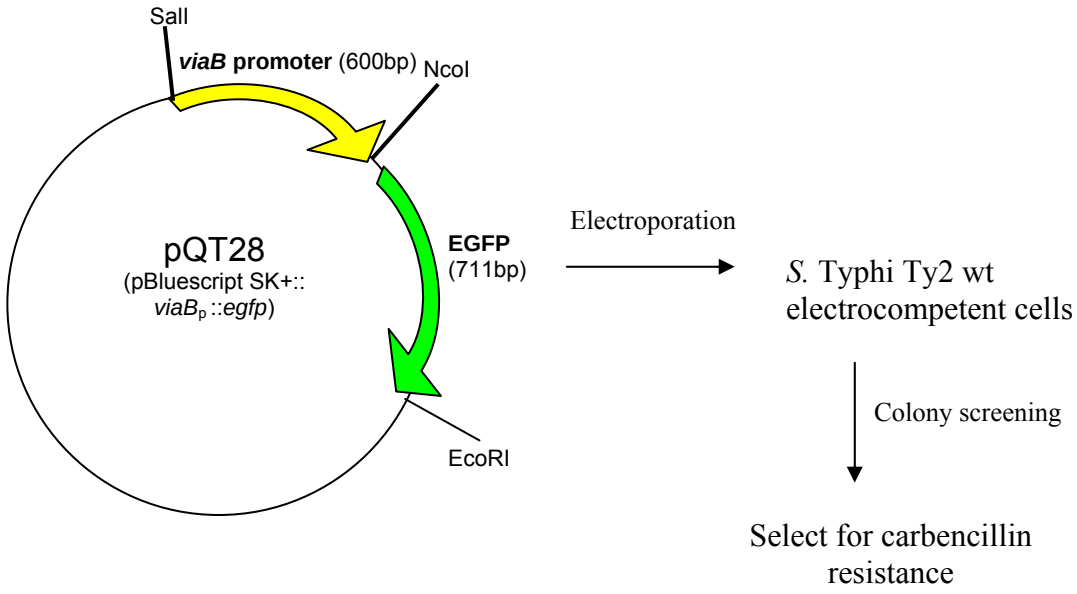

Figure 4. General schematic illustrating the generation of QT81 (*S.* Typhi Ty2::pBluescript::*viaB*$_p$:: *egfp*). The plasmid construct (pQT28) was electroporated into S. Typhi Ty2 wild type electrocompetent cells. Colonies positive for carrying the plasmid were selected on Carb^R plates. Plasmid extraction was subsequently performed to confirm the presence of the plasmid.

To confirm the phenotype *ompR* as a regulator of Vi expression, pQT28 was electroporated into QT74, giving rise to QT114. This strain was grown in different osmolarity conditions in broth and subjected flow cytometry analysis.

Cell culture. T84 cells are a human cell line of colon carcinoma cells that can be polarized upon seeding the cells on the apical compartment of Transwell plates and adding medium to the basolateral compartment. Over the duration of a week, the cells develop a transepithelial resistance that allows them to mimic the conditions of the human intestinal epithelium (shown in Figure 5). T84 cells were seeded at 5 x 10^5 cells/ well and once they developed a transepithelial resistance of 500-1500Ω (65), bacteria were added at approximately 0.418 x 10^9 cfu/well (multiplicity of infection was approximately 10:1) to the apical compartment of polarized T84 cells for 1 hour at 37°C in 5% CO_2 to allow invasion. After 1 hour, the supernatant from each well was removed. To compare bacterial gene expression inside and outside mammalian cells, the traditional gentamicin approach is not required (9). Instead, pre-warmed DMEM-F12 media was added to the apical side and incubated for 2 hours at 37°C in 5% CO_2. After the incubation period, the supernatant was collected and extracellular bacteria were harvested via centrifugation. The monolayer was then washed 3 times with ice cold PBS. T84 cells were then harvested and lysed with 1% Triton x-100 and incubated on ice for 10 minutes.

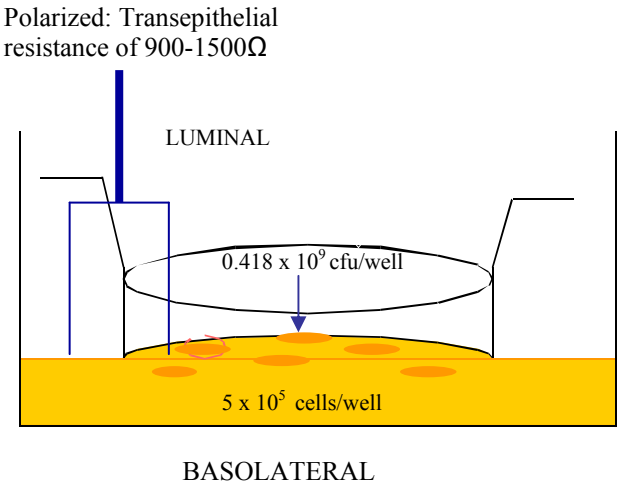

Figure 5. Intestinal culture model epithelia: T84 polarized cells. T84 cells were seeded at 5 x 10^5 cells/ well and after 7-10 days, they develop a transepithelial resistance of 500-1500Ω. Bacteria were added at approximately 0.418 x 10^9 cfu/mL (multiplicity of infection was approximately 10:1) to the apical compartment.

Flow cytometry. Flow cytometry samples were prepared as described previously (9). To characterize QT81 and QT114 in broth, one ml of bacteria culture was harvested by centrifugation at 6000x g for 5 minutes. Bacteria cells were washed twice with PBS to remove LB media. Bacterial pellets were resuspended in 1 ml of 4% paraformaldehyde and incubated for 30 minutes at room temperature. Fluorescence was measured by using a FACS Calibur (Becton Dickinson) and the Cell quest software provided by the supplier. The bacterial population was gated by using side and forward scatter parameters and then analyzed for fluorescence. For the analysis of QT81, the fluorescence intensities were determined for the number of particles (bacterial cells) as follows in culture: QT81 grown in LBH medium, 87759, QT81 grown in SOB medium, 73121, and the negative control Ty2 wild type, 62471. For the analysis of QT114, the fluorescence intensities were determined for the number of particles (bacterial cells) as follows QT114 grown in LBH, 17640, QT114 grown in SOB, 17583, and Ty2 wild type, 19413.

In T84 polarized cell infected with QT81, the flow cytometry results were pooled from experimental infections of two separate transwell plates of polarized T84 cells. The bacterial population was gated with side and forward scatter parameters using samples from the QT81 grown in inducing or low osmolarity (SOB) conditions and uninfected T84 polarized cells treated under the same conditions as infected cells in order to set the region of fluorescence. The results are expressed as the percentage population fluorescing and the mean fluorescence intensity. The number of particles

used to determine fluorescence intensities were: extracellular bacteria, 9704 and the intracellular bacteria with T84 cell lysates, 49812.

Bovine ligated-ileal loop model. Four milk fed 3-4 weeks old calves were obtained from a Texas A&M University cattle herd. Calves were tested for *Salmonella* infection by fecal swabs, enriched in tetraiodothionate broth and plated on XLT4. Calves were tested for leukocytosis and fever prior to experiments. To perform the ligated-ileal loop experiments, calves were anesthetized using propofol induction and isoflurane maintenance for the duration of the experiment (1). A right-flank laparotomy was performed, the jejunum and ileum exposed and loops with lengths ranging from 6-9 cm ligated, leaving 1-cm loops between them. The loops were infected by intralumenal injection of a 4 ml suspension containing approximately 1 X 10^9 CFU/ml of *S.* Typhi strains grown in LBH broth. Loops injected with LBH broth served as a negative control. Loops were excised at 2 and 8 hours post infection to: 1) collect the fluid that has accumulated in the lumen, 2) harvest extracellular bacteria, 6.0 mm biopsy punched tissue samples of the mucosa/submucosa for RNA extraction, and 3) collect tissues for frozen OCT sections. The frozen sections were later cut at 10µm thickness for fluorescence immunohistochemistry and stored at -80ºC.

Fluorescence immunocytohistochemistry. Frozen sections of ileal tissue infected with Typhi grown in LBH and loops inoculated with LBH (negative control) were fixed in methanol. Samples were incubated with a primary anti-Vi rabbit antibody

(1:250) (Difco) overnight in 4ºC. Next day, samples were incubated for 2 hours at room temperature in a humidified chamber with secondary anti-rabbit conjugated to Alexafluor 549 (1:250) (Molecular Probes) in the dark. All antibodies were diluted in antibody dilution buffer (1% BSA in 0.02 M PBS/Tween). Slides were washed 3 times for 10 minutes each in 0.3% Tween/PBS. A drop of prolong- antifade DAPI solution (Invitrogen) was added to each slide. A coverslip was placed over the top and slides were allowed to dry overnight at room temperature. Next day, slides were stored at 4ºC until ready to view. Samples were observed and photographed with an Olympus IX-70 camera.

Real-time PCR. For analysis of intracellular and extracellular Typhi gene expression after one hour post-infection of T84 polarized cells with Typhi wild type, extracellular bacteria were collected from the media and T84 cells were lysed with Triton X-100 to harvest the intracellular bacteria. Samples were stored in ethanol/phenol mRNA stop solution until RNA was extracted with hot acid phenol/chloroform. For analysis of intracellular and extracellular Typhi gene expression in bovine ileal loop infection experiments, extracellular bacteria were harvested from the lumen and kept frozen on dry ice and the ileum tissue was kept frozen on dry ice in TriReagent (Molecular Research Center) at the site of surgery until further processing for RNA extraction. After RNA extraction from ileal tissue, samples were processed subsequently, using the Microbe Enrichment Kit (Ambion) in order to isolate bacterial RNA. All samples prior to Reverse Transcriptase were DNase treated (Ambion DNA-

free kit). Subsequently, 1,000 ng of each sample was retrotranscribed in a 50µl volume (Taqman reverse transcription reagents; Applied Biosystems) and 4µl of cDNA was used for each real time (RT)-PCR. RT-PCR with primers were used to detect the *tviB* gene in bacterial messenger RNA in order to measure Vi expression and *rpoD,* the internal control gene, in *S.* Typhi infected T84 polarized cells using SYBR Green (Applied Biosystems) and the ABI 7500 Real Time PCR System. The *rpoD* gene has been shown to have no significant variation of expression in either Typhi or Typhimurium inside macrophages (23, 25) and T84 polarized cells (this study). RNA samples were analyzed for *prgH* and *fliC* genes to monitor the expression levels of the SPI-1 (T3SS) and flagellin, respectively. For each run, the calculated threshold cycle (C_t) was normalized to the C_t of *rpoD* gene amplified from the corresponding sample, and the data were analyzed using the comparative Ct method (Applied Biosystems). Levels of Typhi gene expression in T84 polarized cells were calculated relative to the inoculum Ty2 wild type grown in LBH. Levels of Typhi gene expression in calves were calculated for each ileal loop infected with Typhi wild type relative to an infected loop with Typhi o*mpR* mutant strain (non-producing Vi strain) collected at the same time point from the same animal. A list of genes analyzed in this study with respective primers is provided in Table 2.

Table 2. Primers for Real-time PCR

Gene	Primer pairs
fliC	5'-CAACCTGGGCAATACCGTAAATAA-3' 5'-CTGCGCGCGAGACATG-3'
iagA	5'-ACGGACAGGGTTATCGGTTTAAT-3' 5'-AAAAGGAAGTATCGCCAATGTATGAG-3'
tviB	5'-ATAATAGGGATCTACGCCAATA-3' 5'-CGCTGGCAGCAAATGGA-3'
prgH	5'-TCATAATCGCCCCTCGCTAA-3' 5'-TCTATGTCGCTGCGCAAAAT-3'
rpoD	5'-GTATGCGTTTCGGTATC-3' 5'-GCTAGGGTGGCGCAGTTTAC-3'

Statistical analysis. For statistical analysis of ratios (i.e. increases in Typhi gene expression or data expressed as percentages), data were transformed logarithmically prior to performance of statistical analysis. A parametric test (paired student's t-test for T84 polarized cell samples and for calf ligated-loop samples) was used to calculate whether differences were statistically significant.

RESULTS

***S.* Typhi carrying the *viaB* promoter fused to *egfp* (enhanced green fluorescent protein) on a plasmid grown in different salt concentrations indicates that the Vi antigen expression is modulated by osmolarity.** GFP (Green fluorescent protein) has been previously applied to study host pathogen interactions and salmonellosis gene expression (9, 11, 100, 101). Here we first wanted to determine whether the *S.* Typhi wild-type isolate Ty2 harboring the *viaB* region promoter fused to *egfp* on a high-copy plasmid (QT81) would demonstrate that Vi antigen expression is modulated by osmolarity and to validate this strain for subsequent T84 polarized cells infection experiments, thus we inoculated SOB (Vi-inducing) and LBH (Vi-suppressing) medium with this strain. As a negative control, SOB medium was inoculated with the Ty2 wild type. The bacterial population was gated by using side and forward scatter parameters and then analyzed for fluorescence. The results are expressed as the percentage population fluorescing and the mean fluorescence intensity.

When QT81 was grown in LBH media (Vi-suppressing) containing carbenicillin (100µg/ml) overnight at 37º C shaking, the number of bacterial cells fluorescing and

level of fluorescence intensity was comparable to that of the negative control. When the same strain was grown in SOB medium (Vi-inducing) containing carbenicillin (100µg/ml) under similar conditions, the majority of cells exhibited fluorescence intensity which was increased by 100 times when compared to the negative control and the strain grown in LBH medium (Figure 6). These results agreed with previous reports that Vi expression of serotype Typhi is affected by osmolarity (114).

S. Typhi expresses the Vi capsular antigen at higher levels intracellularly within T84 polarized cells. To further investigate the location of Vi expression in intestinal epithelial cells *in vitro*, we proceeded by infecting T84 polarized intestinal epithelial cells with QT81. We wanted to determine if Vi expression occurred inside intestinal epithelial cells by infecting T84 polarized cells with QT81. The flow cytometry results were pooled from experimental infections of two separate transwell plates of polarized T84 cells. The bacterial population was gated with side and forward scatter parameters using samples from QT81 grown in inducing or low osmolarity (SOB) conditions and uninfected T84 polarized cells treated under the same conditions as infected cells in order to set the region of fluorescence. The results demonstrated that a larger percentage of bacteria (53.8%) were expressing fluorescence inside polarized T84 cells as opposed to the 22.9% expressed by the extracellular bacteria in the supernatant (Figure 7).

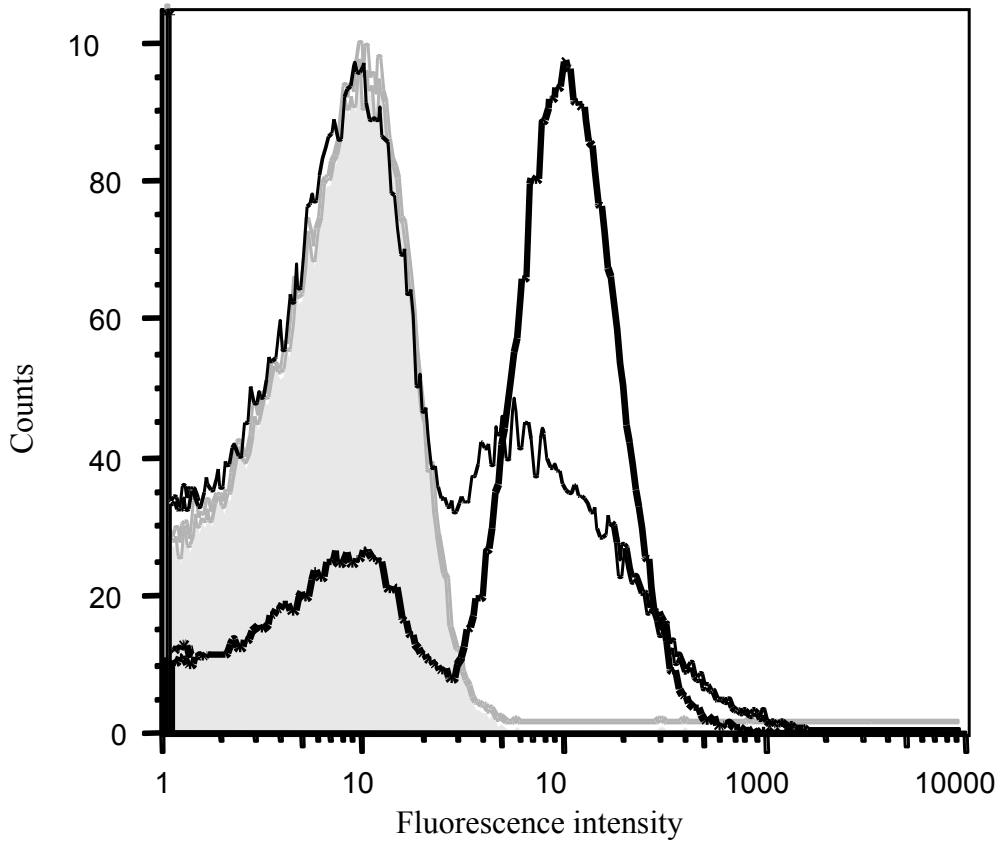

Figure 6. *In vitro* induction of serotype Typhi Ty2 carrying pBlueScript SK+::p*tviA*::*egfp* (QT81). Thick line represents QT81 grown under inducing conditions: low osmolarity (SOB broth). Thin line represents QT81 grown under non-inducing conditions: high osmolarity (LBH broth). The shaded area represents Ty2 wild type without the reporter plasmid under inducing conditions. The results are expressed as the percentage population fluorescing and the mean fluorescence intensity. The number of cells used to determine fluorescence intensity and % GFP positive cells for each sample are as follows: QT81 in LBH, 87759, 28.8%, QT81 in SOB, 73121, 69.2%, and Ty2 wt, 62471, 0.9%.

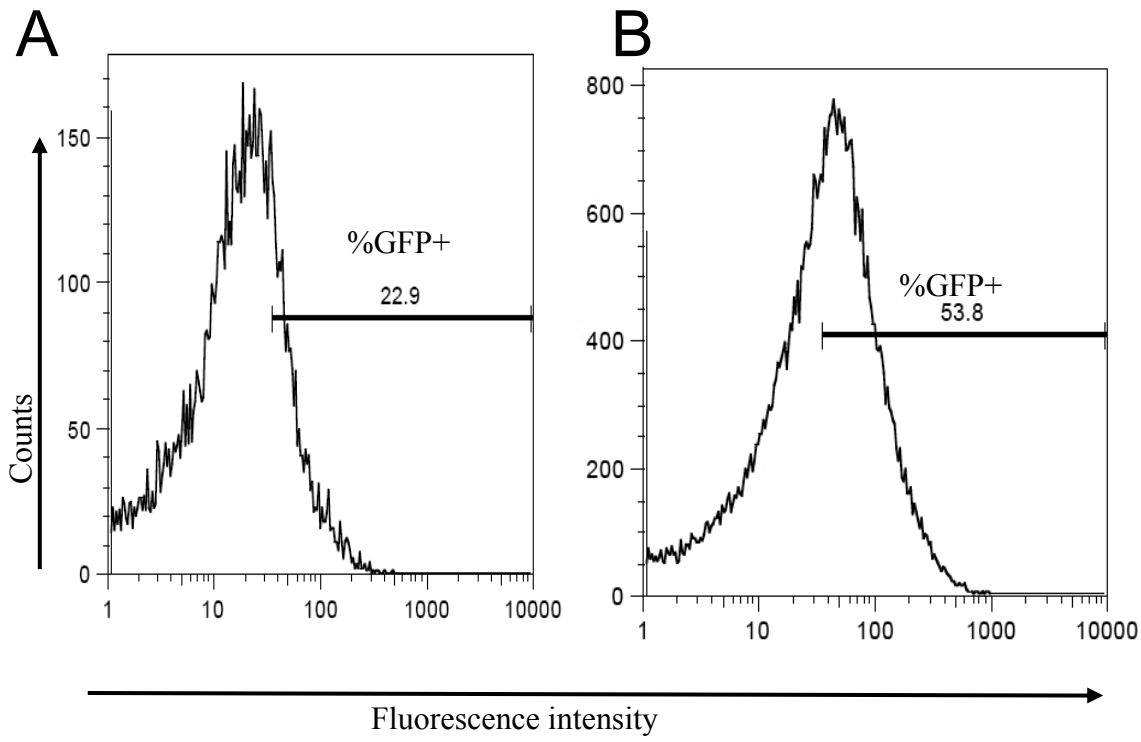

Figure 7. Flow cytometry detection of Vi expression in T84 polarized cells using Typhi Ty2 carrying pBlueScript SK+::*viaB* promoter::*egfp*. (QT81). The number of particles used to determine fluorescence intensities were: extracellular bacteria, 9704 and the intracellular bacteria with T84 cell lysates, 49812.

To further examine Vi expression during infection of T84 cells with QT81, we performed real-time PCR analysis. We monitored the expression of t*viB* and of genes encoded by the SPI-1 T3SS-1 (*fliC*, *prgH*, and *iagA*). T84 polarized cells were inoculated with serotype Typhi grown in LBH medium and for each experiment, bacterial RNA was pooled from infections of 10 separate transwells. The invasion associated SPI-1 and the genes involved in flagellar biosynthesis were previously shown to be upregulated during initial stages of crossing the epithelial cell barrier and down regulated following invasion (30, 35, 39). Levels of serotype Typhi gene expression in T84 polarized cells were calculated relative to the inoculum Ty2 wild type grown in Vi-suppressing medium. The results from the real-time PCR experiments analyzing the expression of *S.* Typhi Vi capsular antigen, flagellin, and invasion genes revealed that *tviB* was expressed significantly higher ($p=0.049$) by intracellular bacteria than by extracellular bacteria, suggesting that the expression of the Vi biosynthesis genes was upregulated after invasion of T84 polarized cells (Figure 8). The *fliC* gene was expressed at higher levels among extracellular bacteria, though this difference was not statistically significant. The *prgH* invasion gene was expressed at significantly higher ($p=0.013$) levels by extracellular bacteria than by intracellular bacteria. Similarly, the *iagA* regulator was expressed at significantly ($p=0.037$) higher levels by extracellular bacteria compared to intracellular bacteria. These findings provided evidence that the Vi antigen was expressed at lower levels during invasion compared to higher levels observed following invasion of epithelial the cells. The inverse was observed for SPI -1 (T3SS) genes, which is in good agreement with previous reports (26, 29).

The transcript levels of *tviB* were expressed as fold increase over those measured in the inoculum culture, which was grown under Vi-suppressing and optimal invasion conditions. The increased level of detected *tviB* expression in extracellular bacteria may be an effect of DMEM-F12 media, which possesses an osmolarity of 150 mM. Thus, the low osmolarity cell culture media may be responsible for inducing Vi expression extracellularly, as suggested in other studies (39). This may be a limitation of the T84 polarized cell culture model in studying Vi expression. However, growing *S.* Typhi in 300 mM NaCl media is known to suppress Vi expression. *S.* Typhi grown under those conditions produces a weak agglutination reaction with Vi antisera (2, 78), indicating that the Vi capsule is expressed at low basal levels.

The osmolarity of the fluid within calf ligated ileal loops is not hyperosmotic. The calf model has been implemented successfully to study the pathogenesis of *S.* Typhi (82, 83). In order to study the expression of the Vi antigen *in vivo*, we inoculated calf ligated-ileal loops with serotype Typhi grown under high osmolarity (300mM NaCL containing LB medium) to mimic the intestinal luminal conditions and to suppress Vi expression, as reported in broth culture experiments (114) .

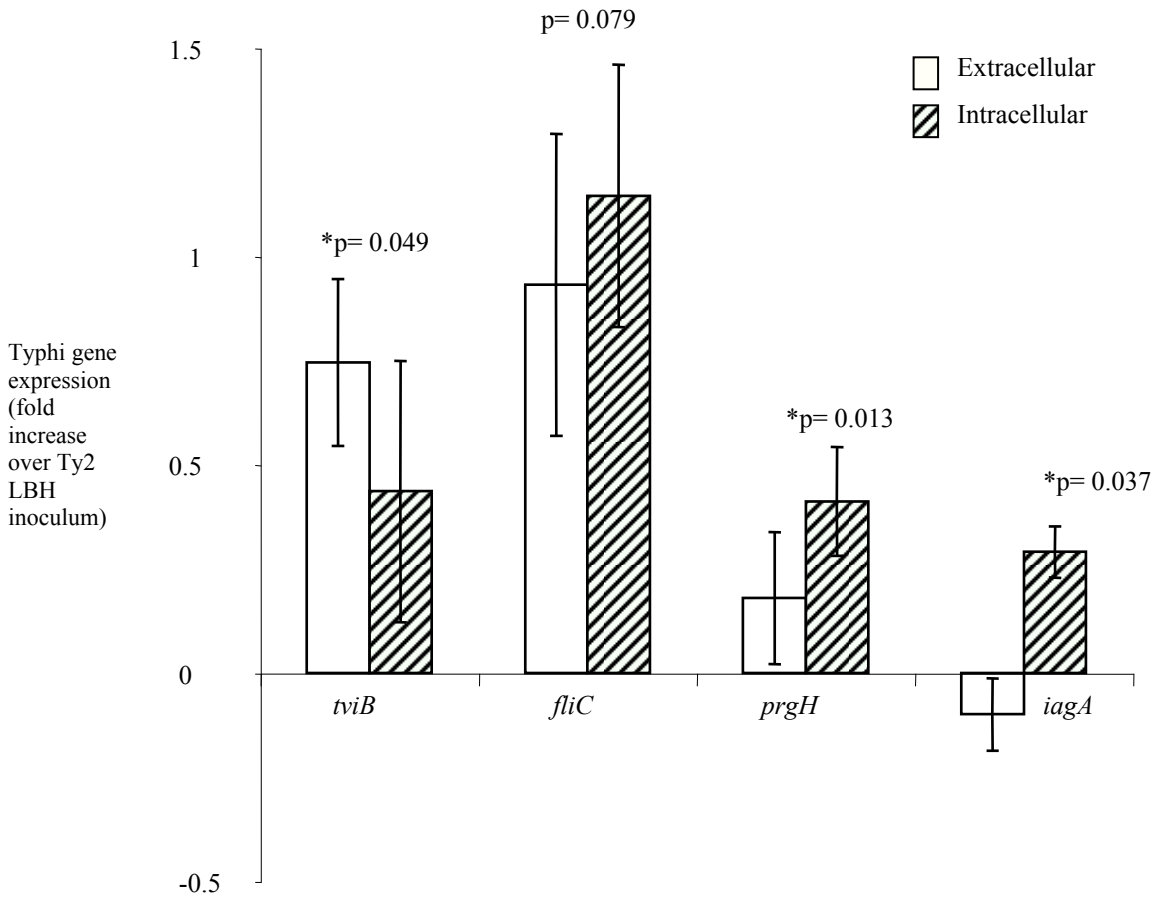

Figure 8. Profile of bacterial gene expression in extracellular and intracellular bacteria harvested from T84 polarized cells infected with serotype Typhi wild type after 1 hour. Expression levels of *tviB*, *fliC*, *prgH*, and *iagA* were determined by real-time PCR. Data are shown as increases of Typhi gene expression relative to the preinoculum grown under Vi suppressing conditions. These data are averages from three independent experiments.

We first wanted to determine the osmolarity level of the intestinal luminal contents at 2 and 8 hours post-infection by measuring the osmolalilty of the luminal fluid using Wescor 5100C Vapor Pressure Osmometer according to manufacturer's instructions. Experimentally only the osmolality can be measured; however the approximate osmolarity values can be determined by conversion (95). A list of osmolality and osmolarity values measured is listed in Table 3.

Table 3. Measured osmolality values.

Sample	osmolality (mmol/kg, mosm/kg)	osmolarity (mosm/L, mmol/L)
LB	409	170 mM NaCl
LBH	631	300 mM NaCl
SOB	104	10mM NaCl
T84 cell media	282	~105 mM
2 hr post-infection	271-298	99.45-114.26 mM
8 hr post-infection	259-275	92.86-101.64 mM
Blood/Tissue	~363 (estimated)[1]	150 mM NaCl

[1]Value was estimated based upon reported osmolarity value of 150 mM NaCl from reference 30.

Measurements of the osmolality of the intestinal fluid revealed that at 2 and 8 hour time points, the intestinal fluid possessed osmolality values of approximately 294 mmol and 268 mmol, respectively, which corresponded to an osmolarity level of ~150 mM. These results agree with previous calf ileal cannula studies in which the ileal fluid osmolality was reported to be similar to that of plasma (280-300 mmol) (18, 61). Nevertheless, the values obtained in our study indicated hypoosmotic conditions, which were predicted to induce Vi expression within the intestinal lumen. This prediction was tested by real-time PCR analysis performed on the extracellular bacteria collected at the time indicated points (Figure 9).

Characterization of a nonpolar *ompR* deletion in *S*. Typhi Ty2. A deletion in *ompR* was previously shown to inhibit Vi synthesis and result in a negative Vi slide agglutination reaction (78). A deletion of the *ompR* gene was constructed in serotype Typhi strain Ty2 and the mutation was verified by Southern Blotting (Figure 10A). The inability of the *S.* Typhi *ompR* mutant to express the Vi antigen was verified with Vi antisera (Figure 10B).

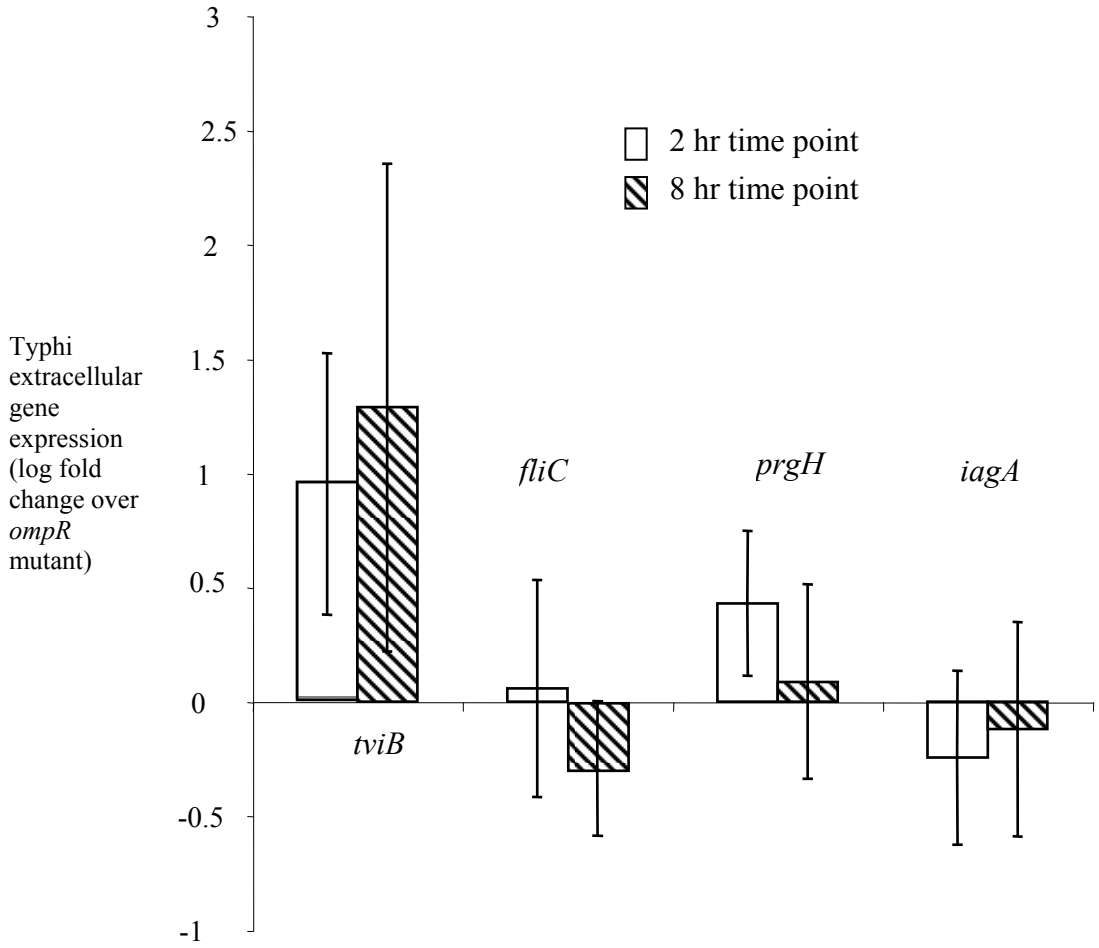

Figure 9. Profile of extracellular bacterial gene expression collected from the luminal fluid at two and eight hours in the calf ligated ileal loops post-infection. Data are expressed as increases in bacterial mRNA levels relative to loops infected with serotype Typhi *ompR* mutant at the same time point. Bars represent geometric means ± standard deviation. These data are averages from four different bovids.

We also constructed a high copy plasmid carrying the whole *ompR* gene (plasmid pQT50) and introduced it into the S. Typhi *ompR* mutant by electroporation. Expression of the Vi capsular antigen in *S.* Typhi was restored and confirmed by Vi agglutination (Figure 10B). Complementation with a low copy plasmid (pwsk29) carrying the *ompR* gene did not restore Vi production when detected by slide agglutination. This finding was consistent with prior *ompR* complementation studies (78). The *ompR* mutant strain was subsequently used in our calf ligated-ileal loop studies.

Vi expression by the *ompR* mutant was further characterized by introducing plasmid pQT28 (carrying a *egfp* reporter gene fused to the promoter of the *viaB* region). The resulting strain (QT114) was grown in culture in media with different osmolarities and *egfp* expression was analyzed by flow cytometry. Bacterial cells were gated using forward and side scatter parameters. The fluorescence intensity emitted by the *S.* Typhi Ty2 wild-type grown under non-Vi inducing conditions (negative control) and strain QT114 grown under Vi-inducing conditions (SOB broth) were similar (Figure 11). QT114 grown in LBH also exhibited similar levels of fluorescence to the Ty2 wild type (data not shown). These data indicated that *ompR* encoded a positive regulator of the *viaB* locus, which was consistent with previous reports (78).

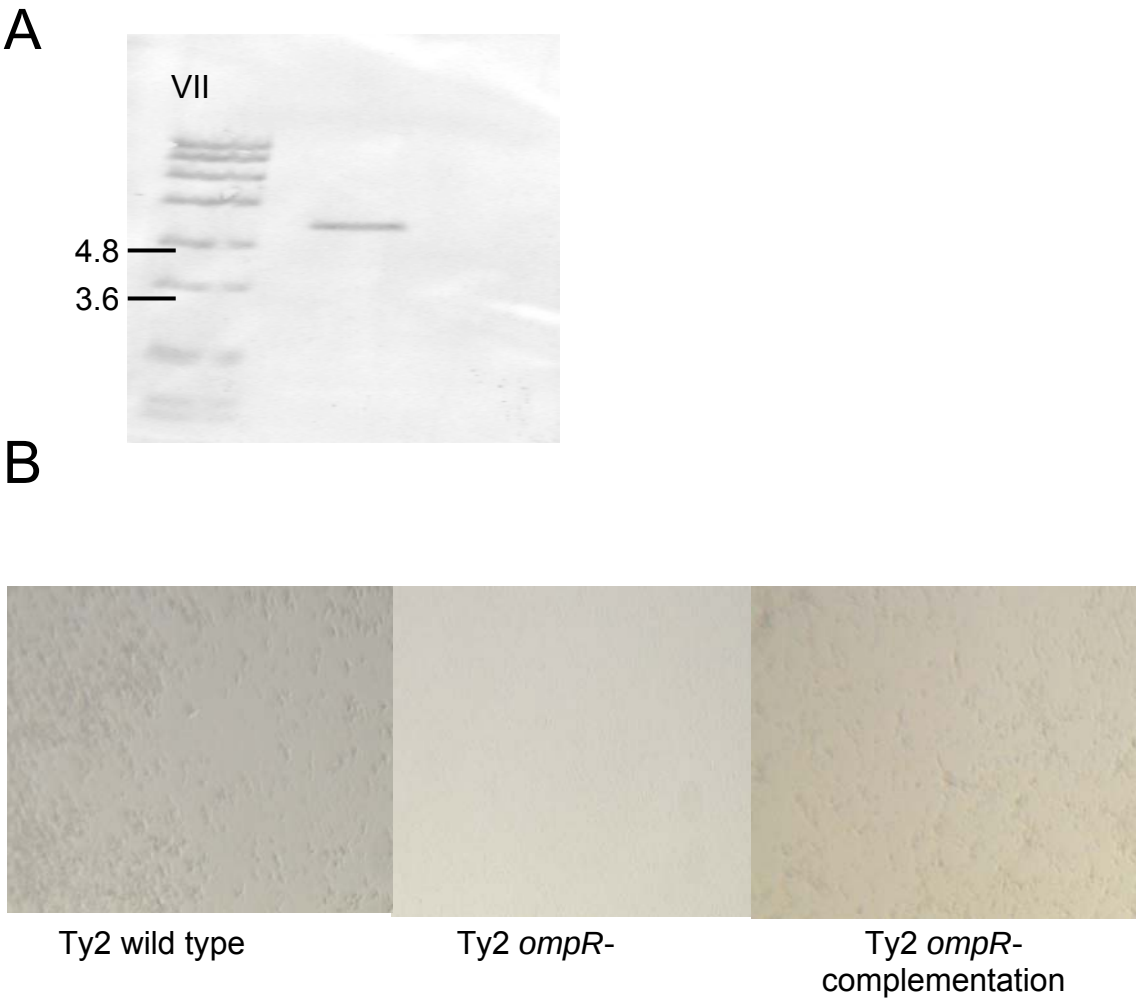

Figure 10. Characterization of serotype Typhi *ompR* mutant. (A) Southern blot with *ompR* specific probe. (B) Agglutination reactions with Vi antisera.

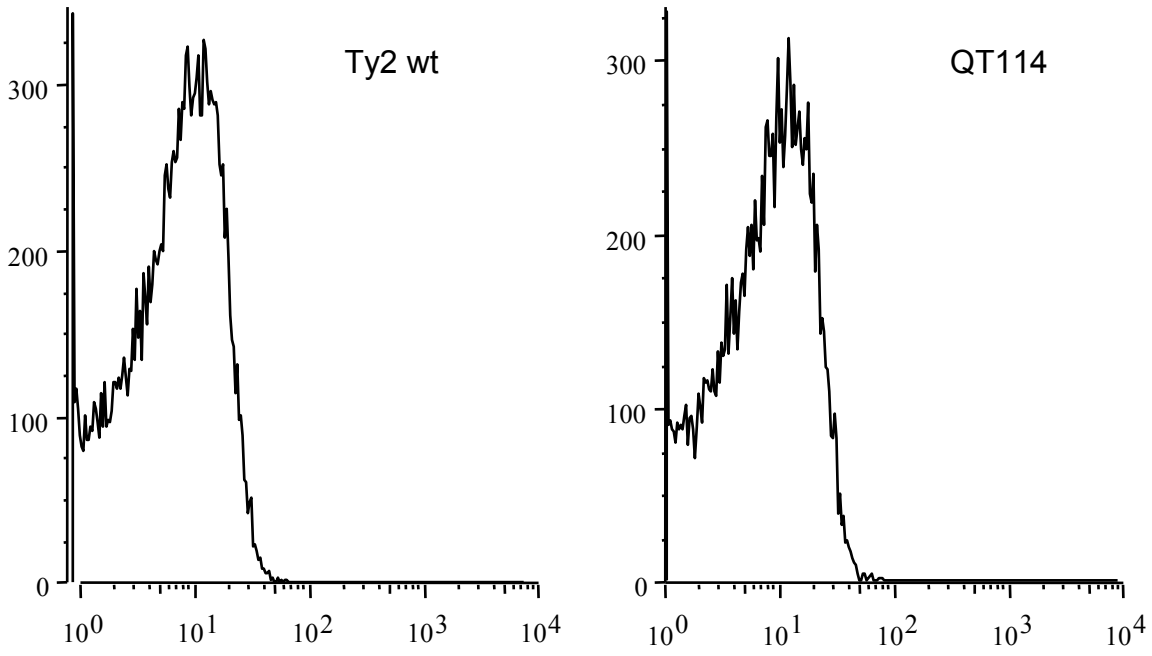

Figure 11. Flow cytometry analysis of QT114 grown under Vi optimal expressing conditions (SOB). Ty2 was used as a negative control. The numbers of cells used to determine fluorescence intensity for each sample are: QT114 in SOB, 17583 and Ty2 wt, 19413.

The Vi capsule is expressed after invasion of the bovine intestinal epithelium. The mammalian small intestinal lumen has a reported osmolarity of 300 mM NaCl (~676 mOsm) and greater (47, 69). However, we detected hypoosmotic conditions within the lumen of bovine ileum (Table 3), which has been shown previously in calf ileal studies post-treated with hypertonic and isotonic solutions (18, 61), suggesting that the intestinal lumen is not consistently hyperosmotic. It has also been demonstrated that in the human small intestine, except shortly after eating, the luminal content osmolarity is the same as plasma as a function of a normal intestinal permeability mechanisms (27).

Nonetheless, we reasoned that to study increases in bacterial gene expression in the tissue, Vi-expression may be compared to that of a *S.* Typhi culture grown in 300 mM NaCl broth (Vi-suppressing conditions), the reported osmolarity of intestinal contents (47, 69). In order to illustrate Vi expression, bacterial gene expression for each loop inoculated with serotype Typhi wild type was analyzed as log fold increase over the *S.* Typhi *ompR* mutant, which is deficient for Vi expression, collected at the same time point.

We performed real-time PCR analysis to investigate the expression of the Vi antigen, by monitoring *tviB*, and genes encoded by SPI-1 T3SS-1 (*fliC*, *prgH*, and *iagA)* in calf ileal tissue following invasion at two and eight hours post-infection. At 2 and 8 hours, expression of *tviB* among intracellular bacteria within calf tissue was significantly higher than in the inoculum (Figure 12). All the invasion-associated genes were down

regulated in tissue at 2 h, and 8 h when compared to expression levels detected in the *ompR* mutant, suggesting that OmpR may be a positive regulator for invasion.

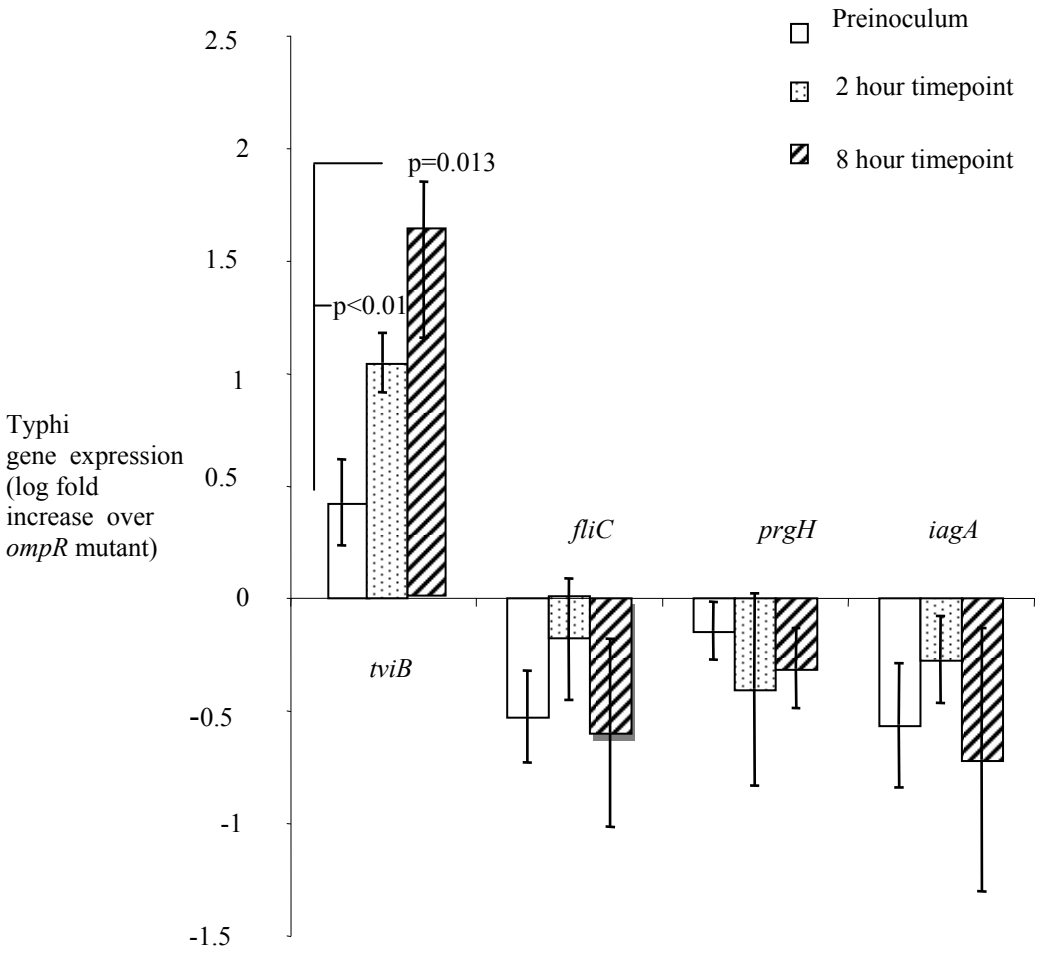

Figure 12. Profile of bacterial gene expression in the preinoculum and bovine ileal tissue infected with serotype Typhi wild type determined by Real-time PCR at two and eight hours post infection. Data are expressed as increases in bacterial mRNA levels relative to loops infected with serotype Typhi *ompR* mutant at the same time point. Bars represent geometric means ± standard deviation. These data are averages from four different bovids. Statistical significances of differences are provided.

We wanted to directly detect Vi production within frozen sections of calf intestinal tissue and to identify the location of capsule expression at 2 hours and 8 hours post infection. We first labeled the infected tissue with primary anti-Vi antibody and then labeled the tissue with a secondary anti-rabbit goat antibody conjugated to Alexafluor 594, which emitted an orange-red emission and the nuclei of cells were stained with DAPI. As a negative control, uninfected intestinal tissue at corresponding time points were subjected to similar labeling procedures. No Vi antigen production was detected in the uninfected ileal tissue (Figure 13C and 13F). Ileal loops infected with *S.* Typhi strain Ty2 grown under Vi-suppressing conditions after 2 hours revealed Vi expressing bacteria along the intestinal villi tips where they appeared to penetrate the intestinal epithelial barrier and enter the lamina propria (Figure 13A and 13B). The two component regulatory systems, OmpR EnvZ and RcsBC, activate Vi antigen expression when *S*. Typhi encounters decreasing osmolarity in tissue or blood (2, 103). Thus, our findings support the concept that the production of the Vi antigen may occur while bacteria pass through the intestinal epithelium. Fluorescent staining of bovine Peyer's patch sections revealed large numbers of Vi-expressing bacteria in the mantle and in the area between germinal centers (Figure 13D and 13E). Since Peyer's patch are large lymphoid aggregations consisting of both T and B cell areas within the small intestinal mucosa, these data suggested that the Vi antigen may be expressed intracellullarly within dendritic cells. Reports suggest that murine Peyer's patch dendritic cells are important in antigen presentation for T-cell proliferation and T-cell help for B-cell responses (92, 93), leading us to reason that dendritic cells may serve a similar immune function in the

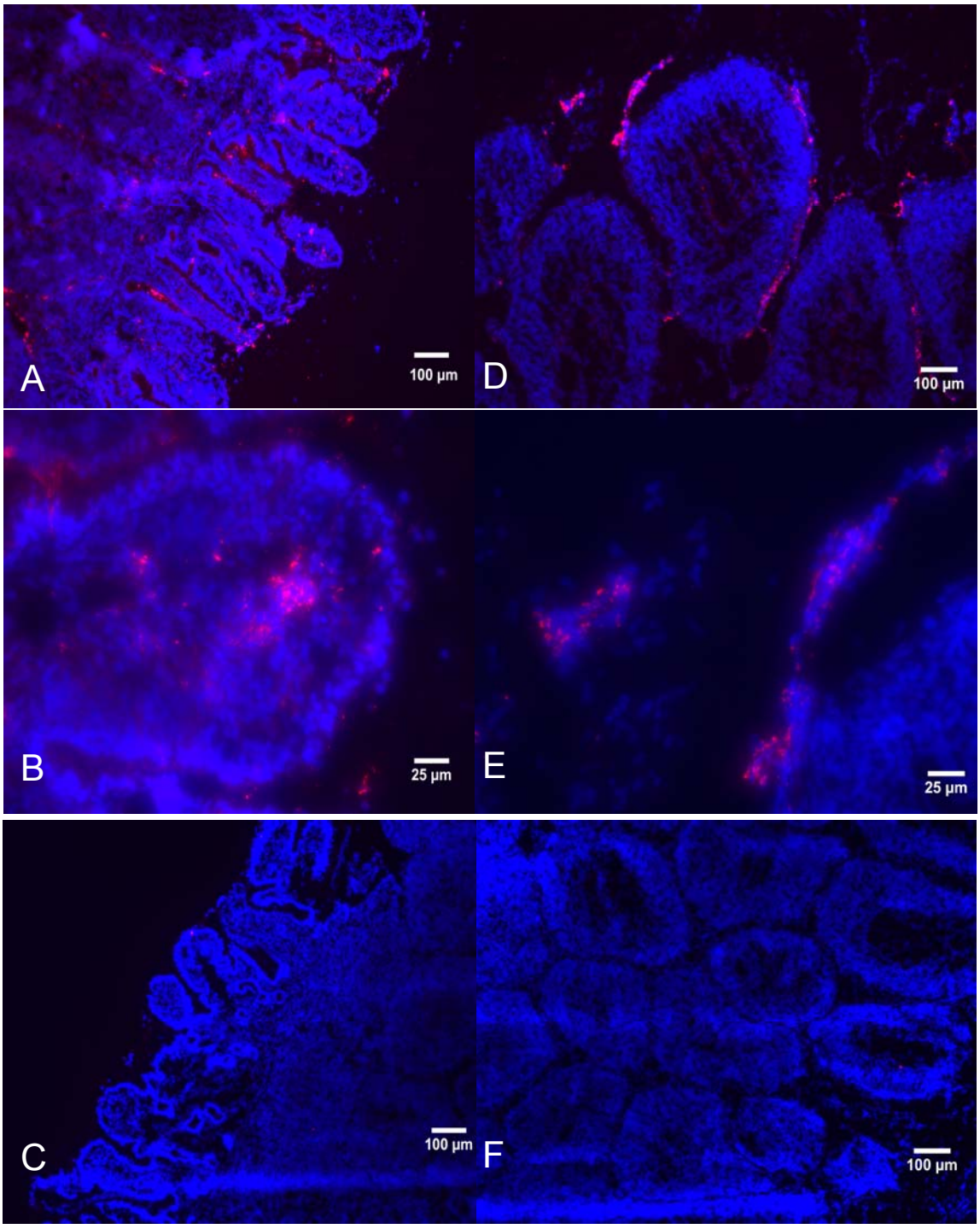

Figure 13. Fluorescence immunohistochemistry detection of Vi antigen expression in bovine ileal tissue. Frozen calf ileal tissue in OCT medium were sectioned at 10µm and primarily stained with anti-Vi rabbit antibody and secondarily with goat anti-rabbit antibody conjugated to Alexfluor 549. Cell nuclei were stained with DAPI. Slides were viewed and images were taken with an Olympus IX-70.

bovine Peyer's patch when encountering enteropathogens. At 8 hours, our findings were similar but the numbers of invasive bacteria expressing capsule within the tissue were elevated. These findings illustrated that the Vi capsule was expressed following invasion of the gut mucosa, which supported a role of the capsule during bacterial host cell interaction in tissue.

DISCUSSION

The expression of the Vi capsular antigen in serotype Typhi infection presents a possible explanation for the different clinical manifestations observed in humans compared to other serotypes of *Salmonella*. There are two major hypotheses on the regulation of Vi expression in the intestinal environment. One theory suggests that the Vi is produced in the intestinal lumen and may inhibit bacterial adhesion and invasion of intestinal epithelia (2, 71). The second theory suggests that the Vi is suppressed in the lumen and activated following invasion to allow the organism to evade host innate immunity (80, 82). Current research on the regulation of the Vi antigen supports the latter hypothesis that the Vi antigen may be expressed after invading nonphagocytic cells and following uptake by macrophages. To further test this hypothesis, we investigated the regulation of Vi antigen expression *in vivo* by performing direct and indirect experimental techniques to detect the Vi antigen in intestinal model epithelia (T84 polarized cells) and *in vivo* in the bovine ligated- ileal loop model.

The infection of T84 polarized cells with *S.* Typhi grown under Vi-suppressing conditions and analyzed by flow cytometry and real-time PCR demonstrated that the Vi

antigen was expressed at higher levels by intracellular bacteria. These results, along with previous findings that capsulated *S*. Typhi have a significant reduction in the amount of inflammatory cytokine secretion compared to a non-capsulated mutant (42, 80), support the view that the Vi antigen has a role following invasion of intestinal epithelial cells. Consistent with this concept, our analysis of T3SS-1 gene expression with real-time PCR indicated increased transcription in extracellular bacteria and a down regulation of these genes after invasion.

Studies on the regulatory mechanisms responsible for Vi capsular expression revealed that the expression of the biosynthesis genes is regulated by osmolarity. Though it is believed that the small intestinal lumen is hyperosmotic due to the influx and digestion of foodstuffs and the presence of sodium salts and bile acids, we reported that the fluid collected from lumen of the calf ligated-ileal loops at designated time points was hypoosmotic, which is considered to be a normal physiological occurrence as seen in prior calf ileal cannula studies measuring the osmolality of luminal fluid (61) (Table 3). Previous experiments have shown that large variations in osmolality occur within the lumen due to the mechanistic effects of the villus countercurrent exchanger, which mobilizes water and electrolytes (33, 34). This counter current exchanger maintains the plasma osmolality of the blood and leaves the fluid surrounding the villus close to isotonicity, regardless of the lumen osmolality. As a result, the hypoosmotic fluid, which can also be considered isotonic, within the lumen may account for inducing Vi expression in the extracellular bacteria (Figure 4). Nevertheless, these data agree

with previous findings and support the concept that Vi expression is regulated by osmolarity *in vivo*.

Under conditions of high osmolarity, which are thought to mimic conditions encountered in the intestinal lumen, the Vi antigen is suppressed (78), while flagella and the T3SS-1 genes are activated (2). When *S.* Typhi wild type is cultured in 300 mM NaCl containing Luria-Bertani broth (LBH), the expression of the Vi antigen is decreased, but the secretion of invasion proteins (SipB, SipC, SipA) are increased (114). Under the same growth conditions, *S.* Typhi wild type is highly invasive and destructive towards epithelial and M cells of rat's Peyer's patches (114). These data suggest that the Vi antigen may be repressed in the intestinal lumen and possibly allow serotype Typhi to express a more invasive phenotype as it penetrates the intestinal epithelium. Here we show that the extracellular bacteria in the lumen of the calf ligated ileal loops expressed *tviB* at significantly higher levels while invasion and flagellin genes (*prgH, iagA, fliC*) were expressed at lower levels in intracellular bacteria when compared to expression levels of a serotype Typhi *ompR* mutant.

Peyer's patches are large lymphoid aggregations in the small intestinal mucosa that are comprised mainly of T cells, B cells, macrophages, and dendritic cells. The surface epithelium contains M cells that transfer antigen from the lumen into the Peyer's patch. We directly detected Vi production in the bovine intestinal mucosa by using anti-Vi antigen antibodies and labeling the antibodies with an orange-red fluorophore. We observed Vi-expressing bacteria during the initial stages of penetration of the intestinal epithelium as well as following invasion in the areas of the intestinal villi and Peyer's

patches. Bacteria entering the patch via M cells are taken up by macrophages and presented to T lymphocytes. Interestingly, the Vi antigen was detected in the dendritic cell-rich areas between the germinal centers, which suggest that Vi antigen expression occurs within dendritic cells. These results illustrate the presence of Vi expression *in vivo*, but the exact cell types involved remain unknown. The Vi capsule has been shown to be expressed inside macrophages *in vitro* (17) and during human infection, as indicated by the detection of anti-Vi antibodies in patient serum and the fact that vaccinations with the Vi antigen confers protection against typhoid fever (49, 50, 62, 85) In addition, CD18-expressing phagocytes have been shown to transport *S.* Typhimurium from the intestine to systemic circulation (106). Therefore, it is plausible that CD-18 phagocytes may also be important in systemic pathogenesis of *S.* Typhi.

In conclusion, this chapter reports that Vi expression is detected following invasion *in vivo*, in T84 polarized cells and *in vivo* in the bovine intestinal mucosa. Furthermore, we demonstrated that T3SS-1 genes, responsible for mediating invasion, act paradoxically to *viaB* encoding locus of the Vi antigen.

CHAPTER III

VI-SUPPRESSED AND VI-DEFICIENT MUTANT STRAINS OF *SALMONELLA* TYPHI PROMOTE A HYPERINVASIVE PHENOTYPE TOWARDS THE BOVINE INTESTINAL MUCOSA

INTRODUCTION

Salmonella Typhi causes a severe systemic infection of the reticulendothelial system, which is communicable by ingesting contaminated food or water. Because it is solely a human pathogen, there is a lack of suitable animal models available to study the disease. Different cell culture systems and the *S.* Typhimurium murine model have been used extensively to study the pathogenesis of *S.* Typhi (26, 35, 41, 82, 91). However, a limitation to using cell culture systems is that studies performed in infected hosts can markedly differ from what has been expected based on *in vitro* and cell culture work (5) and a consequence to using the murine model is that *S.* Typhimurium does not cause typhoid fever in humans, but instead causes a localized gastroenteritis resulting in diarrhea. Recent studies have successfully used the calf model of enterocolitis to study the pathogenic mechanisms of *S.* Typhimurium (84, 89, 98, 99) and *S.* Typhi (82, 83).

The mechanisms used by *S.* Typhimurium and *S.* Typhi for invasion and for intracellular trafficking are quite similar (26, 29, 84). The invasion-associated Type III secretion system (T3SS-1), encoded by Salmonella pathogenicity island 1 (SPI-1), allows *S.* Typhimurium to invade intestinal epithelial cells *in vitro* (26, 29). Once *S.* Typhimurium invades, like *S.* Typhi, it induces membrane ruffling and cytoskeletal

rearrangements upon contact with HeLa cell surfaces. Ruffling induced by *S*. Typhi and *S.* Typhimurium is then accompanied by macropinocytosis and aggregation of cell surface class I MHC (26).

Nevertheless, the ability for *S*. Typhi to only infect humans and produce disease must mean that there are virulence factors present in *S*. Typhi but absent in *S.* Typhimurium. Whole genome sequencing has uncovered that *S.* Typh*i* possesses a *viaB* locus encoding region for a linear polymer of α-1,4 2-deoxy-2-N-acetylgalacturonic acid variably O-acetylated at the C3 position (10, 40), most notably recognized as the Vi capsular antigen. Studies with the *viaB* mutant have shown that the presence of the Vi capsular antigen of *S*. Typhi blocks the recognition of pathogen associated molecular patterns (PAMPs) and inhibits toll-like receptor (TLR) signaling in intestinal epithelial cells (80). Calf ligated-ileal loop experiments using the *viaB* mutant resulted in an increased inflammatory response, supporting the idea that the *viaB* region plays a role in reducing intestinal inflammation *in vivo* (82).

The growth of *S.* Typhi in high osmolarity (300 mM NaCl containing LB broth, LBH) has been demonstrated to suppress the production of the Vi antigen, but the secretion of invasion proteins (SipB, SipC, SipA) are increased (2, 114). Under the same conditions, *S.* Typhi wild type is highly invasive and destructive towards epithelial and M cells of rat's Peyer's patches (114). A *viaB* mutant has been shown to secrete more effector proteins and exhibit increased invasiveness compared to the wild type strain using rat ileal loops and the human intestinal epithelial cell line Intestinal 407 (71). These findings suggest that Vi deficient and Vi suppressed strains may promote a

hyperinvasive phenotype. The *ompR* regulon in *S.* Typhi has been identified as a regulator of Vi biosynthesis and that one of its signals for regulation may be osmolarity (78). Furthermore, mutants in *ompR* have been found to be impaired in virulence, preventing them from colonizing epithelial cells in tissue culture (4).

Here we compared the invasive characteristics of Vi suppressed and Vi deficient mutants of *Salmonella* Typhi in polarized T84 cells and the bovine ligated ileal loop model. Also, we wanted to investigate whether or not a deleted *ompR* mutant, that no longer agglutinates Vi antisera, would induce similar inflammatory lesions and invasion properties as the *Salmonella* Typhi grown in high osmolarity and the *viaB* mutant.

MATERIALS AND METHODS

Bacterial strains and culture. *S.* Typhi strain Ty2 was obtained from the American Type Tissue Culture Collection (ATCC 19430). Strains were cultured aerobically in Luria-Bertani broth containing 300mM NaCl, (LBH) for optimal Vi suppression supplemented with the following antibiotics: carbenicillin, 100 mg/L or kanamycin 100 mg/L, unless otherwise noted. For T84 cell infection experiments, each strain was grown overnight at 37ºC shaking in LBH broth with appropriate antibiotics. The next day 1:1000 dilution of overnight culture was made and bacteria were grown until OD_{600}= 1.0-1.5 (late log phase) for optimal Vi suppression without antibiotics. Bacteria were added at a concentration of 0.418 x 10^9 cfu/well. For bovine ligated ileal loops, each strain was grown overnight at 37ºC shaking in 4 ml of LBH broth with appropriate antibiotics. A volume of 0.04 ml of overnight culture was used for

inoculation of 4 ml of LBH broth without antibiotics, and bacteria were grown until OD_{600}= 1.0-1.5 (late log phase) for optimal Vi suppression. Subsequently, the culture was used as the inoculum, and the numbers of CFU were determined by plating serial 10-fold dilutions on LB plates. *S.* Typhimurium, IR715 is a fully virulent, nalidixic acid resistant strain derivative of isolate ATCC 14028 (96). IR715 was used as a comparator for fluid accumulation in bovine ligated loops at 8 hours, as previously done (83, 84). Strains used in this study are listed in Table 4.

Table 4. Strains used in this study

Strain	Description	Reference and/or source
S. Typhi		
Ty2	*Salmonella* Typhi wild type	ATCC 19430
QT74	Ty2::*ompR*-::Kan^R	This study
QT113	Ty2::*ompR*-:: Kan^R carrying pBluescript SK+::*ompR*::$Carb^R$	This study
STY2	Ty2::Δ*tviABCDEvexABCDE*:: Kan^R	(Raffatellu, 2005)
S. Typhimurium		
IR715	Nalidixic acid-resistant derivative of bovine wild type isolate ATCC 4028	(Stoiljkovic, 1995)

Analysis of secreted protein. The precipitation of bacterial proteins in the supernatant of *Salmonella* growing in culture has been performed previously (84, 85). In brief, bacteria were grown under SPI-1 inducing conditions as described above. The cells were pelleted by centrifugation and 2 ml of the supernatant was collected for each sample. The supernatants were filtered (0.45 μm pore size) and then the proteins were precipitated with 0.25% Trichloroacetic acid for 1 hour on ice. The samples were pelleted by high-speed centrifugation (14,000 x *g*) for 30 minutes. The pellet was washed with cold acetone and resuspended in PBS. The proteins were then boiled in sodium dodecyl sulfate (SDS) for 5 min, and an aliquot of each sample was separated by SDS-10% polyacrylamide gel electrophoresis (SDS-PAGE).

Real-time PCR. For analysis of Typhi gene expression in culture grown bacteria, samples in broth were pelleted and extracted with hot acid phenol/chloroform. All samples prior to Reverse Transcriptase were DNase treated (Ambion DNA-free kit). Following, 1,000 ng of each sample was retrotranscribed in a 50μl volume (Taqman reverse transcription reagents; Applied Biosystems) and 4μl of cDNA was used for each real time (RT)-PCR run. Gene expression was detected using SYBR Green (Applied Biosystems) and the ABI 7500 Real Time PCR System. The *rpoD* gene has been shown to have no significant variation of expression in either Typhi or Typhimurium inside macrophages (16, 22). RNA samples were analyzed for *prgH* and *fliC* genes to monitor the expression levels of the SP-1 (T3SS) and flagellin, respectively. For each run, the calculated threshold cycle (C_t) was normalized to the C_t of *rpoD* gene amplified from the

corresponding sample and the data were analyzed using the comparative Ct method (Applied Biosystems). Levels of Typhi gene expression were calculated relative to the Ty2 wild type grown in LB (Vi expressing conditions). Primers used in this study are listed in Table 5.

Table 5. Primers used in this study

Primers	Forward 5' to 3'	Reverse 5' to 3'
fliC	CAACCTGGGCAATACCGTAAATAA	CTGCGCGCGAGACATG
iagA	ACGGACAGGGTTATCGGTTTAAT	AAAAGGAAGTATCGCCAATGTATGAG
prgH	TCATAATCGCCCCTCGCTAA	TCTATGTCGCTGCGCAAAAT
rpoD	GTATGCGTTTCGGTATC	GCTAGGGTGGCGCAGTTTAC

Cell culture and invasion assay. T84 cells are a human cell line of colon carcinoma cells that can be polarized upon seeding the cells on the apical compartment of Transwell plates and adding medium to the basolateral compartment. Over the duration of a week, the cells develop a transepithelial resistance that allows them to mimic the conditions of the human intestinal epithelium. T84 cells were seeded at 5 x 10^5 cells/ well and once they developed a transepithelial resistance of 500-1500Ω (65),

bacteria were added at approximately 10^7 cfu/well (multiplicity of infection was approximately 10:1) to the apical compartment of polarized T84 cells for 1 hour at 37°C in 5% CO_2 to allow invasion. Invasion assays were performed by using gentamicin protection assays described previously (84, 85). Each well was washed 5 times with sterile PBS to remove extracellular bacteria and medium containing 0.1 mg gentamicin/ml was added for 90 minutes incubation at 37°C in 5% CO_2. Intracellular bacteria were quantified by spreading serial 10-fold dilutions of T84 cell lysates (1% Triton x-100) on LB agar plates to determine the number of colony forming units (cfu). Invasion results were averaged from infection experiments of 3 separate transwell plates of polarized T84 cells.

Bovine ligated-ileal loop model. Four milk fed 3-4 weeks old calves were obtained from Texas A&M University cattle herd. Calves were tested for *Salmonella* infection by fecal swabs, enriched in tetraiodothionate broth and plated on XLT4 (Difco). Calves were tested for leukocytosis and fever prior to experiments. Bovine ligated ileal loop surgery has been described previously (1, 83). In brief, the calves were fasted 24 hours prior to surgery. Calves were induced using propofol (Abbott Laboratories), followed by placement of an endotracheal tube and then maintained on isoflurane (Abbott Laboratories) for the duration of the experiment (1). A right-flank laparotomy was performed, the jejunum and ileum exposed and loops with lengths ranging from 6-9 cm ligated, leaving 1-cm loops between them. The loops were infected by intralumenal injection of a 4 ml suspension containing approximately 1 X 10^9

CFU/ml with *S.* Typhi strains grown in LBH broth. Loops injected with LBH broth served as a negative control. Loops were replaced into the abdominal cavity until collection at 2 and 8 hour time points. Each bacterial strain was tested in four different animals.

After surgical removal of the loops, the fluid in accumulated in the loops was measured and samples were collected for bacteriology and histopathological analysis. The fluid accumulated was normalized to the weight of the tissue (in grams) before statistical analysis. Two 6.0 mm biopsy punches were obtained from intestinal tissue and incubated in PBS containing 0.1 mg/liter gentamicin for 90 minutes. Tissue samples were then homogenized in PBS, serially diluted, and plated on LB plates containing appropriate antibiotics for determining CFU. Data on bacterial CFU were normalized to the length of the loop and the CFU present in the inoculum prior to statistical analysis.

Histopathology. Tissue samples were fixed in formalin, processed according to standard procedures for paraffin embedding, sectioned at 5μm, and stained with hematoxylin and eosin. Inflammatory changes were scored from 0 to 5 according to the following criteria: 0, no inflammation; 1, mild inflammatory changes characterized by multifocal intravascular margination and mild perivascular infiltration of neutrophils in the lamina propria and submucosa; 2, mild to moderate inflammatory changes, characterized by mild to moderate multifocal to coalescent or diffuse infiltration of neutrophils in the lamina propria and submucosa, associated with mild to moderate edema; 3, moderate inflammatory changes, characterized by moderate multifocal to

coalescent or diffuse infiltration of neutrophils in the lamina propria and submucosa, associated with moderate edema; 4, moderate to severe inflammatory changes, characterized by mild to severe multifocal to coalescent or diffuse infiltration of neutrophils in the lamina propria and submucosa, associated with mild to severe edema; and 5, severe inflammatory changes characterized by diffuse severe infiltration of neutrophils in the lamina propria and submucosa, associated with severe edema and/or multifocal hemorrhage and epithelial erosion. Slide reading and grading were conducted blindly by two boarded veterinary pathologists.

Statistical analysis. For statistical analysis of ratios (i.e. increases in Typhi gene expression or data expressed as percentages), data were transformed logarithmically prior to performance of statistical analysis. A parametric test (paired student's t test for T84 polarized cell samples and for calf ligated loop samples) was used to calculate whether differences were statistically significant.

RESULTS

Hypersecretion of invasion proteins in the supernatant of Vi suppressed and Vi mutant strains of *S.* Typhi. Previous studies have demonstrated that a *viaB* deleted mutant strain secreted greater amount of proteins in the supernatant (71) and Vi-suppressed S. *Typhi* grown in LBH broth have a secretion pattern similar to the *viaB* deleted mutant (114). We wanted to compare the amounts of secreted proteins in the

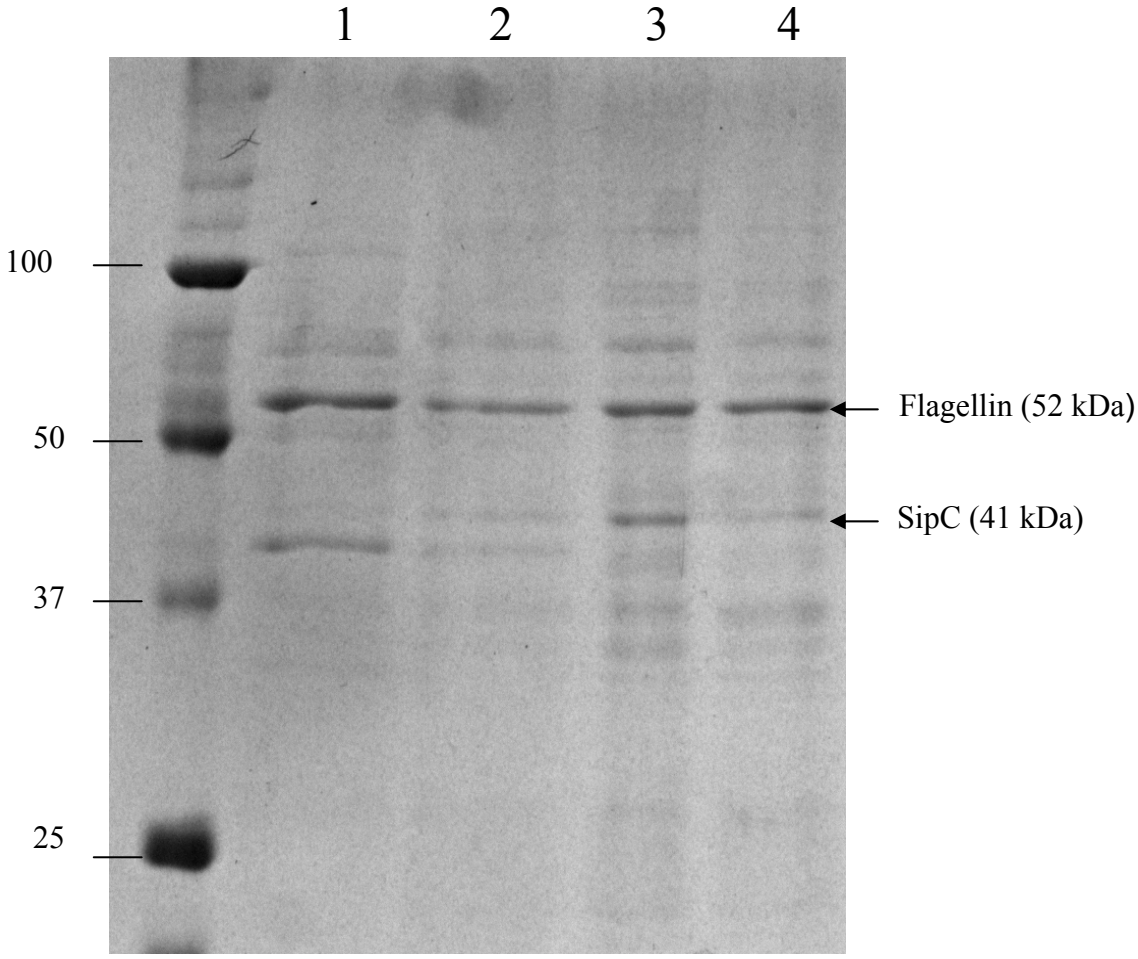

Figure 14. Invasion proteins secreted in the supernatant of Vi suppressed and Vi mutant strains of *S.* Typhi. Lane 1- Ty2 wt grown in LB, 2- Ty2 wt grown in LBH, 3-Ty2 *viaB* mutant (STY2), 4- Ty2 *ompR* mutant (QT74).

supernatant of broth media among *S.* Typhi Ty2 grown in LB (Vi-expressing), *S.* Typhi Ty2 grown in LBH (Vi-suppressing), QT74, and STY2 (Figure 14). After growth in LBH or LB broth for 4.5 hours, secreted proteins of *S.* Typhi were collected and separated on a 12% SDS-PAGE gel. A prominent flagellin 52 kDa protein was secreted in comparable amounts in all the strains. However, SipC, a type III invasion associated protein, appeared to be secreted the most in STY2. SipC in both QT74 and Ty2 wild type grown under Vi-suppressing conditions seem to be produced at similar levels. Ty2 wild type grown in LB broth, in which Vi antigen is expressed, secreted the least amount of SipC in the supernatant. These findings are in agreement with previous studies (114) and support the concept that the regulatory mechanisms responsible for Vi antigen expression and the expression of the SPI-1 (T3SS) may act paradoxically. Essentially, when the Vi antigen is expressed, the Type III secretion system may be down-regulated; whereas when the Vi antigen is suppressed, then the SPI-1 (T3SS) may be up-regulated.

Invasion associated genes of the T3SS are expressed at relatively higher levels in Vi suppressed and Vi mutants grown in culture. We wanted to confirm that the Vi mutants and Typhi wild type grown in LBH would transcribe invasion genes at higher levels than Typhi grown in LB. Regular Luria Bertani broth contains 170 mM NaCl and has an osmolarity near that of blood and plasma. Thus, under this growth condition, Vi production is detectable by antisera. Using Real-time PCR, we found that *fliC*, *prgH*, and *iagA*, genes associated with the

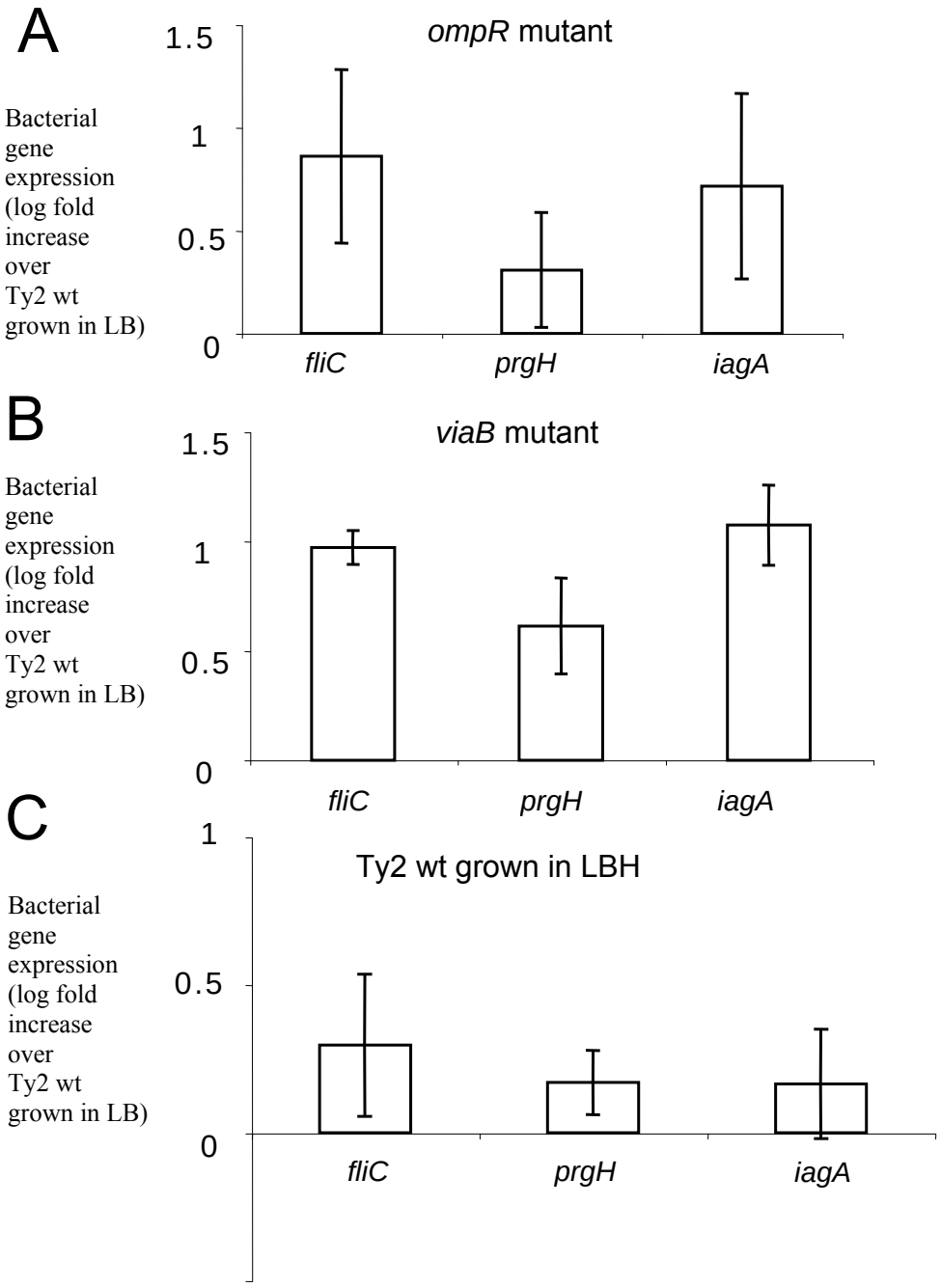

Figure 15. Profile of invasion associated genes expressed in Vi suppressed and Vi deficient strains grown in culture. Bacteria were grown in culture to late log phase (OD_{600}=1.0-1.5). RNA was extracted via hot phenol chloroform. Expression levels of *fliC*, *prgH*, and *iagA* were determined by real-time PCR. Data are shown as increases of gene expression relative to Ty2 wild type grown in Vi expressing conditions (LB). These data are averages from three independent experiments.

T3SS-1 and important in invasion tended to be elevated compared to the Typhi Vi expressing strain (Figure 15). The invasion associated SPI-1 and flagellar biosynthetic genes were previously shown to be up regulated under high osmolarity conditions in recent *in vitro* studies (2). In addition, experiments with a *viaB* deleted mutant also revealed increase invasion in cell culture systems (71, 114) and the calf ligated-ileal loop model (83), perhaps associated with the inactivation and repression of Vi antigen expression when encountering hyperosmotic stimuli. Our results supported prior findings as we also demonstrated that the growth of QT74, STY2, and *S.* Typhi Ty2 wild type grown in LBH medium (Vi-suppressing conditions) tended to express *prgH*, *fliC*, and *iagA* at higher levels than *S.* Typhi Ty2 wild type grown in LB medium (Vi-expressing conditions), though these data were not found to be statistically significant (Figure 15).

***S.* Typhi cultured in high osmolarity and Vi- deleted mutants are hyperinvasive in T84 polarized cells and the bovine intestinal mucosa.** We wanted to further investigate the invasiveness of these strains in cell culture as well as an *in vivo* system. T84 cells are a human colon carcinoma cell line that upon polarization mimic the conditions of intestinal epithelial cells (20). This intestinal model epithelia has been used successfully to study invasion of *Salmonella* (84, 85). Previous invasion experiments have revealed that a *viaB* mutation introduced in to *S.* Typhi results in increased invasion (83, 114). To the author's knowledge, no invasion experiments for the an *ompR* deletion has been performed in T84 polarized cells. Therefore, we

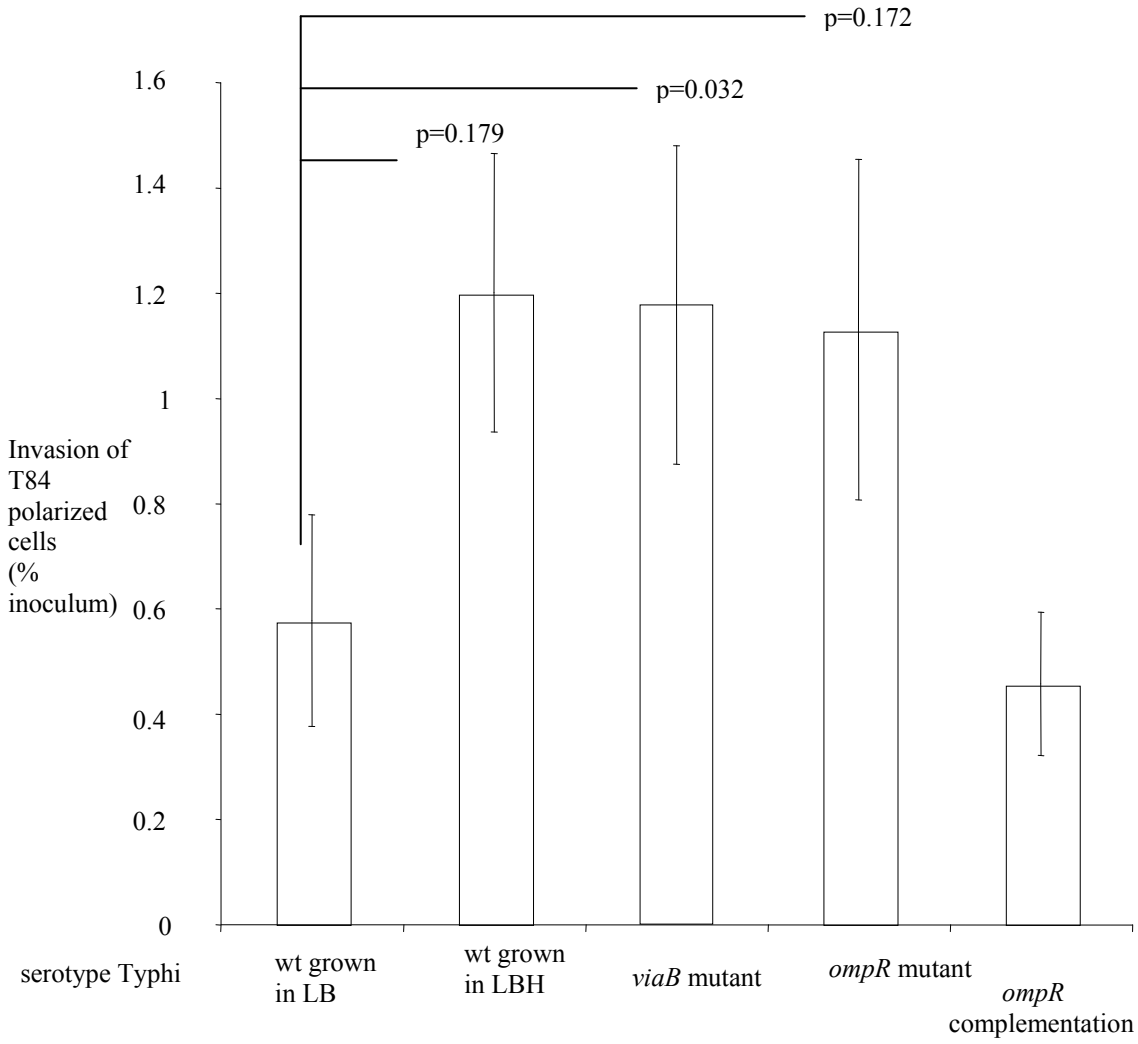

Figure 16. Bacterial invasion of T84 polarized cells after 1 hour of invasion. CFU recovered from each experiment was expressed as a percentage of CFU recovered from each well relative to the inoculum. Data are expressed as geometric means (bars) ± standard deviations from three independent experiments. Statistical significance of differences between groups is given.

performed invasion assay in T84 polarized cells to determine if the ompR mutant invaded at a higher percentage than the Vi-expressing wild type and to confirm increased invasion among *S.* Typhi grown under hyperosmotic conditions and the *viaB* mutant. We also used the *ompR* complementation strain to verify our findings.

S. Typhi Ty2 wild type grown in LBH medium, QT74, and STY2 (p-value= 0.032) was recovered in higher numbers from the T84 polarized cells than *S.* Typhi Ty2 wild type grown in LB medium (Figure 16). We used QT113, which introduced the *ompR* gene on a plasmid in QT74. The results illustrated comparable levels of invasion between *S.* Typhi Ty2 wild type grown in LB and the fully complemented *ompR* mutant, supporting the role of *ompR* as a negative regulator of *S.* Typhi invasion.

Subsequently, we investigated whether the increased invasion associated with suppressing Vi antigen expression can also be demonstrated in the calf ileal-loop model. We inoculated calf loops with the same strains used in the T84 polarized cell experiments: *S.* Typhi Ty2 wild type grown in LBH broth, STY2, QT74, and *S.* Typhi Ty2 wild type grown in LB medium, which contains 170 mM NaCl and allows Vi expression. At two hours post-infection, *S.* Typhi grown under hyperosmotic conditions and both of the deleted Vi antigen mutants exhibited significantly higher percentages of invasion then *S.* Typhi grown in LB (Figure 17A). Next, we wanted to determine if there was a difference in invasion between *S.* Typhi grown in LBH, the *ompR* mutant and *viaB* mutant. We found no statistical significance between *S.* Typhi grown in LBH and the *ompR* mutant (p=0.26) and the *viaB* mutant (p=0.12). We compared the results

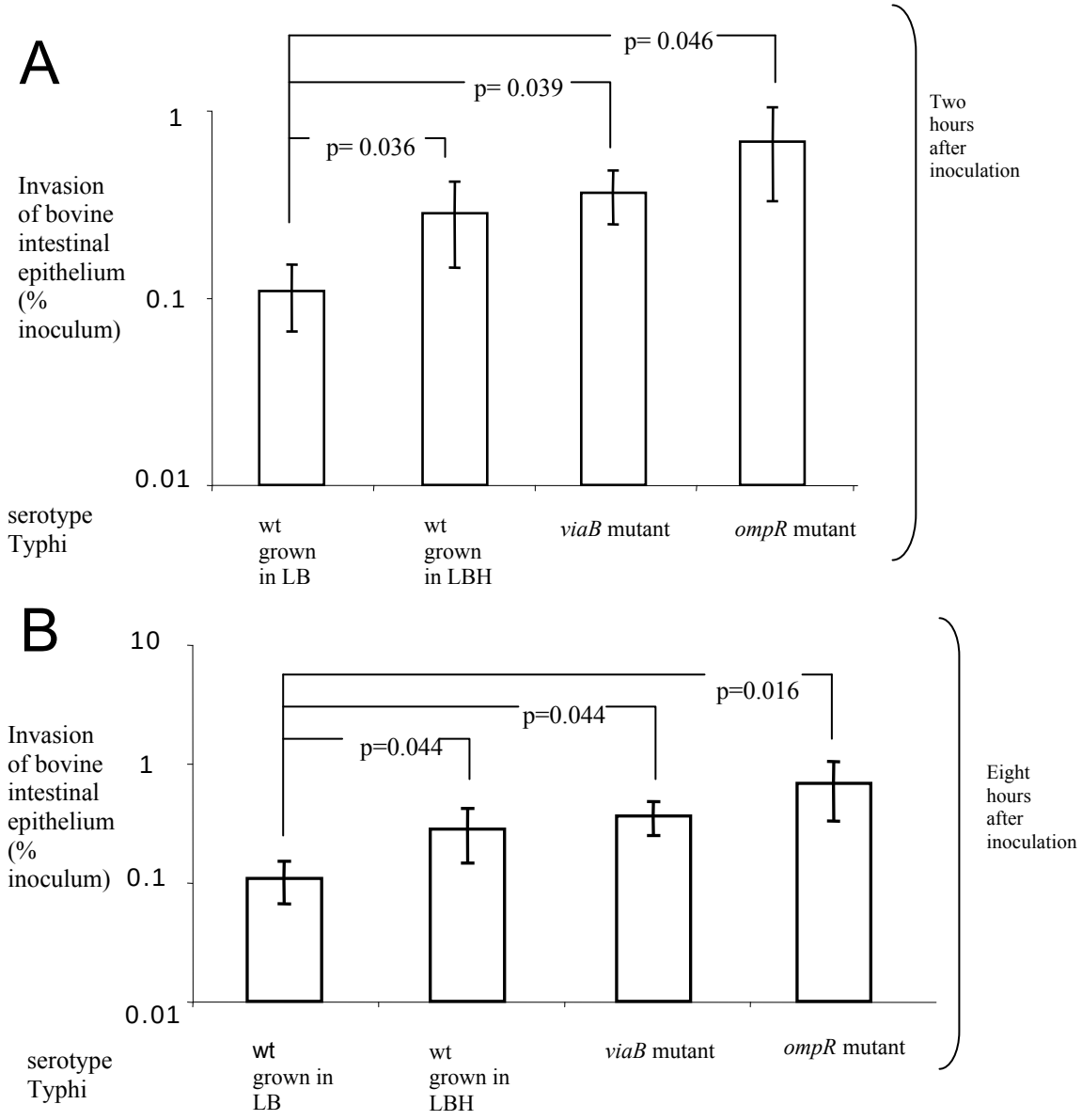

Figure 17. Bacterial invasion in bovine ligated ileal loops (A) at 2 hours and (B) at 8 hours post-infection. Bacteria was recovered from tissue (6.0 mm biopsy punches) and subsequently incubated with gentamicin to kill extracellular bacteria. CFU recovered from each animal was expressed as a percentage of CFU recovered from each loop relative to the inoculum. Data are expressed as geometric means (bars) ± standard deviations from experiments performed with four different animals. Statistical significances of differences between groups are given.

of invasion between the *ompR* and *viaB* mutant, the p-value calculated was 0.06, which indicates no significance as well. Therefore, we found no statistical difference in the amount of invasion between these groups at the 2 hour time point, revealing that *S.* Typhi grown in LBH and the Vi mutants had similar levels of invasion in bovine ileal tissue.

At 8 hours post-infection, *S.* Typhi grown in high osmolarity and both of the deleted Vi mutants also exhibited significantly higher percentages of invasion then Typhi grown in LB (Figure 17B). We compared *S.* Typhi grown in LBH, the *ompR* mutant and *viaB* mutant in invasion at this time point. There was no statistical difference in *S.* Typhi grown in LBH and the *ompR* mutant ($p= 0.14$) and the *viaB* mutant ($p=0.56$). Then, we compared the results of invasion between both of the mutants, *ompR* and *viaB*, the p-value calculated was 0.16, which indicates no significance. Again, we found that there was no statistical difference in the amount of invasion between these groups. Thus, at the 8 hour time point, *S.* Typhi grown in LBH and the Vi mutants also invaded bovine tissue at similar levels.

We used the Ty2 wild type strain carrying a high copy plasmid carrying the whole *ompR* gene in the calf experiments. However, this strain was not used in the analysis of our calf experiments for the 8 h time point because the number of cfu recovered from calf tissue was very minimal, suggesting that over time, the plasmid was lost due to instability or lack of selective pressure. This was confirmed with a growth curve in LB without antibiotics and subsequently plating cfu over time on LB plates containing carbenicillin (100 μg/ml), to check for the presence of the plasmid. The

optimal time point, in which the number of cfu still retained the plasmid was the highest, was at an OD600= 1.2 at 4 hours. At approximately 6 hours, the number of colonies retaining the plasmid dropped dramatically; less than ~ 38 % of the cfu recovered at the 4 hour time point. By the 8 hour time point, the numbers recovered were ~11% of the amount at the 4 hours time point.

Vi deficient and suppressed strains of serotype Typhi cause increase fluid accumulation in bovine ileal loops. Bovine ligated-ileal loops were inoculated with *S. Typhi* wild type, *viaB* mutant, *ompR* mutant, and LBH as a control loop. For comparative purposes, an LB loop was included to illustrate that LBH would not induce considerable osmosis into the lumen and samples were expressed relative to IR715, as previously done (81, 85). Fluid accumulation (a surrogate of diarrhea) is a marker of host response in salmonellosis infections. After 8 hours after infection, fluid amounts elicited by each strain were measured. *S. Typhi* wild type grown in Vi suppressing medium, *viaB* mutant, and *ompR* mutant expressed significantly higher ($p<0.05$) levels of fluid accumulation then the LBH control loop. The *viaB* mutant has been previously shown to produce increase fluid accumulation and cause increase inflammation compared to wild type in the calf model. In this case, we analyzed for statistically significant differences in the amount of fluid accumulation between serotype Typhi grown in LBH, the *viaB* mutant, and the *ompR* mutant. The fluid accumulated with

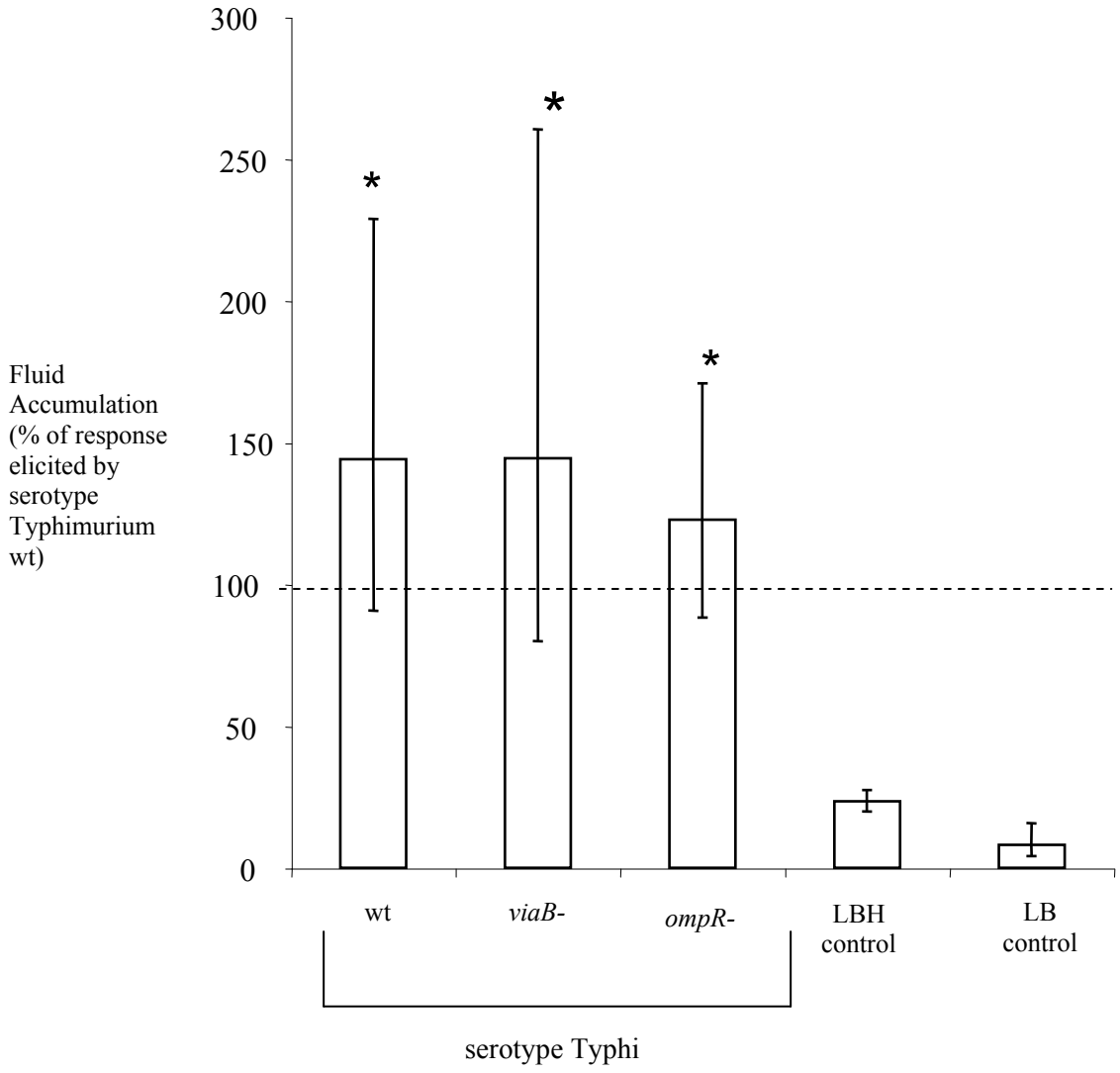

Figure 18. Fluid accumulation in the bovine ileum at eight hours post-infection of ligated ileal loops with indicated strains of serotype Typhi. Data are expressed as percentages of the response elicited by serotype Typhimurium wild type. Data on fluid accumulation were normalized to the weight of the tissue (in grams). Bars represent geometric means with ± standard deviation. Asterisks (*) indicate that differences in percent fluid accumulation are statistically significant ($p<0.05$) when compared to LBH control loop. These data are averages from four different bovids.

serotype Typhi grown in high osmolarity was not significantly higher (p=0.11) than the *ompR* mutant grown in LBH. Typhi grown in LBH was not significantly higher (p=0.17) than the *viaB* mutant grown in LBH. In comparing the *ompR* and *viaB* mutant, the amount of fluid was not statistically different (p=0.79). Interestingly, when performing the student's t-test to compare the strains with serotype Typhimurium, there was no statistically significant difference in the amount of fluid accumulated as well. Together, these data support the idea that the level of host response correlates with the lack of Vi expression.

Histopathology of bovine ileal tissue reveals increased inflammation amongst non-expressing Vi capsular strains compared to Vi expressing wild type. Gross pathological sections were observed at both 2 and 8 hour time points for bovine ligated-ileal loops inoculated with *S.* Typhi Ty2 wild type grown in LBH, *S.* Typhi Ty2 wild type grown in LB, STY2, QT74, *S.* Typhimurium IR715 wild type, or with sterile LBH broth. Histopathological changes in the bovine ileum in response to infection were graded blindly by two board certified veterinary pathologists based on a scale from 1 to 5, as mentioned in the material and methods section at both two hour and eight hour time points. However, more pathological changes were observed at the eight-hour time point. Therefore, pathology scores were determined based on changes for the eight-hour time point. The average pathological scores assigned for each strain at the 8 hour time point were: 4.5, 2.5, 4.8, 4.5, 4.9, and 1, respectively.

Sections of ileum infected with *S.* Typhi Ty2 wild type grown in LBH revealed moderate to severe changes in the epithelium, characterized by epithelial detachment, blunting and erosion, which agrees with previous studies that demonstrate the induction of invasion genes and the destruction of the intestinal epithelium with *S.* Typhi under high osmolarity growth conditions (114). Only mild increases in neutrophilic counts were detected in the lamina propria and submucosa of the ileum whereas the numbers of macrophages were markedly increased (Table 6 and 7). Sections of bovine Peyer's patches illustrated mild to moderate increases in neutrophil and moderate mononuclear cell counts. Our pathological findings were similar to previous *S.* Typhi cultured in LBH infection experiments involving rat (114) and murine (51) ileal Peyer's patches.

Table 6. Histologic changes of calf ileal loops infected with *S.* Typhi Ty2 wild type grown in LBH medium (Vi-suppressing conditions) at 2 hours

Calf #	Changes in Epithelium	Changes in Villi/Crypts	Neuts	Monos/ Macs	Lymphs	Other Pathological Findings
1	0 erosion	1 blunting	1	1	1	Some dilated lacteals
2	0 erosion	1 blunting	1-2	1	1	Some luminal necrotic debris
3	0 erosion	1 blunting	1	1	1	None
4	0 erosion	1 blunting	1	1	1	None

Table 7. Histologic changes of calf ileal loops infected with *S.* Typhi Ty2 wild type grown in LBH medium (Vi-suppressing conditions) at 8 hours

Calf #	Changes in Epithelium	Changes in Villi/Crypts	Neuts	Monos/ Macs	Lymphs	Other Pathological Findings
1	2 erosion 2 exocytosis	3 blunting	3-4	2	1	Margination of neutrophils in submucosal blood vessels
2	1 erosion	2-3 blunting 2 crypt abscessation	2	1	1	Some luminal necrotic debris, neutrophils, fibrin
3	4 erosion, epithelial necrosis	4 blunting 2 early crypt abscessatiom	2	2-3	1	Pseudomembrane Formation Bacterial colonies in submucosa
4	4 erosion	4 blunting 2 crypt abscessation	1-2	2	2	Pseudomembrane formation

Histopathological analysis of ileum infected with *S.* Typhi Ty2 wild type grown in LB revealed normal contracted intact epithelium with a mild increase in neutrophils (Table 8 and 9). The lack of infiltrating neutrophils and the presence of a predominate mononuclear cell infiltrate within the intestine is characteristic of typhoid fever infections (36, 53, 72, 75, 95).

Table 8. Histologic changes of calf ileal loops infected with S. Typhi Ty2 grown in LB at 2 hours

Calf #	Changes in Epithelium	Changes in Villi/Crypts	Neuts	Monos/ Macs	Lymphs	Other Pathological Findings
1	1 erosion	2 blunting	2	2	1	None
2	1 erosion	2 blunting	2	1	1	Neutrophils in lacteals
3	1 erosion	2 blunting	2	2	1	None
4	1 erosion	1 blunting	1	1	1	None

Table 9. Histologic changes of calf ileal loops infected with S. Typhi Ty2 grown in LB at 8 hours

Calf #	Changes in Epithelium	Changes in Villi/Crypts	Neuts	Monos/ Macs	Lymphs	Other Pathological Findings
1	1 erosion	1 blunting	1	1	1	Reactive endothelium lining submucosal blood vessels
2	2 erosion	1 blunting	2	2	1	Some luminal necrotic debris
3	1 erosion	1 blunting	2	2	1	Some luminal necrotic debris
4	2 erosion	1 blunting	2	2	1	None

Sections of ileal loops inoculated with STY2 demonstrated moderate to severe changes in the epithelium infrastructure characterized by blunting and fusion of the epithelium (Table 10 and 11). There was a loss of glandular structures along with a predominant neutrophil infiltrate in the lamina propria with signs of early crypt abscess development. Large numbers of neutrophils were also present within the interstitium of lymphoid follicles (Peyer's patch). Our results confirm previous data in bovine ligated-ileal loops, in which the viaB mutant induced more inflammatory changes when compared to the wild type (83). In rat ligated-ileal loop experiments infected with a Vi-deficient strain (GIFU10007-3), similar morphological destructive changes in intestinal follicle-associated epithelium were observed (114).

Table 10. Histologic changes of calf ileal loops infected with S. Typhi Ty2 Δ*viaB* at 2 hours

Calf #	Changes in Epithelium	Changes in Villi/Crypts	Neuts	Monos/ Macs	Lymphs	Other Pathological Findings
1	0 erosion	0-1 blunting	1	1	1	Some dilated lacteals
2	0 erosion	1 blunting	1	1	1	Some luminal necrotic debris, neutrophils, fibrin
3	0 erosion	0-1 early crypt abscess formation 2 blunting	0-1	2	1	None
4	0 erosion	1 blunting	1	1	1	None

Table 11. Histologic changes of calf ileal loops infected with S. Typhi Ty2 Δ*viaB* at 8 hours

Calf #	Changes in Epithelium	Changes in Villi/Crypts	Neuts	Monos/ Macs	Lymphs	Other Pathological Findings
1	2 erosion 2 exocytosis	3 blunting 1-2 crypt abscessation	3	2	1	Pseudomembrane formation
2	2 erosion 2 epithelial detachment 2 exocytosis	3-4 blunting 2 crypt abscessation	2	2	1	Pseudomembrane formation
3	3-4 erosion	4 blunting 2 early crypt abscessation	1	2-3	1	Pseudomembrane formation
4	2 erosion	2-3 blunting 1 crypt abscessation	2	2-3	2	Pseudomembrane formation

To the author's knowledge, no previous experiments have been conducted to examine the intestinal pathology induced by an *ompR* deletion, a possible positive regulator for Vi antigen expression and negative regulator for invasion of *S.* Typhi. Calf ligated-ileal loops were inoculated with QT74, an *ompR* mutant we generated in this study. After two and eight hours of infection, the intestinal epithelium displayed moderate blunting and fusion of the intestinal epithelium along with a predominant neutrophil infiltrate within the lamina propria (Table 12 and 13). Fibrin and neutrophils were present within the intestinal lumen (pseudomembranous formation). There were signs of early crypt abscess formation and submucosal edema. Multiple sections revealed engorgement of blood vessels with neutrophils as well as rod-shaped bacteria inside neutrophils, as a result of phagocytosis. The lack of Vi antigen production and the activation of the invasion-associated genes have been previously reported (2, 79, 104, 114). This provides a possible explanation for the inflammation and subsequent destruction to the intestinal epithelial integrity induced by S. Typhi Vi-deficient strains (114).

Table 12. Histologic changes of calf ileal loops infected with S. Typhi Ty2 Δ*ompR* at 2 hours

Calf #	Changes in Epithelium	Changes in Villi/Crypts	Neuts	Monos/ Macs	Lymphs	Other Pathological Findings
1	1 erosion 1 exocytosis	1 blunting	2-3	2	1	Reactive endothelium and margination of neutrophils in arterioles
2	1 erosion	1 blunting	1-2	1	1	None
3	3 erosion	3 blunting	1	2	1	None
4	0 erosion	1 blunting	0	0	0	None

Table 13. Histologic changes of calf ileal loops infected with S. Typhi Ty2 Δ*ompR* at 8 hours

Calf #	Changes in Epithelium	Changes in Villi/Crypts	Neuts	Monos / Macs	Lymphs	Other Pathological Findings
1	2 erosion 2 exocytosis	2-3 blunting 3 crypt abscessation	3	2-3	1	Pseudomembrane formation
2	2 erosion	2-3 blunting 2 crypt abscessation	2-3	2	1	Bacterial emboli Pseudomembrane formation
3	2-3 erosion	2-3 blunting 2 crypt abscessation	2	2-3	1	Bacteria in deep submucosa
4	2 erosion	2 blunting 2 crypt abscessation	3	2	1	Pseudomembrane formation

Studies infecting bovine ligated ileal loops with *S.* Typhimurium IR715 wild type, an *in vivo* model for human gastroenteritis, have previously demonstrated the inflammatory deleterious effects on gut tissue (27, 91). Results from inoculating calf ligated-ileal loops with IR715 served as our positive control in these studies for host inflammatory changes. Sections of ileum illustrated blunting and fusion of the intestinal epithelium with a predominant neutrophil infiltrate in the lamina propria as neutrophils marginated from the vascular supply into tissue (Table 14 and 15). There were also signs of submucosal edema, congested lymphatics, and necrosis. All these finding were in good agreement with previous reports (91, 100, 113).

Table 14. Histologic changes of calf ileal loops infected with *S.* Typhimurium IR715 wild type at 2 hours

Calf #	Changes in Epithelium	Changes in Villi/Crypts	Neuts	Monos/ Macs	Lymphs	Other Pathological Findings
1	4 erosion 3 exocytosis	3 blunting	3-4	2	1	Margination of neutrophils in arterioles
2	3 erosion	3 blunting	3	2	1	Early crypt abscess formation
3	3 erosion	3 blunting	4	2	1	Margination of neutrophils in arterioles
4	3 erosion	4 blunting	3	2	1	Margination of neutrophils in arterioles

Table 15. Histologic changes of calf ileal loops infected with *S.* Typhimurium IR715 wild type at 8 hours

Calf #	Changes in Epithelium	Changes in Villi/Crypts	Neuts	Monos/ Macs	Lymphs	Other Pathological Findings
1	4 erosion 3 exocytosis	3 blunting	3-4	2	1	Margination of neutrophils in arterioles
2	4 erosion	3 blunting	4	2	1	Crypt abscess formation
3	4 erosion	3 blunting	4	2	1	Crypt abscess formation
4	4 erosion	4 blunting	4	2	1	Margination of neutrophils in arterioles

Negative control loops inoculated with LBH broth had no significant pathological findings. Most sections revealed submucosal edema with a normal intact epithelium, with few neutrophil infiltrates.

Table 16. Histologic changes of uninfected LBH control calf ileal loops at 2 hours

Calf #	Changes in Epithelium	Changes in Villi/Crypts	Neuts	Monos/ Macs	Lymphs	Other Pathological Findings
1	0 erosion	0 blunting	1-2	1	1	None
2	0 erosion	0 blunting	1-2	1	1	None
3	0 erosion	1 blunting	1	2	1	None
4	0 erosion	2 blunting	0-1	2	1-2	None

Table 17. Histologic changes of uninfected LBH control calf ileal loops at 8 hours

Calf #	Changes in Epithelium	Changes in Villi/Crypts	Neuts	Monos/ Macs	Lymphs	Other Pathological Findings
1	0 erosion	0 blunting	1	1	1	None
2	0 erosion	0 blunting	1	1	1	None
3	0 erosion	0 blunting	1	1	1	None
4	0 erosion	0 blunting	1	1	1	None

DISCUSSION

The Vi capsular antigen of *S.* Typhi is a significant virulence factor important in the pathogenesis of typhoid fever. Studies have shown that the Vi capsule may exhibit anti-inflammatory effects (81, 83). Much focus has recently been placed on studying the regulators and environmental conditions that modulate the Vi antigen expression. Expression of the Vi antigen is controlled by the *viaA* and *viaB* chromosomal loci (37). *In vitro* studies have demonstrated that the infection of T84 polarized cells with a *viaB* mutant of *S.* Typhi causes an increase in IL-8 levels compared to infection with *S.* Typhi wild type, suggesting that the presence of the capsule causes decreased inflammatory cytokine production (81). *ompR* and *tviA* are two genes that are located on the *viaB* locus. Another gene, *rcsB*, located on the *viaA* locus is thought to act as a positive regulator of Vi expression through *tviA* (105). More recently, the *rpoS* gene, a master regulator in the general stress response and required for survival under extreme stress conditions and for virulence in *S.* Typhimurium, has been reported to act as another regulator of Vi polysaccharide synthesis (88).

In this study, our main goal was to examine the role of the *ompR* regulon *in vitro* with T84 polarized cells, an intestinal culture model system that has not been used extensively to study *S.* Typhi and the bovine ligated ileal loop model, an animal model that has been successfully implemented to *S.* Typhi pathogenesis (81, 84).

The involvement of the *S.* Typhi *ompR* gene in regulating the biosynthesis of the Vi capsule has been previously demonstrated, and it has been suggested that this regulation may be responsive to osmolarity environmental signals (78). However, the

function of *ompR* during invasion of *S.* Typhi remains unclear. In *Shigella flexne*ri, *ompR* mutants were shown to be defective in their ability to invade epithelial cells (4). The evaluation of the invasion properties of a *S.* Typhi *ompR* mutant have been reported in one study to not impair the ability of invasion of epithelial cells, but does not divulge any data indicating increased levels of invasion (78). Currently, experimental studies are underway to evaluate the current hypothesis that the lack of Vi antigen production may up-regulate the expression of the T3SS-1 invasion genes and cause hyperinvasion among these strains. Studies have shown that Vi-deficient strains and Vi-suppressed strains result in increased invasion compared to the wild type and destruction to murine Peyer's patches (71, 114). Our results from performing the invasion assay in T84 polarized cells showed increase invasion among Vi-suppressed strains and Vi-deficient strains, including a *S.* Typhi *ompR* mutant we constructed, compared to the wild type Vi-expressing strain. Using the same strains and inoculating them into bovine ligated-ileal loops, we also recovered higher bacterial numbers among the Vi-lacking strains. Histopathological examination of infected bovine ileal tissue with Vi mutant strains revealed moderate to severe inflammation along with high neutrophilic infiltration.

Our other experimental results pertaining to the hypersecretion of invasion-associated proteins in the supernatant of culture medium agree with previous studies (114). Our protein expression analysis revealed that the *ompR* mutant secretes moderately increased levels of flagellin and SipC. Real-time PCR analysis of invasion genes in different osmolarity growth conditions and among different Vi regulator mutants also supports the increased invasion theory when repressing or inactivating Vi

expression. Fluid accumulation in the intestinal (a surrogate of diarrhea) is a valid measurement of the host inflammatory response induced by each bacterial strain (111). The measured fluid collected in the bovine intestinal lumen at 8 hours revealed that the *ompR* deletion mutant results in similar levels of inflammation to *S.* Typhi grown in LBH and the *viaB* deletion mutant. Thus, it is logical to propose based on previous data and our findings that deleting the *ompR* gene in *S.* Typhi, which inhibits Vi capsule production, may subsequently up-regulate the SPI-1 (T3SS-1) invasion genes, resulting in equivalent invasion and damaging effects to the intestinal epithelium.

Possible explanation for any differences in invasion analysis results may be a consequence of laboratory techniques, including bacterial growth conditions; inoculation at different growth phases; inoculum size; and inoculum composition used. Divergent findings in the interaction between *Salmonella* invasion and murine Peyer' patch has been reported (14, 51, 77). Clark demonstrated that the infection of murine ileal-ligated loops with *S.* Typhimurium results in M cells damage and follicular-associated epithelium dependent upon inoculum composition (14). Our T84 infection (65, 80) and bovine ligated ileal-loop experiments (82, 84) were conducted based on previous methodology. In brief, our strains were grown overnight at 37ºC shaking in appropriate broth, LB or LBH medium with or without appropriate antibiotics. The next day 1:1000 dilution of overnight culture was made and bacteria were grown until OD_{600}= 1.0-1.5 (late log phase) for optimal Vi suppression without antibiotics. Subsequently, the culture was used as the inoculum, and the numbers of CFU were determined by plating serial 10-fold dilutions on LB plates.

In conclusion, our data confirm the concept that the Vi polysaccharide of *S.* Typhi behaves as a negative regulator in the invasion process under hyperosmotic growth conditions, which is proposed to mimic the conditions of the intestinal lumen in mammals. This is further supported by increased invasion demonstrated among Vi regulator mutants in this investigation and others. The Vi-suppressed *S.* Typhi is similar to the Vi-deficient mutants in its increased invasiveness in T84 polarized cells bovine ligated-ileal loops and destruction the bovine intestinal epithelium and Peyer's patch.

CHAPTER IV

SUMMARY AND CONCLUSIONS

Typhoid fever is an acute, systemic infection of the reticuloendothelial system caused by *Salmonella enterica* serovar Typhi, which is responsible for an estimated 16 million illnesses and 600,000 deaths worldwide, annually (68). *S.* Typhi is highly host adapted to humans and higher nonhuman primates. Thus, there is a lack of animal models suitable to study the molecular mechanisms of this pathogen as well as its host-pathogen interactions. The pathogenesis of typhoid fever is commonly studied using *Salmonella enterica* serovar Typhimurium infection in mice as an animal model. The intestinal pathology caused by *S.* Typhimurium in mice resembles that of typhoid fever patients. However, human infection with *S.* Typhimurium causes a localized enterocolitis with a massive neutrophil influx and subsequent diarrhea. Differences in disease manifestations in humans between *S.* Typhi and *S.* Typhimurium indicate that *S.* Typhi possesses distinct virulence factors important in its pathogenesis. The calf model has been used previously to study the pathogenic mechanisms of typhoidal salmonellosis (82, 83). Thus, the calf model is a good animal model that allows us to perform comparative studies investigating *S.* Typhi virulence factors that are absent from *S.* Typhimurium and those that may be responsible for allowing *S.* Typhi to cause typhoid fever in humans.

S. Typhi possesses an important virulence factor, recognized as the Vi antigen, which is not present in the *S.* Typhimurium genome. Current preventative typhoid fever

vaccines are formulated based on the polysaccharide capsule and confer a 65-75% protective immunity. Previous studies demonstrated that a non-capsulated *S.* Typhi produces a stereotypic inflammatory host response resulting in CXC IL-8 secretion and neutrophilic infiltration, resembling the inflammatory cascade elicited by *S.* Typhimurium (80, 82). The results from these experiments suggest that the Vi capsular antigen may be responsible for preventing the host innate immune response in the intestinal mucosa. The lack of inflammation seen with typhoidal salmonellosis also raises the possibility that the Vi antigen may exhibit properties of an anti-inflammatory drug. However, Vi polysaccharide expression in response to the intestinal environmental stimuli and the regulators involved in its biosynthesis and export has not been examined extensively in cell culture models or in a suitable animal model. Therefore, the goal of these studies was to unravel the mechanisms of Vi capsule expression and to understand the function of the regulators that modulate its expression using human colonic epithelial cells and the bovine ligated-ileal loop model.

In the first chapter, we implemented direct and indirect methods to localize and detect Vi antigen expression within intestinal epithelial cells using T84 polarized cells and the bovine ligated-ileal loop model. We reported that *tviB*, a gene necessary for Vi production in *S.* Typhi, was significantly up-regulated intracellularly within T84 polarized cells using real-time PCR. Infection experiments conducted in the same cell line with a *S.* Typhi carrying a *gfp*-expressing plasmid fused to the *viaB* promoter, revealed relatively increased numbers of intracellular Vi-expressing bacteria compared to the numbers of extracellular bacteria using flow cytometry analysis. These findings

help support our hypothesis that the Vi antigen is up-regulated following invasion of intestinal epithelial cells. Along with previous data showing a *viaB* mutant eliciting decreased levels of IL-8 production in human epithelial cells (82), it provides evidence that the location of Vi expression may in fact serve to prevent TLR recognition.

To determine whether this occurred *in vivo*, we inoculated calf ligated ileal loops and studied Vi gene expression with real-time PCR and detected Vi production via fluorescence immunohistochemistry. Again, we discovered that *tviB* was expressed at levels significantly higher in calf tissue after invasion compared to the inoculum grown under Vi-suppressing conditions. The presence of the Vi capsular antigen was detected in bacteria along the intestinal villi tips where they appeared to penetrate the intestinal epithelial barrier and enter the lamina propia. The expression of the Vi capsule along the tips of the villi and essentially in the lumen may be a result of the hypoosmotic stimuli of the luminal fluid since it is has been shown that Vi is expressed under hypoosmotic growth conditions (78, 103). On the other hand, it may be that the Vi capsule is expressed in the lumen, which would be in support of other research findings that indicate the capsule may reduce bacterial adhesion and invasion of intestinal epithelium (2, 71). This explanation, however, is not supported by the majority of the current studies on capsule expression. Thus, the reason for Vi expression detected at the tips of the villi remains unclear.

The detection of Vi production in the area of the lamina propria does suggest that the Vi may function in masking innate immune recognition in the lamina propria since the location of TLRs have been identified on the basolateral pole of intestinal epithelial

cells (31). Additionally, the lamina propria contains numerous phagocytes that express a wide range of PAMP receptors on their surfaces. Fluorescent staining of bovine Peyer's patch sections revealed large numbers of Vi-expressing bacteria in the mantle and in the area between germinal centers. Since Peyer's patch are large lymphoid aggregations consisting of both T and B cells areas within the small intestinal mucosa, these data suggested that Vi antigen may be expressed intracellularly with dendritic cells. The dendritic cells may function in antigen presentation for T cell- proliferation and T-cell help for B-cell responses. The Vi capsule has been shown to be expressed inside macrophages *in vitro* (17) and during human infection. In addition, CD18-expressing phagocytes have been previously shown to be important in the dissemination of *S.* Typhimurium infection (102), so it is possible that Vi-expression may occur within CD-18 cells. However, no studies have definitely shown the exact cells types in which Vi-expression occurs. Our results illustrated that the Vi capsule was expressed following invasion of the gut mucosa, which supported a role of the capsule during bacterial host cell interaction in tissue. This coincides with earlier calf ligated-ileal loop studies in which a capsulated *S.* Typhi reduced IL-17 expression in the bovine intestinal mucosa (82). Our data along with the experimental findings from that study help to put forth the belief that Vi expression occurs after invasion and blocks the host immune system. With previous data and our experimental findings in this chapter, the potential role of the Vi polysaccharide capsule and its regulated expression are summarized in Figure 19.

Two distinct processes contribute to the hyperosmotic conditions within the intestinal lumen. First, the digestions of foodstuffs in which macromolecules are

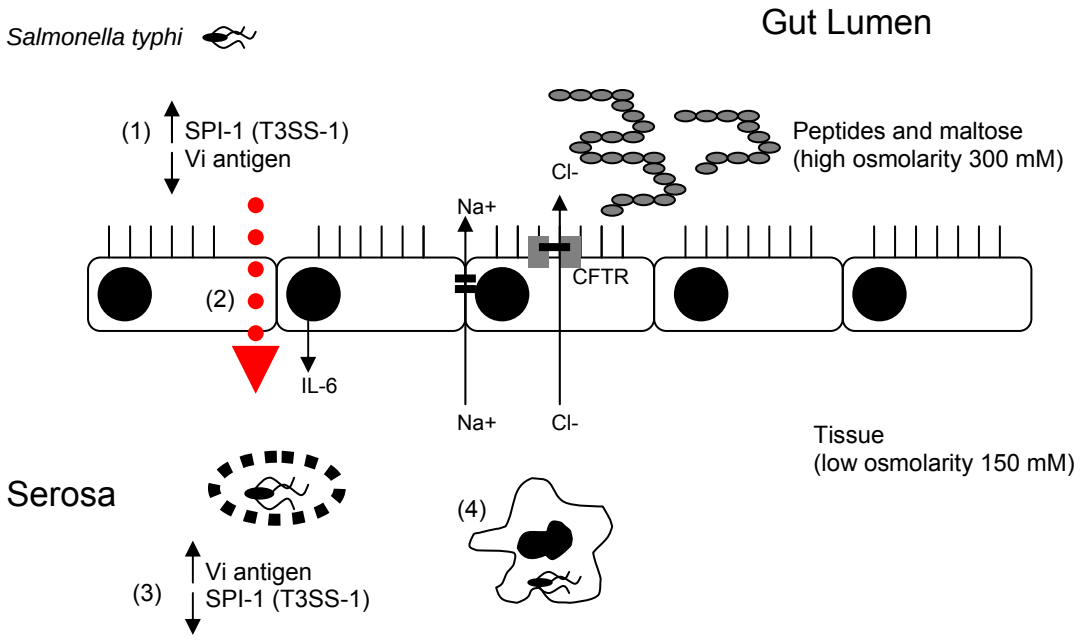

Figure 19. Summary of events occurring within the gut lumen and gut-associated tissues with *Salmonella typhi* (Vi+) infection. (1) Exposure to the hyperosmotic conditions of the gut lumen upregulates SPI-1 and promotes a hyperinvasive phenotype. Vi-expression is down-regulated, which further contributes to increase secretion of SPI-1 effectors. (2) *Salmonella typhi* invades intestinal epithelial cells and induces IL-6 secretion. (3) Exposure to the hypoosmotic conditions within tissue downregulates SPI-1 and Vi-expression is upregulated. (4) The invading bacteria are taken up by macrophages, dendritic cells (M cells), and lymphocytes within gut-associated tissue and disseminated throughout the body.

enzymatically broken down into thousands of smaller molecules, each osmotically active and causing a dramatic increase in the osmolarity of the chyme. Secondly, crypt cells actively secrete electrolytes into the lumen. *S.* Typhi gains access to the intestinal lumen and is exposed to the hyperosmotic conditions, causing an up-regulation of SPI-1 (Type III secretion system) genes and the suppression of Vi antigen genes. *S.* Typhi invades the intestinal mucosa and induces IL-6 secretion from intestinal epithelial cells. After invasion, *S.* Typhi encounters hypoosmotic conditions within the tissue. At this point, SPI-1 is down-regulated and Vi antigen expression is activated. The expression of the Vi antigen in the lamina propria may be acting to mask PAMP recognition by phagocytes and preventing a TLR-mediated response. This is consistent with the lack of IL-8 production and neutrophil recruitment seen with typhoid fever infections. The invading bacteria are eventually taken up by macrophages, dendritic cells, and lymphocytes within the gut-associated lymphoid tissue and disseminated systemically.

In the second chapter, we wanted to particularly study the involvement of the *ompR* regulon during invasion and its role in mediating Vi expression using human intestinal epithelial cells and the calf-ligated ileal loop model. *Salmonella* Typhi possesses a functional invasion associated Type III secretion system (T3SS-1) important during the initial stages of infection of intestinal epithelial cells, similar to the mechanisms present in *S.* Typhimurium. Recent studies have shown that that *S.* Typhi grown under conditions of high osmolarity *in vitro* switches off Vi antigen expression (78) whereas the type III secretion system genes become up-regulated (2). The

mechanism by which this occurs still remains uncertain, though it appears that the expression of the Vi capsule and invasion-associated genes behave in an opposite manner. Results from experiments with non-expressing Vi strains displayed increased invasion towards intestinal epithelial cells (71, 114). These findings support the concept that Vi-suppressed and Vi mutant strains of *S.* Typhi promote an increased invasive phenotype, perhaps through the downregultion of Vi expression. OmpR is a positive regulator in the synthesis and expression of the Vi capsule. A single study reports that an *ompR* deletion in *S*. Typhi inhibits Vi production and the strain was not impaired in its ability to invade intestinal epithelial cells, but did not assess whether this strain displayed increased invasion (78). As a result, we wanted to compare the invasiveness characteristics between *S.* Typhi grown under hyperosmotic conditions, a *viaB* mutant, and an *ompR* mutant. We also wanted to determine the effects of these strains in eliciting inflammation in the calf model, a good animal model for studying typhoid fever pathogenesis.

We report that *S.* Typhi grown under Vi -suppressing conditions, the *viaB* mutant, and the *ompR* mutant all illustrated increase invasion in polarized T84 cells and bovine ileal tissue. Analysis of invasion-associated genes encoded by SPI-1 with real time PCR also revealed increased levels of expression in *fliC*, *iagA, prgH* in the *ompR* mutant strain. These data help to support our hypothesis that increased invasion among these strains may be due to the deficiency in Vi antigen expression and the regulation responsible for controlling SPI-7 and SPI-1 may function conversely. The findings complement the theory that Vi expression maybe down-regulated in the intestinal lumen

in response to hyperosmotic stimuli in order to promote a hyperinvasive phenotype, allowing *S.* Typhi to invade deeper into tissues.

Fluid accumulation in previous calf ligated-loop studies is observed as a marker of inflammation elicited by *S.* Typhimurium and *S.* Typhi as it corresponds to the number of bacteria that have invaded the gut mucosa (82-84). The *viaB* mutant was previously demonstrated to have more fluid accumulation relative to the wild-type *S.* Typhi capsulated strain and similar levels of fluid accumulation to a *S.* Typhimurium capsule-expressing strain, once more suggesting the presence of the capsule may be responsible for inhibiting inflammation (82). Fluid accumulation in loops inoculated in this investigation with Vi-deficient and Vi-suppressed strains were comparable to *S.* Typhimurium and were significantly higher than the LBH uninfected control loop. This indicates the number of bacteria that invaded were comparable among the different strains. Interestingly, all the *S.* Typhi strains used were Vi-lacking and *S.* Typhimurium does not possess a Vi capsule, providing a potential rationale for the increase invasion among Vi negative strains. Histopathological evaluation of ileal tissue has also been performed to characterize inflammation induced by *S.* Typhimurium and *S.* Typhi in earlier experiments (82, 90). The *S.* Typhi *viaB* mutant (STY2) has been shown to trigger more inflammation than its capsulated wild-type (82). The histopathological inflammatory lesions produced in this study by the Vi-deficient and suppressed strains were quite similar. The strains appear to elicit more inflammation than the capsulated strain. Our data further supports the notion that Vi- suppressed and Vi mutants of *S.* Typhi exhibit similar levels of increased invasion and inflammation, perhaps

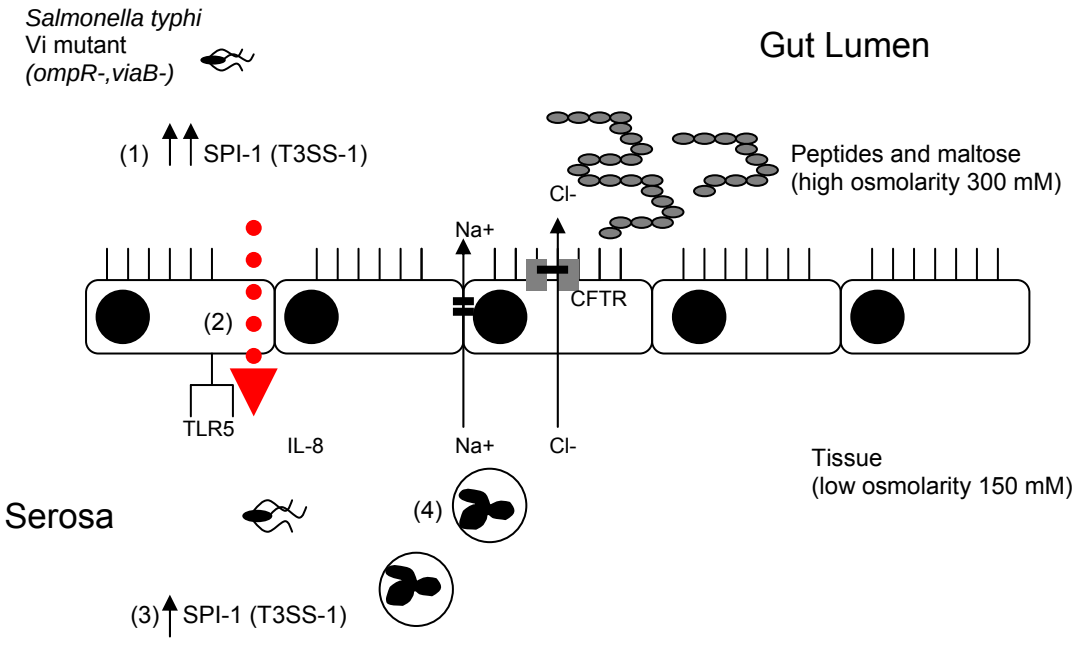

Figure 20. Summary of events occurring within the gut lumen and gut-associated tissues with *Salmonella typhi* (Vi-) infections. (1) Exposure to the hyperosmotic conditions of the gut lumen upregulates SPI-1 and promotes a hyperinvasive phenotype. The lack of Vi expression further contributes to increase secretion of SPI-1 effectors. (2) *Salmonella* invades intestinal epithelial cells and PAMPs are recognized by TLRs on the basolateral surface and induces IL-8 secretion. (3) *Salmonella* continues to express SPI-1 after encountering hyposmotic conditions in tissue. (4) IL-8 recruits neutrophils to the site of infection.

mechanistically through the inactivation of the Vi antigen. With previous reports and our experimental findings in this chapter, the characteristics of the Vi negative strains and their regulated expression are portrayed in Figure 20.

In summary, the non-capsulated *S.* Typhi strains are exposed to the hyperosmotic conditions within the intestinal lumen. Subsequently, the SPI-1 (T3SS-1) genes are upregulated and allow increased invasion. Following invasion, the Vi negative strains do not express a Vi capsule, so PAMPS are recognized by the innate immune system, including TLR5 and TLR4, stimulated by flagellin and LPS, respectively. The bacteria are also recognized by a wide variety of other PAMP receptors located on phagocytes circulating in the lamina propria. Upon activation of the TLR response, neutrophils are recruited to the site of infection and IL-8 is secreted.

Extensive investigation has been conducted to characterize the regulation of the Vi expression *in vitro*; however, studies have shown that the gene regulation in infected hosts can markedly differ from what has been expected based on *in vitro* and cell culture work (5, 55, 56) stressing the need for *in vivo* studies to understand *Salmonella* virulence gene regulation fully.

Polysaccharide capsules are found on the surface of a wide range of gram-negative bacteria. Capsules have a significant role in determining access of certain molecules to the cell membrane, mediating adherence to surfaces, and increasing tolerance of desiccation. Furthermore, capsules of many pathogenic bacteria impair phagocytosis (63) and reduce the action of complement-mediated killing. Consistent

with this belief, the Vi-antigen was shown to impede uptake of *S*. Typhi by human neutrophils (64). Thus, the Vi capsular antigen is likely to be major virulence determinant of *S.* Typhi. With this concept in mind, continued efforts should concentrate on the investigation of novel role(s) that the Vi-antigen may serve and the regulators that modulate its expression at the host-pathogen interface.

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APPENDIX

HISTOLOGY SLIDES FROM THE INOCULATION OF CALF-LIGATED ILEAL LOOPS

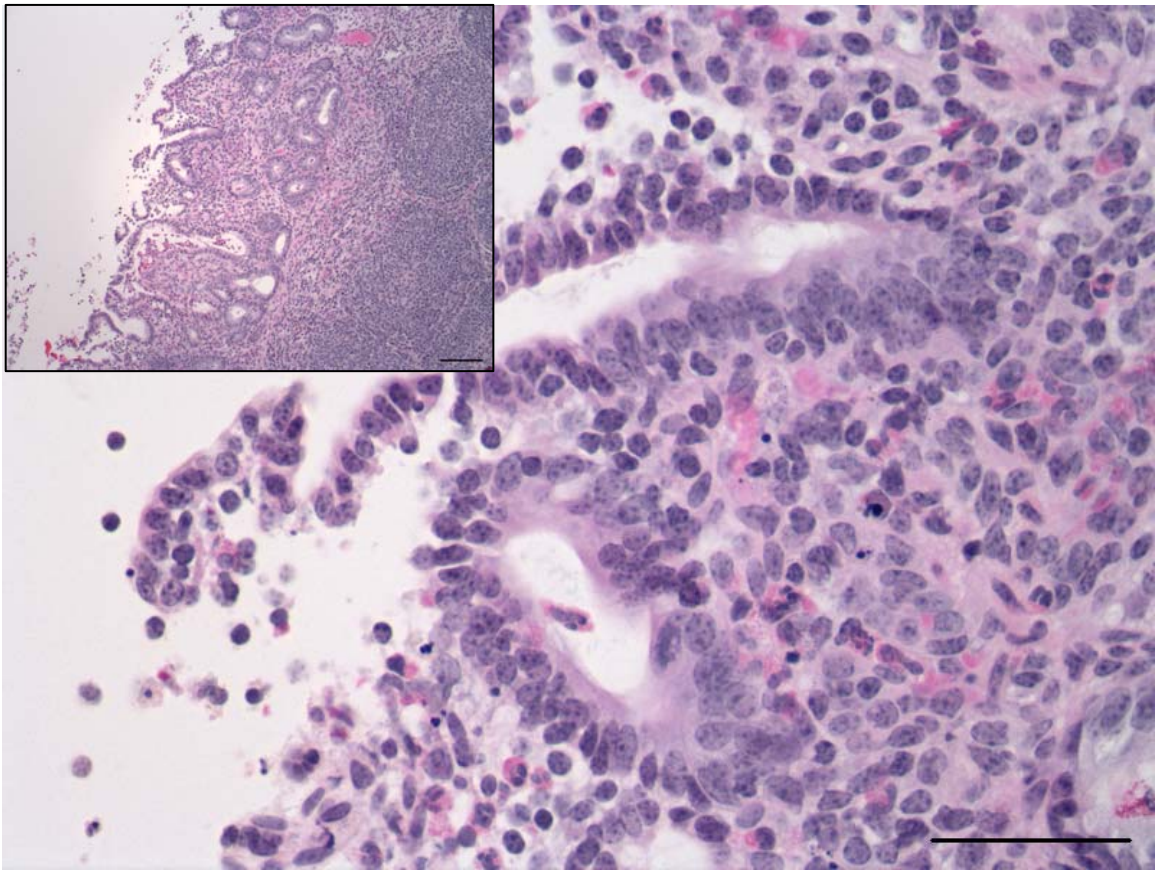

Figure A-1. H&E stain of bovine intestinal epithelium (ileum) from calf# 2, 8 hours post-inoculation with *S.* Typhi Ty2 *viaB* mutant. The tissue exhibits moderate blunting, erosion, and epithelial detachment, with moderate neutrophilic and mononuclear infiltration. Some luminal necrotic debris containing neutrophils and fibrin present in the lumen (pseudomembranous formation).

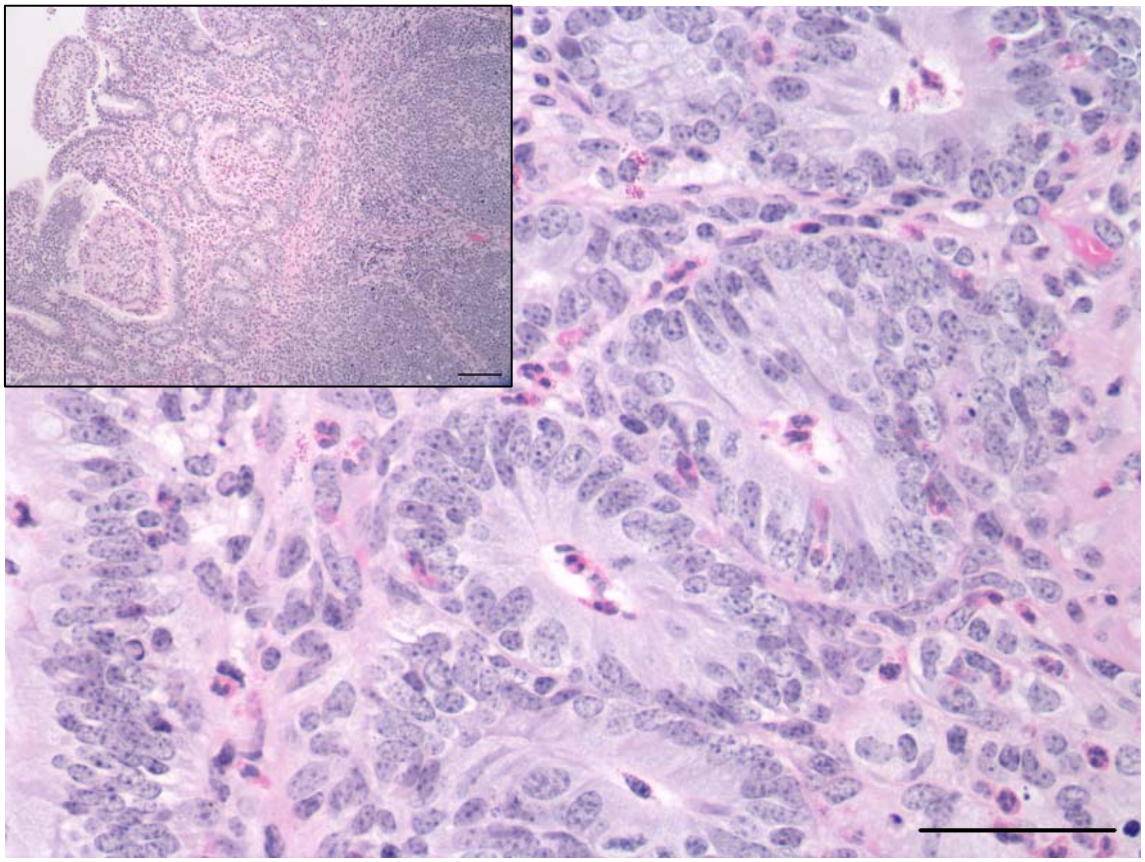

Figure A-2. H&E stain of bovine intestinal epithelium (ileum) from calf# 2, 8 hours post-inoculation with *S.* Typhi wild type Ty2 grown in LB broth. The tissue exhibits mild blunting and epithelial detachment, with a mild neutrophilic and mononuclear infiltration.

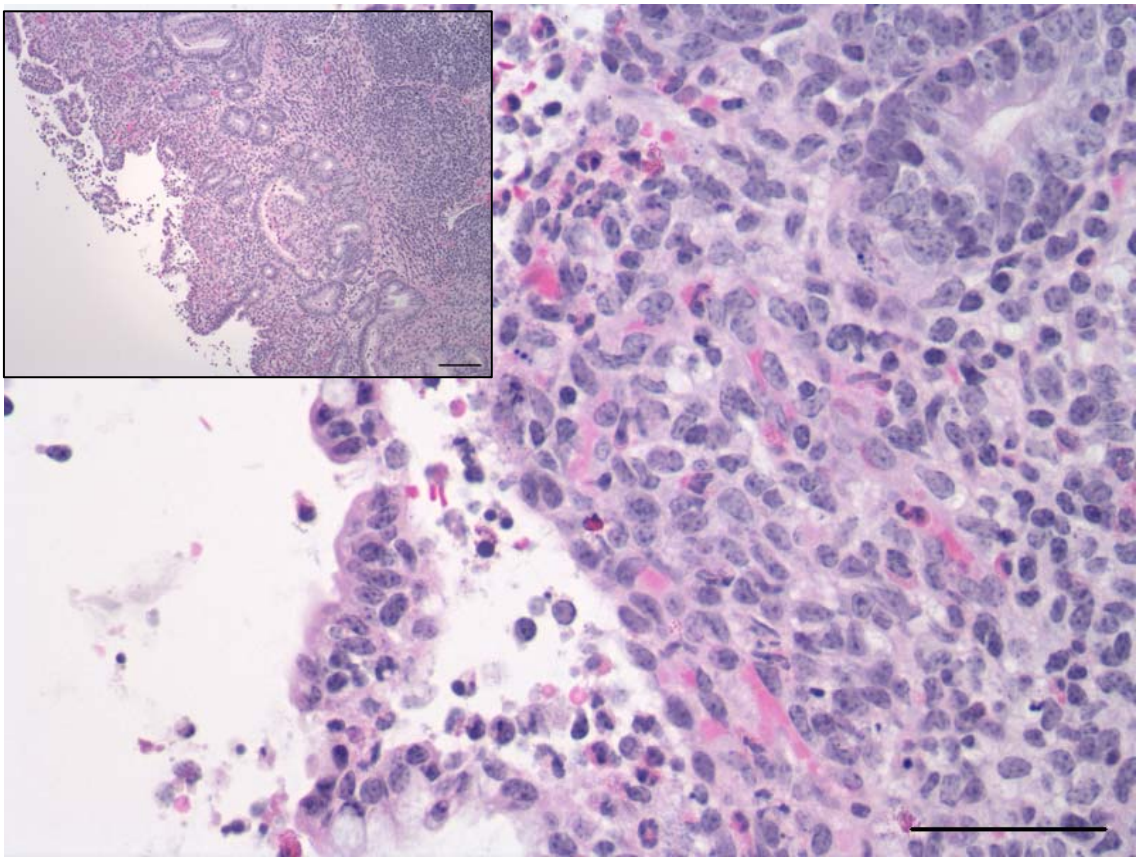

Figure A-3. H&E stain of bovine intestinal epithelium (ileum) from calf# 2, 8 hours post-inoculation with *S.* Typhimurium wild type (IR715). The tissue exhibits severe blunting, erosion, and epithelial detachment, with a moderate neutrophilic and mononuclear infiltration. The endothelium is reactive and possesses marginating neutrophils in the submucosal blood vessels. Occasional abscess formation is present.

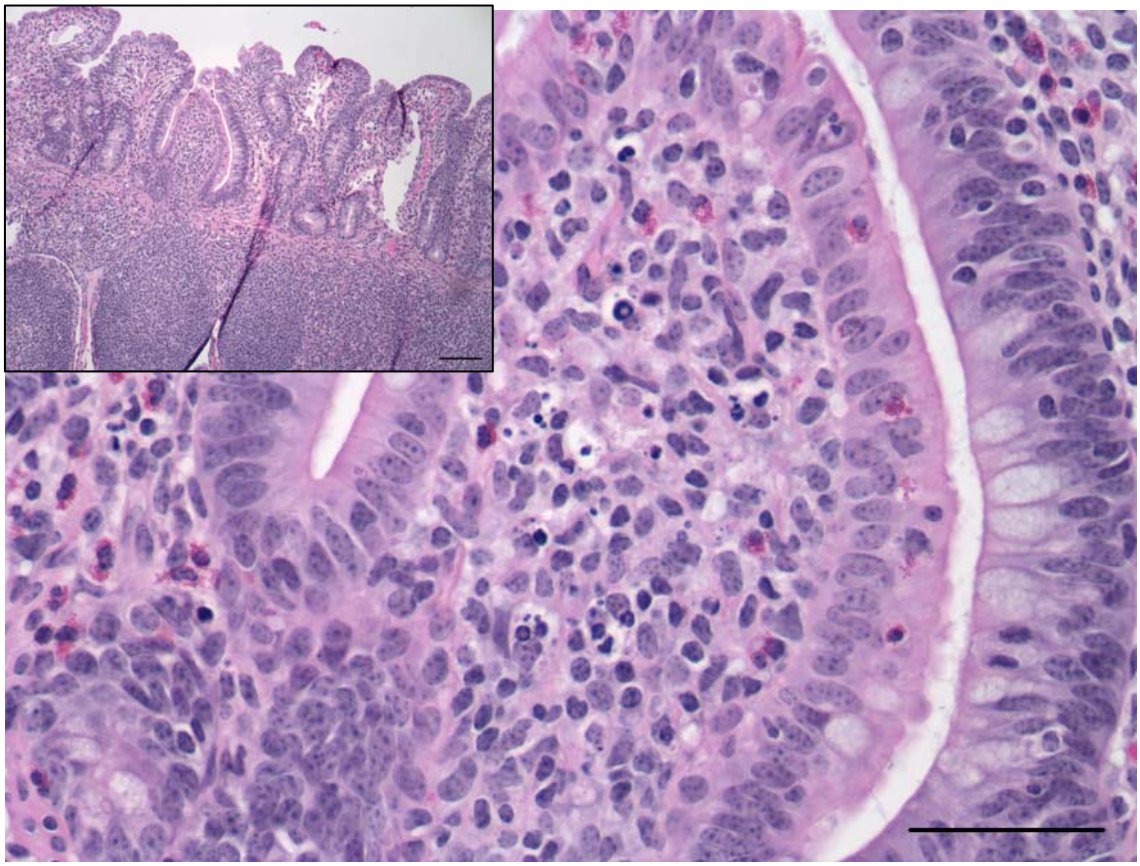

Figure A-4. H&E stain of bovine intestinal epithelium (ileum) from calf# 3, 8 hours post-inoculation with *S.* Typhi Ty2 wild type grown in LBH broth. The tissue exhibits moderate blunting, erosion, and epithelial detachment, with a mild neutrophilic and mononuclear infiltration. Mononuclear cell necrosis in the dome villi.

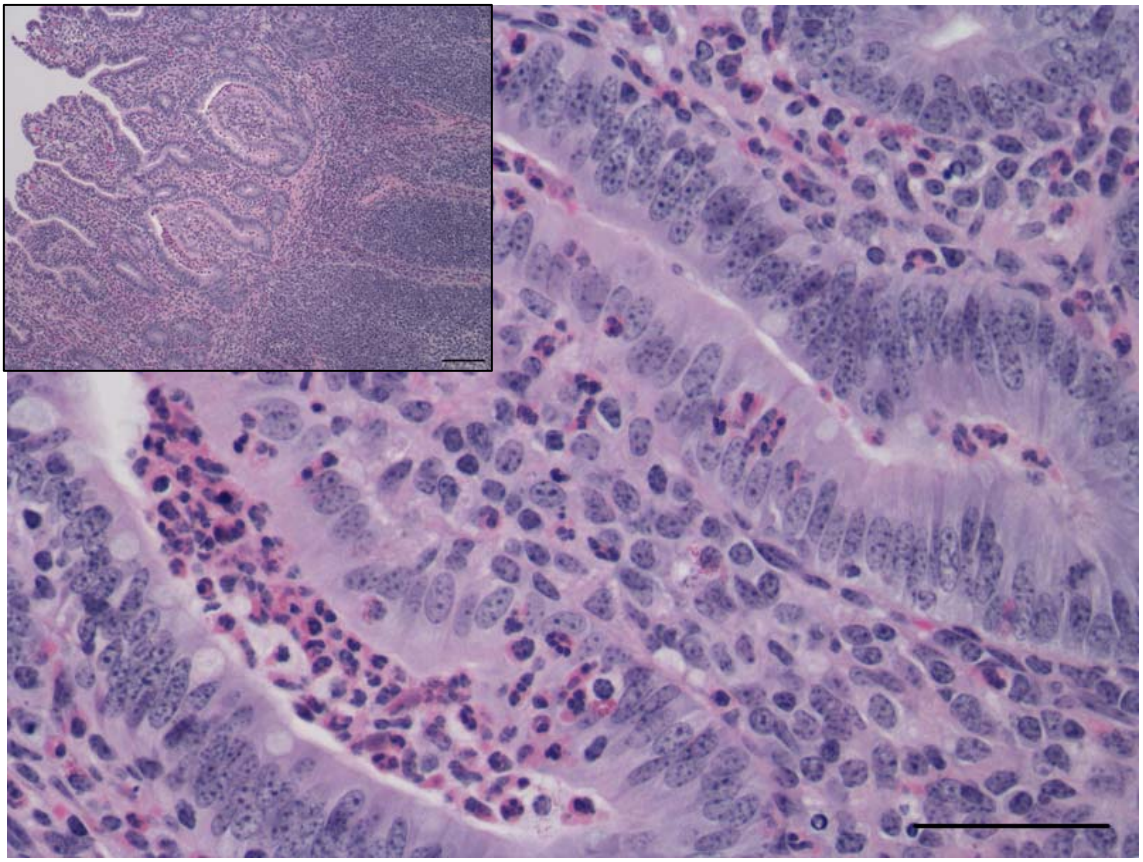

Figure A-5. H&E stain of bovine intestinal epithelium (ileum) from calf# 1, 8 hours post-inoculation with *S.* Typhi Ty2 wild type grown in LBH broth. The tissue exhibits moderate blunting, erosion, and epithelial detachment, with a mild neutrophilic and mononuclear infiltration. Margination of neutrophils in the submucosal blood vessels and transmigration of neutrophils over the dome villi. Early crypt abscess formation is present.

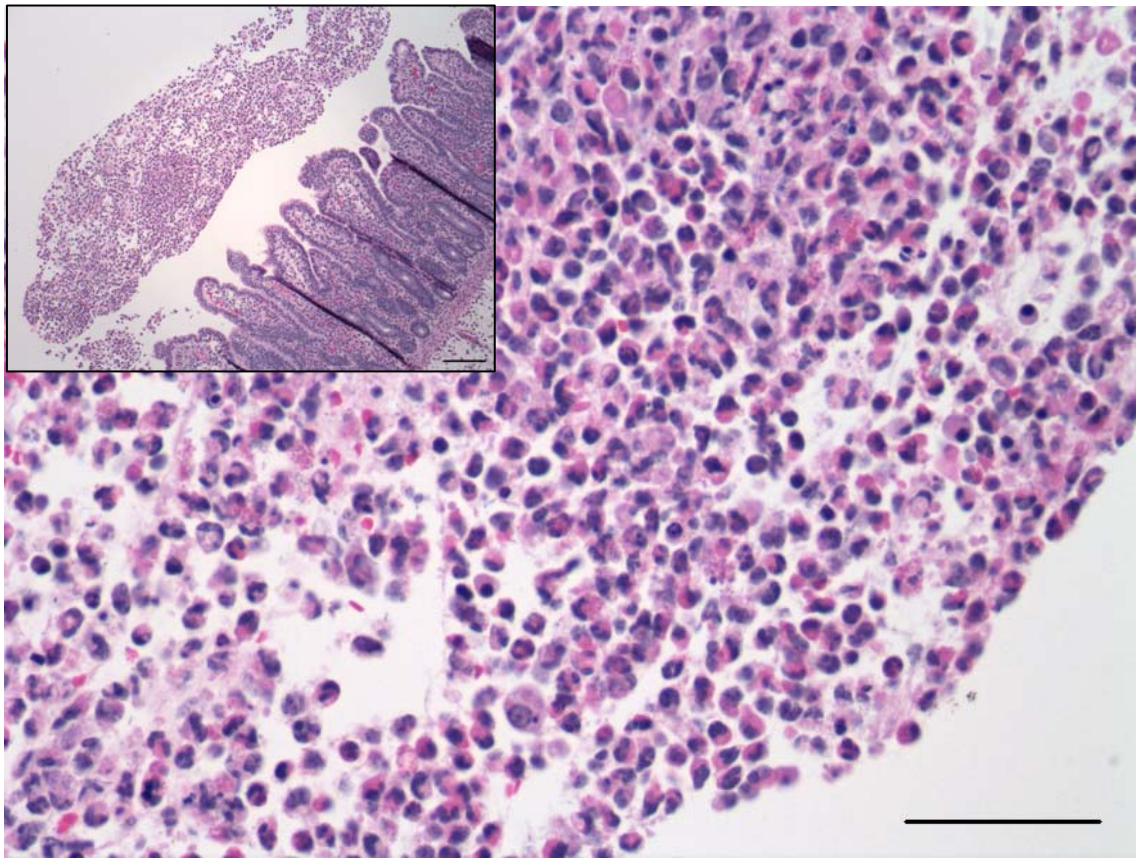

Figure A-6. H&E stain of bovine intestinal epithelium (ileum) from calf# 1, post-inoculation with *S.* Typhi Ty2 grown in LBH broth. The tissue exhibits severe blunting, erosion, and epithelial detachment, with a moderate neutrophilic and mild mononuclear infiltration. Prominent pseudomembranous formation containing bacteria present in the lumen.

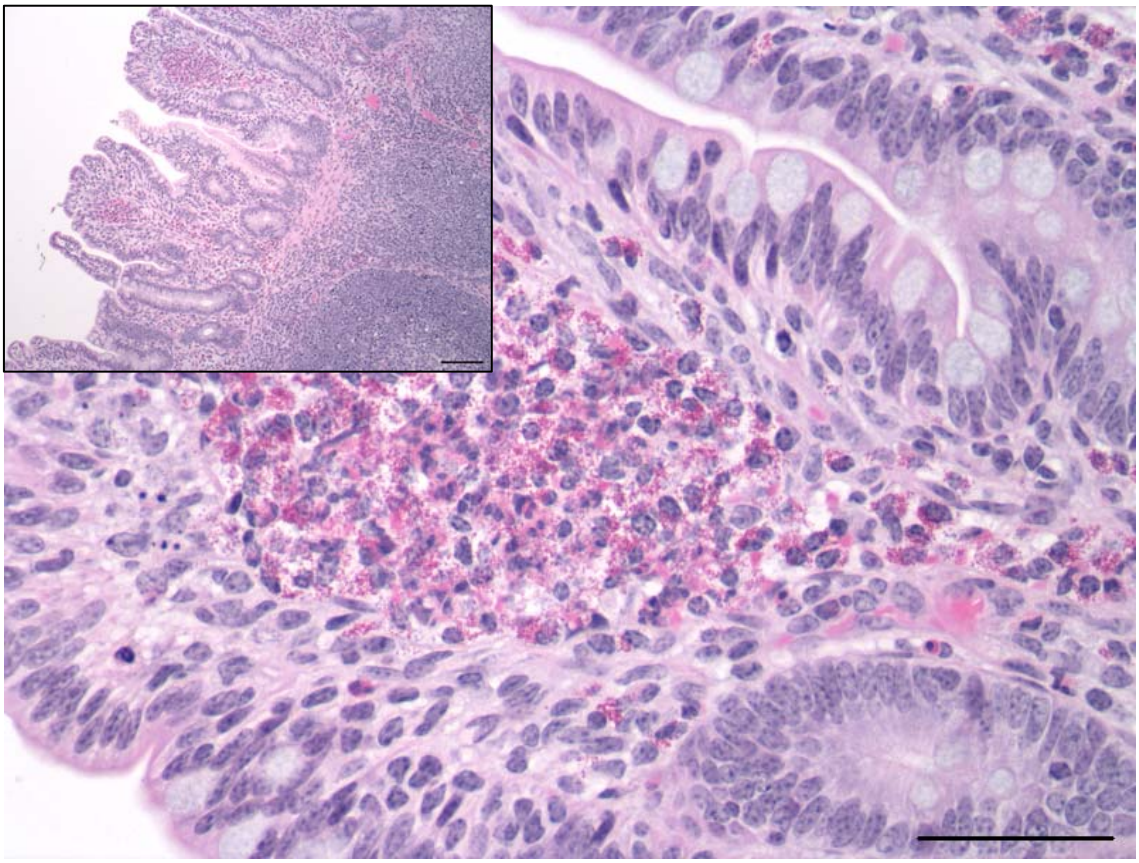

Figure A-7. H&E stain of bovine intestinal epithelium (ileum) from calf# 2, post-inoculation with *S.* Typhi Ty2 grown in LB broth. The tissue exhibits mild blunting, erosion, and epithelial detachment, with a mild neutrophilic and mononuclear infiltration. Plugs of neutrophils within the submucosa of thin-walled vessels. Neutrophils and globular leukocytes inside lacteals. Presence of reactive endothelium and margination of neutrophils in arterioles.

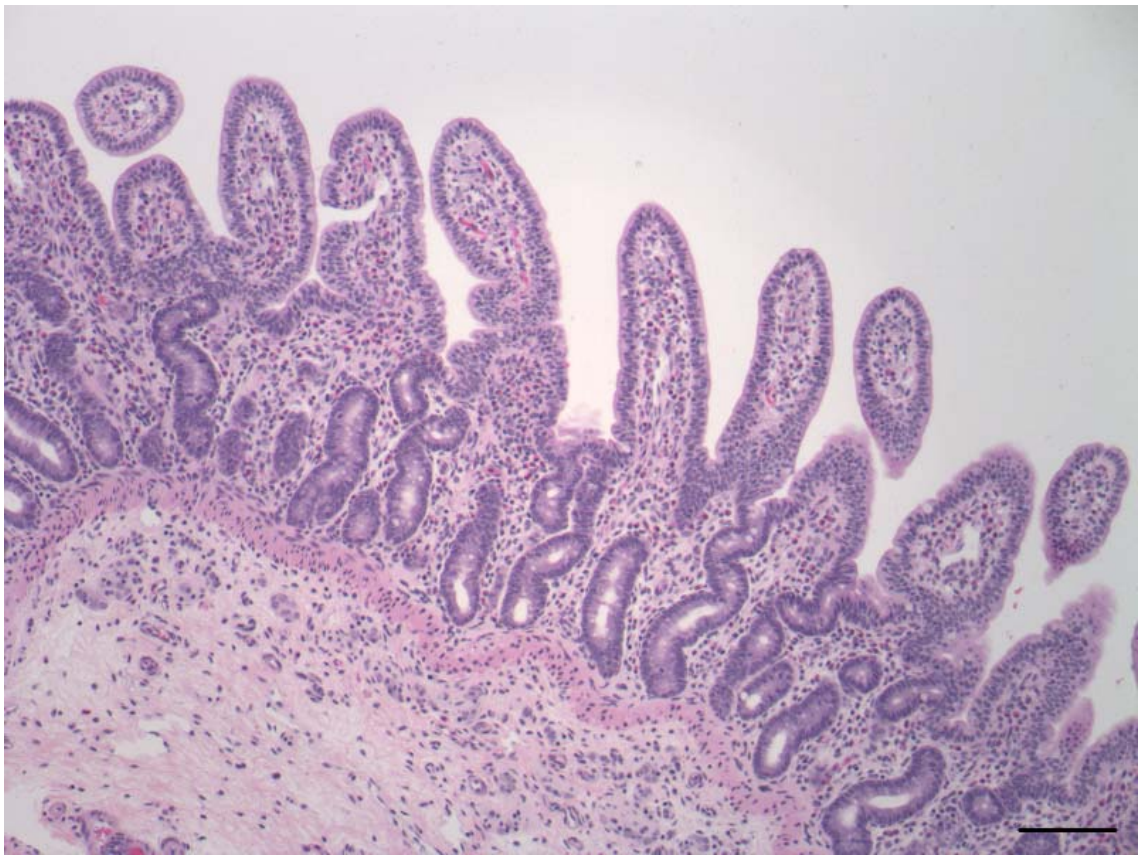

Figure A-8. H&E stain of bovine intestinal epithelium (ileum) from calf# 1, 8 hours post-inoculation with LBH broth (uninfected control loop). No pathological lesions present.

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