Effect of anti-lipopolysaccharide IgA antibodies on the virulence and physiology of salmonella enterica and shigella flexneri

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Effect of Anti-Lipopolysaccharide IgA Antibodies on the Virulence and Physiology of
Salmonella enterica and Shigella flexneri

A Dissertation
presented to the Faculty of the University at Albany, State University of New York
in partial fulfillment of
the requirements for the degree of
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School of Public Health
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Stephen J. Forbes
2009
Effect of Anti-Lipopolysaccharide IgA Antibodies on the Virulence and Physiology of

*Salmonella enterica* and *Shigella flexneri*

by

Stephen J. Forbes

2009

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Abstract

Secretory IgA (SIgA) antibodies directed against the serotype-specific, O-antigen of lipopolysaccharide (LPS) are the primary determinants of mucosal immunity to enteric bacterial pathogens, including of *Salmonella enterica* serovar Typhimurium and *Shigella flexneri*. While the singular importance of SIgA in preventing enteric infections is recognized, the underlying mechanisms by which these antibodies protect the mucosal epithelium remains poorly understood. In this study, I demonstrated that Sal4, a protective, anti-LPS specific monoclonal IgA, is a potent inhibitor of *S. Typhimurium* flagella-based motility and type three secretion (T3S) mediated entry into epithelial cells. Sal4’s effects on motility and invasion occurred rapidly (<15 min), and were independent of agglutination. I also present evidence the protective anti-LPS, monoclonal IgA, IgAC5, reduced *S. flexneri* T3S of IpaB and IpaC. Concurrent with the IgA-induced reduction in T3S, there was a decrease in both the proton motive force and ATP levels in both bacterial species examined. I also report that Sal4 and IgAC5 have effects on the bacterial envelope. Scanning, transmission, and cyro-electron microscopy revealed dramatic antibody-mediated alterations in the topology of the outer membrane (OM) of *S. Typhimurium* and *S. flexneri*. Furthermore, Sal4-treatment caused a ~5 fold increase in O-antigen, and elevated levels of lipid A, released into culture supernatants, consistent with the loss of LPS. Correspondingly, the OM of antibody-treated bacteria was ~50% more permeable than control cells. Based on these data, I proposed that Sal4 binding to the LPS destabilizes the outer leaflet of the OM of *S. Typhimurium*, thereby compromising the integrity of the bacterial envelope, disrupting bioenergetics by physical and/or mechanical stress, and arresting both flagella-based motility and T3S. This study
reveals a previously unrecognized capacity of SIgA to “disarm” enteric pathogens in mucosal secretions, thereby preventing colonization and invasion of the intestinal epithelium.
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Chapter 1

INTRODUCTION

The gastrointestinal epithelium is the interface between two different environments, the sterile lamina propria and underlying tissues, and the bacterial colonized intestinal lumen. The intestinal epithelium, which consists of a single layer of polarized epithelial cells joined side-by-side by tight junctions, and estimated to exceed 400 m², is inherently vulnerable to infection by a wide range of pathogenic microorganisms, including viruses, bacteria and parasites (Russell et al., 1999). Diarrheal diseases remain a leading cause of morbidity and mortality in developing countries, and disproportionately affect children under the age of five. Enteric infections also represent an increasing problem in the United States, primarily due to the emergence of multi-drug resistance. Vaccines capable of eliciting local immunity within the gastrointestinal tract offer the greatest promise for controlling these diseases (Holmgren and Czerkinsky, 2005; Neutra and Kozlowski, 2006). A major obstacle to vaccine or therapeutic development is the relatively little is known about the specific determinants, or correlates, of protective immunity. Of particular importance are secretory antibodies, as these are the sole component of the adaptive immune system capable of intercepting pathogens on the mucosal surfaces of the intestinal tract and thereby preventing the onset of infection. Although SIgA antibodies directed against the serotype-specific, O-antigen of lipopolysaccharide (LPS), are the primary determinants of mucosal immunity to Shigella, Salmonella, and Vibrio cholerae (Apter et al., 1993a; Chowers et al., 2007; Iankov et al., 2004; Iankov et al., 2002b; Phalipon et al., 2002; Phalipon et al., 1994), little is known
about how these antibodies actually prevent bacterial colonization and invasion of intestinal epithelial cells.

**Part I: Salmonella and Shigella**

**Salmonella Public Health Relevance**

Bacteria of the genus *Salmonella*, with over 2,500 serotypes, are Gram-negative, motile bacilli, that are found in a wide range of environments (WHO). In developed countries, this genus is most commonly associated with gastroenteritis (or salmonellosis), which is acquired primarily through the consumption of contaminated water or foods. Contamination is normally associated with beef, poultry, milk, and egg products, but is also found with vegetables (CDC). Two serovars, *Salmonella enterica* serovar Typhimurium and *Salmonella enterica* serovar Enteritidis account for half of the salmonella infections in the United States (CDC). Typically, symptoms begin 12 to 72 hours after consuming contaminated food or water, and include fever, abdominal cramps, and diarrhea. Illness typically lasts between 4 and 7 days (CDC). In developing countries, *Salmonella* infections are a leading cause of enteric disease, which collectively are responsible for approximately 2 million deaths annually. There are no vaccines against non-typhoid *Salmonella*. In many regions infection with *Salmonella enterica* serovar Typhi are endemic. *S. Typhi* is the causative agent of typhoid fever, a potentially life threatening disease that involves the spread of bacteria across the intestinal epithelium and into the systemic compartment (CDC, ; WHO). Infections caused by *S.Typhi* alone are estimated to cause more than 16 million cases of typhoid fever and half a million deaths each year (Pasetti et al., 2000). Although there are two *S. Typhi*
vaccines on the market, a Vi polysaccharide vaccine and a live-attenuated vaccine (Zhang et al., 2008), their efficacy is relatively low and in need of improvement. While S. Typhi is a pathogen specific for humans, infection of mice with S. Typhimurium elicits a disease that mimics the disease symptoms of typhoid fever in humans. S. Typhimurium proliferates in the intestinal mucosa of mice, and spreads systemically to the liver and spleen (Collins et al., 1966; Haraga et al., 2008). Also, a new model in the mouse allows for S. Typhimurium to be studied as a cause of gastroenteritis, which is the disease it causes in humans (Hapfelmeier and Hardt, 2005). Both of these models provide powerful systems to understand both the pathogenesis and mucosal immunity of this organism.

S. Typhimurium Virulence Factors

The virulence factors of S. Typhimurium are numerous and underlie its capacity to successfully colonize and invade the intestinal epithelium (Fig. 1.1). Factors that induce virulence gene expression in S. Typhimurium include low levels of oxygen, high osmolarity, and temperature (37ºC) (Ellermeier and Slauch, 2003). There are several types of fimbriae, both chromosomally and plasmid encoded, involved in adherence. Lipopolysaccharide (LPS) is the major constituent of the outer leaflet of the bacterial outer membrane (OM). In particular, the O-antigen component of LPS forms a protective coat surrounding the bacterium, which confers resistance against antimicrobial agents present in intestinal secretions (Dann and Eckmann, 2007; Nikaido, 2003). S. Typhimurium is highly motile due to the presence of flagella, which act in concert to propel the bacterium through liquid and viscous environments (Berg and Turner, 1993; Carsiotis et al., 1984; Ilino, 1974). This motility is postulated to enable the bacterium to
penetrate the thick mucus coat that covers the intestinal mucosa, as well as to promote contact with epithelial cell surfaces (Jones et al., 1994; Schmitt et al., 2001). Motility has also been shown to be important for invasion in vitro (Jones et al., 1992; Jones et al., 1981; Khoramian-Falsafi et al., 1990). *S. Typhimurium* are able to specifically invade both villus enterocytes, and microfold cells (M cells) present in the follicle associated epithelium overlaying the Peyer’s patches (Cossart and Sansonetti, 2004). Invasion is mediated by the *Salmonella* Pathogenicity Island 1 (SPI-1) encoded type III secretion system (T3SS), effectors, and translocon molecules (Cossart and Sansonetti, 2004; Galan and Wolf-Watz, 2006; Haraga et al., 2008). Survival of *S. Typhimurium* in epithelial cells and macrophages is mediated by the *Salmonella* Pathogenicity Island 2 (SPI-2) encoded T3SS, effectors, and translocon molecules (Cossart and Sansonetti, 2004; Shea et al., 1996). SPI-2 facilitates intracellular replication of the bacteria with *Salmonella* containing vacuole (SCV). The SCVs are vacuoles redirected from the normal phagosomal pathway, preventing fusion with the lysosome, which is dependent on secreted protein SpiC (Cossart, 2005). Additionally, the self-transmissible plasmid, pSLT, is found in all virulent strains, and encodes the *spv* genes that are needed for survival in macrophages (Ahmer et al., 1999).
Figure 1.1 Invasion by *S. Typhimurium* is a multi-step process involving several virulence factors. (Step 1) Survival and Growth: *S. Typhimurium* ingested via the oral route proliferates within the small intestine; (Step 2) Motility: the bacterium possesses flagella that allows it to ‘swim’ through the mucus layer covering the epithelium; (Step 3) Attachment: *S. Typhimurium* is thought to bind (at least transiently) to epithelial surfaces by pili; (Step 4) Invasion: *S. Typhimurium* promotes its own uptake into epithelial cells by secretion of bacterial ‘effector’ molecules through a type III secretion system (T3SS); (Steps 5-6) Intracellular replication and dissemination: *S. Typhimurium* grows within epithelial cells and sub-epithelial macrophages, and spreads systemically.
Motility

Motility is considered a Salmonella virulence factor (Carsiotis et al., 1984), and is postulated to enhance pathogenesis by enabling the bacteria to penetrate the thick mucus coat of the intestinal mucosa. The flagella based motility of \textit{S. Typhimurium} promotes contact with epithelial cell surfaces \textit{in vivo} in mice (Jones et al., 1994; Schmitt et al., 2001). Motility is vital for \textit{S. Typhimurium} to invade epithelial cells \textit{in vitro}, as evidenced by the fact that non-motile mutants are 20-50 times less invasive than wild type controls (Jones et al., 1992). The importance of motility in \textit{S. Typhimurium} \textit{in vivo} is less clear. It has been reported that there is no significant difference in invasion between wild type and nonmotile mutant strains, as determined by colony forming units, in mouse organs in the systemic infection model (Lockman and Curtiss, 1990). However, other studies have data indicating that motility is significant in sustaining infection in the intestine in mice (gastroenteritis) (Hapfelmeier and Hardt, 2005). Additionally, a nonmotile mutant in mice has been shown to be more virulent systemically in the mouse typhoid model (Schmitt et al., 2001; Weinstein et al., 1984). A potential reconciliation of these conflicting results, is that motility has been found to be important in causing gastroenteritis, but not for systemic (invasive) infection (Stecher et al., 2008; Stecher et al., 2007).

\textit{S. Typhimurium} typically has 6-7 peritrichous flagella (Ilino, 1974). Each flagellum of \textit{S. Typhimurium} consists of a basal body spanning the inner membrane (IM) and OM, with a hook and a helical filament extending out from the OM (\textbf{Fig. 1.2}) (Berg, 2003; Ilino, 1974). The flagellar hook emanating from the OM is a flexible structure that is 55 nm long and 20 nm in diameter (Blocker et al., 2003; Minamino and Pugsley,
The flagellar filament is up to 10 µm long and can assume 12 different polymorphic forms in response to elastic strain while still retaining function (Berg, 2003; Turner et al., 2000). The energy source that drives the flagella is the flux of protons from the periplasm to the cytoplasm, through the rotator-stator mechanism that composes the flagellar motor (Berg, 2003). The power and torque of the flagellar motor is sufficient to rotate a cell tethered by a single flagellum (Berg and Turner, 1993; Washizu et al., 1993).

The normal chemotactic motility of *S. Typhimurium* consists of ‘runs’ (*i.e.*, directional forward movement), and ‘tumbles’ (*i.e.*, a period of random reorientation with little net displacement) (Berg, 2003; Turner et al., 2000). Motility in *S. Typhimurium* occurs when the flagellar motors turn counterclockwise, causing the flagellar filaments to bundle together (Berg and Anderson, 1973). The “tumbles” between ‘runs’ result from the reversal of the flagellar motor, clockwise rotation, in which the filaments leave the bundle and spread out (Berg and Anderson, 1973). As few as one or two filaments out of the bundle can result in a tumble (Turner et al., 2000).

**Type 3 Secretion System Mediated Invasion of Epithelial Cells**

In *S. Typhimurium* the T3SS is responsible for bacterial invasion of the epithelium (Cossart and Sansonetti, 2004; Galan and Collmer, 1999; Galan and Curtiss, 1989). The deletion of the SPI-1 locus leads to a greater than 10 fold decrease in invasion *in vitro* (Ellermeier et al., 2005; Forbes et al., 2008). The T3SS consists of a basal body spanning the IM and OM, and a needle-like structure complex extending 80 nm from the OM (*Fig. 1.2*) (Blocker et al., 2003; Minamino and Pugsley, 2005). Contact of the needle complex, with epithelial cells, results in a translocation pore formation in the host.
Figure 1.2

Figure 1.2 Inner and Outer Membrane of S. Typhimurium.

Like all Gram-negative bacteria, S. Typhimurium, has two membranes, the phospholipid inner membrane (IM) and an asymmetrical outer membrane (OM) (above, left). Between the IM and OM is the periplasmic space, which contains a thin structural layer of peptidoglycan (shown in green and red). The LPS in the outer leaflet of the OM can be broken down into the membrane anchored lipid A, the LPS core, and the O-antigen repeats (above left, shown in light green). Spanning the IM and OM are the T3SS (left, dark blue), and the flagellar basal body and hook (right, dark blue). The flagellar filament is up to 10 µm long (not shown). An IgA (Sal4) is shown for size comparison, is 20-30 nm in length (top left, red with blue tips)
membrane. This allows an array of effector proteins to be injected into the host cell cytoplasm, which results in cytoskeletal rearrangements and macropinocytosis of the bacteria (Cossart and Sansonetti, 2004; Galan and Wolf-Watz, 2006). In S. Typhimurium, the translocase complex is composed of SipB, SipC, and SipD. The effector molecules translocated through the pore commandeer the host actin machinery, initially inducing membrane ruffling and macropinocytosis, followed by a return to an unperturbed membrane structure. The C-terminal domain of SipC initiates actin nucleation, which is further enhanced by SopE1 and SopE2 (Cossart, 2005). The SopE proteins are exchange factors for Rac-1 and Cdc42 GTPases (Cossart, 2005). SopB and SigD stimulate actin rearrangements that results in the macropinocytic pocket, while SipA stabilizes actin filaments. Closing of the macropinocytic pocket and internalization is due to the actin depolymerization activity of SptP (Cossart, 2005). SptP regulates MAPK and GTPase activating protein (GAP) activity, opposing SopE activity. Both SptP and SopE are TTSS secreted proteins, and are injected together. SptP is a more stable protein than SopE, which is rapidly degraded (Cossart, 2005).

The Membranes of Gram-Negative Bacteria

The cell envelope of S. Typhimurium consists of two membranes, there is a phospholipid IM and an asymmetrical OM, composed of a phospholipid inner leaflet and a LPS containing outer leaflet (Fig. 1.2). Between the IM and OM is the periplasmic space, which contains a thin structural layer of peptidoglycan (Meroueh et al., 2006). The LPS can further be broken down into the membrane anchored lipid A, the LPS core, and the O-antigen repeats. There are typically 35-40 O-antigen repeats per LPS molecule. It is
estimated that there are $3.5 \times 10^6$ LPS molecules per bacterium, occupying ~75% of the OM surface (Nikaido, 1996). The remaining ~25% of the area of the outer leaflet is occupied by proteins (Nikaido, 1996).

LPS is a complex of lipid and carbohydrates. LPS biosynthesis involves greater than 30 proteins, encoded in the *rfa, rfb*, and *rfc* gene clusters (Nikaido, 1996). Lipid A, also known as endotoxin, is composed of two head groups and 6 acyl chains. One head group has 2 acyl chains, while the other has 4 acyl chains and 2 KDO (3-deoxy-D-manno-actulosonic acid) residues, which link to the LPS core. The lipid A acyl chains are the primary determinants for Toll-like receptor 4 (TLR4) recognition (Miller et al., 2005; Munford and Varley, 2006). In *S. Typhimurium*, the lipid A can be modified by the PhoPQ two component system. Disruption of the PhoPQ two component system attenuates *S. Typhimurium* *in vivo* (Miller et al., 2005). The core of the LPS is composed of carbohydrates, and can be divided into the inner and outer cores. The core extends less than 5 nm from the OM. Truncation of the core region results in destabilization of the OM, both by increased permeability and lack of normal OMP composition (Vaara, 1992). The core is relatively rigid compared to the O-antigen repeats, which thought to coil and to be flexible structures (Kastowsky et al., 1992). In *S. Typhimurium* strain ATCC14028, the O-antigen consists of mannose, rhamnose, galactose, and abequose in the form

\[
\{ \rightarrow 2\}[^{\alpha D-Abe(1 \rightarrow 3)}][^{\alpha D-Man(1 \rightarrow 4)}]^{\alpha L-Rha(1 \rightarrow 3)}^{\alpha D-Gal(1 \rightarrow )}\ (Raetz, 1996).
\]

The LPS in *S. Typhimurium* strain ATCC14028 constitutes the O4, O5, and O12 antigens (Luderitz et al., 1966; Slauch et al., 1995). It is estimated that the length of the LPS is approximately 22-70 nm (Kastowsky et al., 1992). This measurement is based on the Ra-LPS (core only) being 4.4 nm in length (with lipid A being 2.4 nm of that distance), and a
single O-antigen repeat ranging from 0.26 nm coiled, and 1.6 nm fully extended (Kastowsky et al., 1992). The standard number of O-antigen repeats is 35-40 (Raetz, 1996), which would make the length of the LPS approximately 22-70 nm.

The asymmetric bilayer of the OM of Gram-negative bacteria serves as a selective barrier between the bacteria and the environment. The O-antigen repeats block entry of many large molecular weight (MW) and hydrophobic molecules (Nikaido, 2003; Nikaido and Vaara, 1985). The length of the O-antigen repeats prevents complement deposition on the lipid membrane, and therefore complement mediated lysis (Holzer et al., 2009), while the hydrophilic nature of the LPS inhibits the entry of large hydrophobic molecules, such as some antibiotics (Vaara, 1992).

The integrity of the lipid and lipid A portion of the membrane provide a barrier against hydrophilic and small MW molecules (Nikaido, 2003; Nikaido and Vaara, 1985). The lipid portion of the membrane barrier is affected both by LPS length (rough mutants), and lipid structure (PhoPQ) (Murata et al., 2007; Nikaido, 2003). The OM was originally thought to be impermeable to some compounds. Recently, it was demonstrated that the protein TolC is utilized to export compounds that diffused across the membranes. The disruption of tolC reversed this affect (Murata et al., 2007).

**Proton Motive Force**

In *E. coli* and *S. Typhimurium*, the proton motive force (PMF) is the energy source behind the electron transport and ATP production, active transport of molecules across the membranes, flagellar rotation, and T3SS (Berg, 2003; Blocker et al., 2003; Paul et al., 2008; Wilharm et al., 2004). A localized transmembrane proton
electrochemical gradient, or PMF ($\Delta \rho$), occurs across the IM, which consists of the proton gradient ($\Delta \rho H$) and the electrochemical potential ($\Delta \Psi$). PMF is defined as $\Delta \Psi - 2.3RT \Delta \rho H/F$ in millivolts ($R =$ gas constant, $T =$ temperature, $F =$ Faraday’s constant) (Kadner, 1996). In *E. coli*, the flagellar motor speed has been shown to vary linearly with PMF, and dissipation of the PMF causes an immediate arrest in motility (Gabel and Berg, 2003). Proton ionophores, such as CCCP (carbonyl cyanide m-chlorophenylhydrazone), that dissipate the PMF cause an immediate arrest in motility (Berg, 2003) and disable T3S (Wilharm et al., 2004). The PMF is known to be perturbed by OM stresses and factors that alter the normal function the OM (Darwin, 2005; Model et al., 1997). For example, infection with filamentous phage or over-expression of translocation defective OM proteins results in dissipation of PMF (Brissette et al., 1990; Kleerebezem et al., 1996). In *E. coli* and *S. Typhimurium* there are measurable physiologic effects associated with loss of PMF, which include: a decrease in $\Delta \Psi$ and $\Delta \rho H$, the inability to grow on lactate, fumarate or succinate as sole carbon sources, and a decrease in ATP levels. ATP also plays a role in T3S through the separation of the effector protein from the chaperone prior to secretion (Akeda and Galan, 2005; Minamino and Namba, 2008).

**Shigella**

Shigella, a gamma proteobacteria, is a close relative of Salmonella. *Shigella flexneri* (*S. flexneri*) is a Gram-negative enteric pathogen capable of infecting the intestinal mucosa of humans and rabbits (Boullier et al., 2009; Phalipon et al., 1995). Shigellosis is a disease characterized by acute inflammation, diarrhea, and in *Shigella dysenteriae*, renal failure. The single most important determinant in the process of
infection and invasion is the bacteria’s ability to penetrate and gain entry to intestinal epithelial cells. Unlike *S. Typhimurium*, *Shigella* as a genus are non motile. Invasion of epithelial cells by *S. flexneri* involves a plasmid encoded T3SS (Cossart and Sansonetti, 2004; Sansonetti et al., 1982). *S. flexneri* has approximately 20 T3SS per cell (West et al., 2005), each with a 60 nm needle that extends beyond the 35 nm LPS layer (West et al., 2005). Upon contact with epithelial cells, effector proteins are injected into the host cell cytoplasm, resulting in cytoskeletal rearrangements and macropinocytosis of the bacteria by the host cell (Cossart and Sansonetti, 2004; Galan and Wolf-Watz, 2006).

The *S. flexneri* T3SS secreted effector proteins, known as the invasion plasmid antigens (Ipa), play multiple roles in the invasion process (Ogawa et al., 2008; Schroeder and Hilbi, 2008). The T3SS first injects IpaB and IpaC into epithelial cells, forming a translocation pore in the host membrane (Picking et al., 2005). This is followed by secretion of the effector proteins IpaA, IpgD, IpgE, and IpgF, which are involved in inducing macropinocytosis (Cossart and Sansonetti, 2004). The proteins IpaA, IpaB, IpaC, and IpaD are conserved within the *Shigella* genus, and are homologs of the Salmonella SipA, SipB, SipC, and SipD (Hermant et al., 1995). The importance of T3SS in the invasion process is evidenced in strains of *S. flexneri* that lack the 220 kb virulence plasmid, pWR100, as these bacteria are non invasive (Sansonetti et al., 1982; Sansonetti et al., 1986).
Part II: Mucosal Immune System and IgA

Both innate and adaptive immunity are important in controlling Gram-negative bacterial infections. Innate immunity consists of a combination of physical and biochemical factors that reduce the likelihood of bacterial survival, colonization and/or invasion of the intestinal epithelium. The low pH (~pH 2) of the stomach can reduce bacterial viability by several logs (Foster, 2004). Epithelial surfaces are also coated with a thick layer of mucus, which acts as a physical barrier to the bacteria due to its high viscosity index. Mucosal secretions are also enriched in a number of antimicrobial factors, which include bile salts, antimicrobial peptides, proteases, peroxidases, lysozyme, and lactoferrin (Wijburg, 6 June 2006). In the distal small intestine and the colon, indigenous microbial flora are present in great numbers (>10^{14} bacteria) and compete with potential pathogens for nutrients and growth factors (Savage, 2005). Finally, the contents of the intestinal lumen are constantly in flux due to intestinal motility, or peristalsis, which promotes transit and expulsion of foreign microbes from the intestinal lumen (Cone, 2005).

While the innate immune system provides a basic non-specific level of protection against microbial infections, Salmonella are surprisingly adept at surviving these insults. The adaptive immune system is crucial in preventing Salmonella infections (Wijburg, 6 June 2006). There are organized lymphoid follicles (i.e., Peyer’s patches) present throughout the gastrointestinal tract, that function in antigen sampling and the producing of antigen-specific B and T cells (Brandtzaeg, 2003; Brandtzaeg and Farstad, 1999; Woof and Mestecky, 2005). This network of immune cells in the intestine is collectively known as the gut-associated lymphoid tissue (GALT) (Fig. 1.3). The GALT is distinct
from the systemic immune system, as antigen-specific IgA can be produced locally in the mucosa, while systemically antigen specific antibodies are not produced (Brandtzaeg et al., 1999).

**Secretory IgA (SIgA)**

A cardinal feature of mucosal B cells is the production of antibodies of the IgA class. Moreover, this IgA is primarily dimeric, consisting of two IgA monomers joined at their C terminus by a small, 15 kDa protein, called the J chain. Dimeric IgA, produced within the intestinal mucosa, is transported across the epithelial barrier by the polymeric immunoglobulin receptor (pIgR), which are located on the basolateral surface of the epithelial cells. Dimeric IgA is released into the intestinal lumen by proteolytic cleavage of the pIgR. A 80 kDa fragment of pIgR, known as the secretory component (SC), remains bound to the pIgA. The SC-IgA complex is collectively referred to as secretory IgA (SIgA) (Brandtzaeg and Farstad, 1999; Woof and Mestecky, 2005).

Each monomer of IgA is composed of two 50 kDa heavy and two 25 kDa light chains (Harlow and Lane, 1988). The heavy chains are associated with each other, through their constant regions (Fc), while the variable region (V_H) associates with the light chain. It is the combination of the V_H and the V_L which forms the binding pocket and determines antibody specificity. Thus, each monomer has two Fv regions, or antigen binding pockets, at the ends of a Y-structure. The Fc portion and the V_H + V_L are each composed of two immunoglobulin folds, to give an IgA molecule of ~14 nm in length (Woof and Kerr, 2006; Woof and Mestecky, 2005). IgA monomers are glycosylated
along the Fc region of the heavy chain. Furthermore, IgA in the mucosa is primarily a dimer, joined by the J chain (15 kDa), which is also heavily glycosylated.

SIgA is the predominant immunoglobulin isotype in tears, saliva, milk, nasal fluid, bile, and intestinal fluid, and is thought to function in protection against infection by microbial pathogens by a number of mechanisms (Woof and Kerr, 2006). Foremost, is the ability of SIgA to promote bacterial agglutination, entrapment in mucus, and clearance by peristalsis, a phenomenon referred to as immune exclusion (Fig. 1.3) (Holmgren and Czerkinsky, 2005). SIgA is also capable of directly binding regions of toxins, viruses and bacteria that are involved in attachment to receptors on epithelial cell surfaces (Silvey et al., 2001; Williams and Gibbons, 1972). Coating of bacteria with SIgA has been hypothesized to make the bacterial surface more hydrophilic, thereby increasing interactions with mucin. Finally, SIgA is postulated to interact synergistically with innate defense molecules to mediate bacterial killing, although the molecular mechanism is unknown (Rogers, 1973). It should be noted that SIgA alone has never been shown to have bacteriocidal properties (Iankov et al., 2004; Michetti et al., 1994; Phalipon et al., 1995).

SIgA antibodies directed against the serotype-specific, O-antigen of LPS, are the primary determinants of mucosal immunity to *Shigella, Salmonella, and Vibrio cholerae* (Apter et al., 1993b; Chowers et al., 2007; Iankov et al., 2004; Iankov et al., 2002b; Michetti et al., 1992; Phalipon et al., 2002; Phalipon et al., 1995; Phalipon et al., 1994). However, little is known about how these antibodies actually prevent bacterial colonization and invasion of intestinal epithelial cells. While it is generally assumed that secretory antibodies function by “immune exclusion,” direct evidence to support this
Figure 1.3 Gut-associated lymphoid tissue (GALT).

Organized lymphoid follicles (i.e., Peyer’s patches) are present throughout the gastrointestinal tract. Specialized M cells function in antigen sampling of the intestinal lumen. Mucosal B cells produce dimeric IgA, which is transported across the epithelial barrier by the polymeric immunoglobulin receptor (pIgR) located on the basolateral surface of the epithelial cells. The dimeric IgA is released into the intestinal lumen by proteolytic cleavage of the pIgR. The pIgR fragment remaining bound to the dimmeric IgA is referred to as the secretory component (SC). The resulting molecule is secretory IgA (SIgA). SIgA in the intestinal lumen can bind bacteria, viruses, and toxins, correlating with protection.
mechanism is lacking (Kraehenbuhl and Neutra, 1992; Phalipon et al., 2002; Williams and Gibbons, 1972).

IgA against *S. Typhimurium* are generated *in vivo* against the O (LPS) and H (flagella) antigens, and the OMPs. The immunodominant epitopes are the O-antigens, particularly the O5 antigen of *S. Typhimurium* (Slauch et al., 1995). In the absence of the O5 antigen, the majority of antibodies are produced against the O4 antigen, followed by the O12 (Slauch et al., 1995). IgA against the O-antigen are sufficient to prevent bacterial invasion of the intestinal epithelium, but are not associated with decreased attachment *in vitro* (Hohmann et al., 1978; Iankov et al., 2002b; Michetti et al., 1992). A monoclonal IgA against the O5 antigen alone is sufficient to confer protection *in vivo* (Michetti et al., 1992), and *in vitro* (Michetti et al., 1994). IgA against the H-antigens, are associated with decreased attachment *in vitro*, but are not protective *in vivo* (Hohmann et al., 1978; Iankov et al., 2002b). The accessibility to the OMPs, the LPS core, and lipid A to IgA has been debated, and the protective capacity is questionable (Barclay, 1999).

**Sal4 and IgAC5**

That SIgA directed against the O-antigen of *S. Typhimurium* are sufficient to prevent mucosal infection, was first demonstrated experimentally by the production and characterization of a collection of B-cell hybridomas isolated from the Peyer’s patches of mice immunized with an attenuated strain of *S. Typhimurium* (Michetti et al., 1992). From this screen, Michetti and colleagues identified Sal4, an anti-O-antigen-specific, dimeric monoclonal IgA, that when delivered into the intestinal lumen by normal pIgR mediated transepithelial transport, was sufficient to protect mice against a lethal oral
challenge with *S. Typhimurium* (Michetti et al., 1992). Using an *in vitro* model system, it was demonstrated that the IgA, Sal4, prevented *S. Typhimurium* from invading polarized epithelial cell monolayers *in vitro* (Michetti et al., 1994). Sal4 did not protect mice against a systemic challenge with *S. Typhimurium*, revealing that the monoclonal antibody’s mechanism of protection was mucosal specific (Michetti et al., 1992). It was subsequently demonstrated that Sal4 recognized the O5 epitope within the bacterial LPS (Slauch et al., 1995). The O5 antigen is defined by the acetylation of the abequose residue of the *S. Typhimurium* LPS at the 2’ position (Kim and Slauch, 1999). When the abequose residue is not acetylated, it is termed the O4 antigen, which is not recognized by Sal4.

SIgA against the O-antigen of *S. flexneri* are protective, as demonstrated by the mouse monoclonal IgA called IgAC5. IgAC5 was produced by immunization of mice orally with *S. flexneri* serotype 5a, strain M90T (Phalipon et al., 2002; Phalipon et al., 1995). Monoclonal IgA, which were found to have a high affinity for LPS, were purified and used in an intranasal challenge of naïve mice. IgAC5, directed against *S. flexneri* LPS was shown to provide protection to challenged mice and rabbits (Boullier et al., 2009; Phalipon et al., 1995). Although IgAC5 alone is sufficient to confer mucosal immunity to *S. flexneri*, the mechanism by which this occurs remains unknown. IgAC5 is neither bacteriocidal nor bacteriostatic, at least *in vitro* (Phalipon et al., 1995).

**Mechanism of Sal4 and IgAC5 Protection**

Immune exclusion, as defined by bacterial agglutination, entrapment in mucus, and clearance by peristalsis, does not explain the protective capabilities of either Sal4 or
IgAC5, particularly *in vitro* (Holmgren and Czerkinsky, 2005). Agglutination, as the first step in immune exclusion, is achieved only at an optimal antibody-antigen ratio. Therefore, agglutination is likely to be a rare occurrence *in vivo*, given that bacterial numbers and SIgA concentrations vary widely along the length of the intestinal tract (Kozlowski et al., 1997). Moreover, immune exclusion cannot account for the capacity of anti-LPS antibodies to prevent infection of intestinal epithelial cells *in vitro*, since neither mucus nor mechanical clearance are present (Apter et al., 1993b; Iankov et al., 2002b; Michetti et al., 1994). Thus, LPS-specific SIgA must have additional functions in mucosal immunity, independent of agglutination, which account for the protective nature of these antibodies, *in vitro* and *in vivo*.

Despite numerous advances in the field, the exact mechanism of protection against infection with *S. Typhimurium* by Sal4 antibodies remains largely unknown. Michetti and colleagues attributed the protective effect of Sal4 to its ability to promote bacterial agglutination (Michetti et al., 1994). They observed that treatment of *S. Typhimurium* with Sal4 at concentrations equal to, or exceeding, 5 µg/ml caused bacterial agglutination, blocked invasion and reduced bacterial attachment to epithelial cell monolayers (Michetti et al., 1994). While agglutination may play a significant role in promoting bacterial entrapment within the mucus layer and thereby hindering access to the epithelial surface *in vivo*, it is difficult to envision how agglutination can account for protection *in vitro* on epithelial cell monolayers that do not produce detectable amounts of mucus (Giannasca et al., 1996). Agglutination is also unlikely to explain Sal4-mediated immunity, as the cross-linking of *S. Enteritidis* or *S. Typhimurium* cells with
anti-flagellin (anti-H) antibodies had no effect on the bacteria’s ability to invade epithelial cells *in vitro* (Hohmann et al., 1978; Iankov et al., 2002b).

The mechanism of protection against infection with *S. flexneri* conferred by IgAC5 remains unknown. The O-antigen of *S. flexneri* is not involved in bacterial attachment or invasion of host cells, as LPS mutants which lack the O-antigen, are fully invasive (West et al., 2005). However, IgAC5, like other anti-LPS antibodies, is capable of inducing bacterial agglutination. It has been suggested that IgAC5 functions by immune exclusion (Boullier et al., 2009; Kraehenbuhl and Neutra, 1992; Phalipon et al., 1995). While immune exclusion may contribute to IgAC5’s protective capacity *in vivo*, it may not be the only mechanism. Furthermore, agglutination *per se* is not sufficient to interfere with invasion, and may actually increase adherence and invasion (Hale and Bonventre, 1979; Hale et al., 1979).

**Hypothesis**

The overall hypothesis of this dissertation is that IgA antibodies have protective functions, independent of immune exclusion, which impact bacterial virulence *in vivo* and *in vitro*. I used the protective IgA antibodies, Sal4 and IgAC5, against *S. Typhimurium* and *S. flexneri*, respectively, to better understand the mechanism by which IgA protects against enteric bacterial pathogens. I proposed that both of these monoclonal IgAs interfere with *S. Typhimurium* and *S. flexneri* pathogenesis, independent of agglutination, prior to or during the attachment and invasion of epithelial cells. Here I put forth evidence that Sal4, at concentrations previously shown to prevent bacterial entry into epithelial cells, is a potent inhibitor of *S. Typhimurium* of flagellum-
based motility, and SPI-1-mediated invasion. In both S. Typhimurium and S. flexneri, evidence is presented of damage to the OM. Additionally, I provide data implicating IgA in a direct effect on microbial physiology, resulting in a reduction in T3S, PMF and ATP levels. These data suggest a possible mechanism to explain Sal4’s protective capacity in vivo and in vivo. These findings challenge our previous assumption, that SIgA is primarily a physical barrier separating host and microbe by immune exclusion, and shows a direct impact of SIgA function in mucosal immunity, independent of agglutination.
Chapter 2

MATERIALS AND METHODS

Bacterial strains and culture conditions.

*Salmonella enterica* serovar Typhimurium and *Shigella flexneri* strains used in this study are listed in Table 2.1. All *S.* Typhimurium strains are derivatives of strain ATCC14028, except for BJ11 and BJ32, which are derived from SL1344, and were a gift from Dr. Brad Jones (University of Iowa). Strains JS93, JS107, JS481, and JS534 were kindly provided by Dr. James Slauch (University of Illinois at Urbana-Champaign). Strain LB142 was a gift of Lynne Becker and Ferric Fang (University of Washington). Strains HN1139 and HN1140 were gifts of Hiroshi Nikaido (University of California Berkeley). *Shigella flexneri* strains M90T (wild type) and BS176 (ΔpWR100), formerly M90Ta, were obtained from Dr. Philippe Sansonetti (Institut Pasteur, Paris, France). *S.*Typhimurium strains were grown on LB agar, and *S. flexneri* strains on Congo red TSA agar. Bacterial strains were routinely cultured at 37°C in Luria-Bertani (LB) broth. Subcultures were grown at 37°C on a rotary shaker to mid-log phase (~0.700). Optical density at 600 nm was taken using an Ultrospec 2100 pro (GE Healthcare, Piscataway, NJ), and adjusted to an OD600 of 0.700. M9 media used in this study lacked thymine, and contained 0.5% glucose or succinate. When necessary, media were supplemented with antibiotics at the following concentrations: kanamycin (50 µg/ml), tetracycline (20 µg/ml), chloramphenicol (35 µg/ml), and ampicillin (100 µg/ml).

*S. Typhimurium* strain SJF2 (ATCC14028 *ArpoN::kan*) was made via P22HTint mediated transduction from SJF1 (LT2 *ArpoN::kan*), which was created using Lambda
Red mediated recombination with pKD13 as the template plasmid and kanamycin antibiotic resistance marker (Datsenko and Wanner, 2000; Maloy, 1996). *S.*Typhimurium strains SJF3 (Δrfc::kan) and SJF4 (ΔrfaL::kan) are isogenic derivatives of ATCC14028 created using Lambda Red mediated recombination with pKD13 as the template plasmid (Datsenko and Wanner, 2000). *S.* flexneri strain SJF31 (ΔtolC::kan) is derived from the wild type strain M90T, and was created using Lambda Red mediated recombination with pKD4 as the antibiotic resistance marker template (Datsenko and Wanner, 2000). The primer sets for mutagenesis and verification are given in Table 2.2. Appropriate insertion of the antibiotic resistance marker was confirmed by PCR.

*S.* Typhimurium strains SJF7, 8, 10, 11, 12, 14, and 32 were created by P22HTint mediated transduction (Maloy, 1996). Strains SJF7 (cheA52 zea::Tn10) and SJF8 (motB275 zea-2::Tn10) were created by P22HTint mediated transduction from BJ11 and BJ32 into ATCC14028, respectively. SJF10 (zjg8101::kan oafA126::Tn10d-Tc fkpA-lacZ) was created by P22 HT int mediated transduction of fkpA-lacZ from strain JS534 into strain JS93. Strains SJF11 (fkpA-lacZ oafA126::Tn10d-Tc) and SJF12 (oafA126::Tn10d-Tc) were created by P22HTint mediated transduction of oafA126::Tn10d-Tc from JS93 into JS534 or ATCC14028, respectively. Strain SJF14 (tolC::kan) was created by P22HTint mediated transduction from HN1139 into ATCC14028. Strain SJF32 (oafA126::Tn10d-Tc tolC::kan) was created by P22HTint mediated transduction of tolC::kan from SJF14 into SJF12. All transductants were selected for on antibiotic plates containing 10 mM EGTA, and tested for pseudolysogens on EBU agar against P22H5 (Maloy, 1996).
Table 2.1  S. Typhimurium and S. flexneri strains used in this study.

<table>
<thead>
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<th>Strain</th>
<th>Description</th>
<th>Source or Reference</th>
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<td>fkpA-lacZ::kan</td>
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<td>phoP-24 tolC::kan</td>
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Antibodies. Antibodies and antisera are listed in Table 2.3. The murine hybridomas secreting monoclonal, polymeric IgA antibodies Sal4 and 2D6 were obtained from Dr. Marian Neutra (Children’s Hospital, Boston, MA) (Michetti et al., 1992). 2D6 is specific for Vibrio cholerae 0395 LPS (Apter et al., 1993b). Purified murine monoclonal, polymeric IgA antibodies IgAC5 and SIgAC5 were obtained from Drs. Blaise Corthésy (CHUV, Lausanne, Switzerland) and Armelle Phalipon (Institut Pasteur, Paris). The murine monoclonal IgA antibody 23D7 directed against ricin subunit A was described previously (Mantis et al., 2006), and was used as an isotype control in these studies. 23D7 has no detectable reactivity with S. Typhimurium as determined by ELISA. The murine anti-phosphoryl choline monoclonal IgA antibody TEPC-15 (Sigma Co) was used as an isotype control for Sal4 in these studies. TEPC-15 has no detectable reactivity with S. Typhimurium as determined by ELISA and was routinely reconstituted at 1 mg/ml and dialyzed against PBS prior to use in motility and invasion experiments. IpaB, IpaC, and IpaD specific monoclonal antibodies were obtained from Dr. Edwin Oaks (The Walter Reed Army Institute of Research, Silver Spring, MD) (Mills et al., 1988). Monoclonal mouse anti-OmpC antibodies CM 104.1 and CM 12.1 (Singh et al., 1996) were a gift of Dr. Lynn Bry (Brigham and Women’s Hospital Boston), and were tested for concentration by sandwich ELISA.

Hybridomas were cultured in CD Hybridoma serum-free, protein-free medium (Gibco-Invitrogen, Carlsbad, CA) without antibiotics, and were maintained at 37°C in a 5% CO2/95% air atmosphere. Hybridoma supernatants were dialyzed against PBS by means of a Slide-a-lyzer (10,000 MW cut-off; Pierce Scientific, Rockford, IL) prior to
<table>
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use. The concentration of IgA in hybridoma supernatants was determined by sandwich ELISA with IgA TEPC-15 as a standard. In some cases antibody concentration of hybridoma supernatants was increased using a protein concentrator column (20,000 MW cut-off; Pierce Scientific, Rockford, IL).

Rabbit polyclonal antisera against Salmonella O-antigens (Group B Factors 1, 4, 12, 27 or 1, 4, 5, 12) and H-antigens (single factor 2 and factor i), *V. cholerae* O1 Ogawa, and *S. flexneri* O-Ag (groups 1-6) were purchased from BD Difco (Franklin Lakes, NJ). All antibody preparations were reconstituted in 0.85% NaCl solution and dialyzed against PBS for >16 h to remove any possible traces of azide, prior to being used in the assays described in this study. Dialysis was performed using 10,000 molecular weight cut-off Slide-A-Lyzer dialysis cassettes.

F(ab)_2 and Fab fragments were produced essentially as described previously (Harlow and Lane, 1988; Harlow and Lane, 1999). Salmonella O antiserum (100 µl) was mixed with Protein A on Sepharose beads (50 µl; Sigma), and incubated with rocking for 2 hours at room temperature. For F(ab)_2 production, the antibody-Protein A bead complexes were collected by centrifugation (2 min at 3,000 x g), washed in PBS, and then suspended in 100 mM sodium citrate (pH 3.5-4.0) buffer containing ~38 units of pepsin bound to agarose (Sigma Co.). Digestion was achieved by incubation at 37°C for 8 hours with gentle rocking. The reaction was stopped by the addition of 3M Tris (pH 8.8). The mixture was subjected to centrifugation (2 min at 3,000 x g) to separate the Fc-Protein A agarose beads (pellet) from the F(ab)_2 products (supernatant). For Fab production, the antibody-Protein A bead complexes were collected by centrifugation (2 min. at 3,000 x g), washed in PBS, and then suspended in digestion buffer consisting of
20 mM EDTA, 20 mM L-cysteine (pH 6.3) and 1 unit of Papain bound to agarose beads. Digestion was achieved by incubation at 37°C for 12 hours with gentle shaking. The Fab products were separated from the Fc-Protein A beads and papain-agarose beads by centrifugation, as described above. Purity of the F(ab)2 and Fab-containing supernatants was determined by SDS-PAGE and Western blotting with goat anti-rabbit IgG-HRP antibody (H + L chain specific, Southern Biotech).

**Viability Assays.** Bacterial were grown overnight and subcultured as described above. The OD600 was adjusted to ~0.700, then diluted 1:10 into M9 + 0.5% glucose supplemented with 10 µg/ml Sal4, IgAC5 or 23D7, in triplicate. At 15 minutes intervals (T = 0-45 min) aliquots were plated onto LB agar to determine CFUs per ml. Alternatively, mid-log phase cultures were washed in PBS and then diluted 1:10 into PBS in a 96-well plate. They were then treated with either Sal4 (5µg/mL), chemical or biological agents, or a combination of both, in triplicate for 1 hour. The agents included: antibiotics included rifampicin, novobiocin, or erythromycin at 10µg/ml), deoxycholate (5%, 10%, or 20%), defensins HNP-1 and Crpt4 (5 µg/ml), and complement (10%, 20% or 50% bovine or goat serum). In order to carry out the classical complement pathway, rabbit serum and anti-O antiserum was used at 5 µg/ml. Chlorphenicol was also added to some assays at a concentration of 300 ng/ml to prevent growth. For media assays, mid-log phase cultures were washed and diluted 1:10 into either LB, M9 + 0.5% glucose, or M9 + 0.5% succinate. After treatment cells were serially diluted in PBS, and 100 µl were spread onto LB agar plates. Plates were incubated overnight 37°C to allow colony formation.
Growth Assays. Bacterial were grown overnight and subcultured as described above. The OD600 was adjusted to ~0.700, then diluted, washed with LB, and diluted (1:10) into LB in a 96-well clear flat bottom plates (BD Falcon, Franklin Lakes, New Jersey). Diluted cultures were then treated in triplicate with either Sal4 (5 µg/ml), chemical or biological agents, or a combination of both, as described above. After treatment, OD readings were taken every 30 minutes for 4 hours at 600 nm using a VersaMax spectrophotometer (Molecular Devices, Sunnyvale, CA).

Bacterial motility assays. For agar motility assays, LB agar plates (0.3 g Bacto-agar per 100 ml LB) containing antibodies (5 µg/ml) were stab inoculated with 1 µl of an overnight culture of S. Typhimurium. The plates were incubated upright at 37°C for 8 hours, and the diameter of bacterial spread was measured every hour. For video light microscopy, bacteria in mid-log phase were diluted into PBS (~1x10^6 CFU in a volume of 10 µl) at room temperature, mixed with the relevant monoclonal or polyclonal antibodies, and then immediately spotted onto a microscope slide. The droplet was mounted with a coverslip (22 x 22 mm), and imaged at room temperature using a Nikon TE2000 inverted microscope equipped with differential interference contrast optics (DIC), a 40X (0.9 NA) objective lens, and an HQ camera (Roper Scientific, Trenton, NJ). The images were captured at approximately 7 Hz with RS Image software (Roper Scientific) at 100 frames of 100 milli seconds each. The movements of individual bacteria over time were tracked in sequential video frames by overlaying the video screen with transparency film and highlighting bacteria with an ink pen. In some cases, bacterial motility was monitored with ImagePro software (Media Cybernetics, Silver Springs,
MD). Typically, there were 30-60 bacteria per field (range, 19-123 bacteria); at this density, bacterial “collisions”, and thereby agglutination, was minimized. For video light microscopy, motility was expressed as percent of control: (% of motile bacteria in experimental sample/ % motile bacteria in control samples) x 100. TEPC-15 (1-10 µg/ml) was used as an IgA isotype control for Sal4 in these experiments. I routinely verified that the percent of motile bacteria in TEPC-15-treated cultures did not change over the course of the experiments. For each experimental condition, I analyzed 30-40 video images, each containing 30-60 bacteria. Three to six replicates were performed per experimental and control conditions. The student’s t test was used to determine statistical significance between control and experimental samples.

**Invasion and attachment assays.** MDCK II cells were obtained from the ATCC (#CCL-34). MDCK II cells were maintained in DMEM supplemented with 10% FBS at 37°C under 5%CO2/95% air atmosphere. MDCK II cells were seeded in 96 well plates (~5x10⁴ cells/well in 0.1 ml) and incubated for 7-10 days, to permit the formation of polarized monolayers. HeLa cells were obtained from the Wadsworth Center cell culture core facility and were maintained as described for the MDCK II cells. HeLa cells were seeded into 96 well plates (~1x10⁵ cells/well in 0.1 ml) and used for invasion assays 24-48 hr after seeding (at 50-90% confluence). Competitive invasion assays were performed essentially as described previously (Michetti et al., 1994). S. Typhimurium strains were grown to mid-log phase in LB with aeration, adjusted with fresh LB to equivalent optical densities (OD600nm ~ 0.7), washed in PBS and resuspended in DMEM. Bacteria were mixed at equal proportions, then diluted 1:10, and (~10⁷ total
CFUs in 0.2 ml). The exact ratio of the two strains was determined by replica plating or plating on media containing X-gal (30 µg/ml). The bacteria were then incubated with Sal4 (5 µg/ml) for either 15 min before being applied to epithelial cells (MOI of 10) in 96-well microtiter plates. As necessary, the microtiter plates were subjected to low-speed centrifugation (10 min at 1000 x g) at 4°C to promote bacterium-epithelial cell contact. The plate was incubated at 37°C for 15-60 minutes. The number of invasive bacteria was determined via the gentamicin protection assay (Michetti et al., 1994). Cell were washed 3 times in Hanks Balanced Salt Solution (HBSS) and incubated for 1 hour with 100 µg/ml gentamicin. Cells were washed 3 times in HBSS then lysed with a 1% Triton X-100 detergent solution in calcium and magnesium free PBS. The ratio of the two strains was determined by replica plating or plating on media containing X-gal (30 µg/ml). The competitive index (CI) was calculated as: 

\[
\text{CI} = \frac{(\% \text{ strain A recovered}/\%\text{strain B recovered})}{(\% \text{ strain A inoculated}/\%\text{strain B inoculated})}
\]

(Ellermeier et al., 2005). The student’s t test was used to determine statistical significance between control and experimental samples.

Non competitive epithelial cell invasion assays were performed in 96-well microtiter plate format, as described previously, with slight modifications (Michetti et al., 1994). S. Typhimurium or S. flexneri strains were grown to mid-log phase in LB with aeration, adjusted with fresh LB to equivalent optical densities (OD600nm ~ 0.7). Bacteria were washed in PBS and resuspended in DMEM, then diluted 1:10 or 1:100 into DMEM. The initial concentration of bacteria was determined by CFUs. The bacteria were then incubated with Sal4 or IgAC5 for 15 minutes before being applied to epithelial cells grown in 96-well microtiter plates at a multiplicity of infection of approximately 10 or 1,
dependent on dilution. The microtiter plates were subjected to low-speed centrifugation (10 min at 1000 x g) to promote bacterium-epithelial cell contact. The plate was incubated at 37°C for 15-60 minutes. The number of invasive bacteria was determined via the gentamicin protection assay, as described previously (Michetti et al., 1994). Cells were washed 3 times in Hanks Balanced Salt Solution (HBSS) and incubated for 1 hour with 100 µg/ml gentamicin. Cells were washed 3 times in HBSS then lysed with a 1% Triton X-100 detergent solution in calcium and magnesium free PBS.

For attachment assays, MDCK cells, HeLa cells, and bacteria were prepared as described above. After bacteria were added to epithelial cells, the plate was incubated for 15 minutes at 37°C, or subject to centrifugation then incubated for 15 minutes at 37°C. The cells were then washed 3 times in cold HBSS to remove unbound bacteria, and then lysed with a 1% Triton X-100 detergent solution. CFUs in cell lysates were determined by plating on LB agar.

The murine macrophage cell line J774A.1 was obtained from the ATCC (TIB-67) and maintained in DMEM with 10% FBS. For macrophage invasion assays, cells were seeded into 96-well microtiter plates (2-5 x 10^5 cells per well in 0.2 ml) and used 18-40 hours later, as described previously (Buchmeier and Heffron, 1989).

Carbonyl cyanide m-chlorophenylhydrazone (CCCP) was employed in some invasion or attachment assays to deplete proton motive force in the bacteria. Bacteria were incubated with CCCP for 10 minutes, centrifuged for 5 minutes, and resuspended in fresh media. Washing the bacteria was necessary to remove the CCCP, which would have otherwise killed the epithelial cells.
**β-galactosidase assay.** β-galactosidase assays were performed as described by Miller (Miller, 1972), and modified to 96 well plate format (Ellermeier et al., 2005; Slauch and Silhavy, 1991).

**Scanning electron microscopy.** Overnight cultures or subcultures of *S. Typhimurium* or *S. flexneri* were diluted 1:50 into a 2 ml total volume containing 5 µg/ml of Sal4, IgAC5, or TEPC-15. The bacteria-antibody mixtures were incubated at room temperature for 15 minutes or 1 hour, and then captured on a 0.2 or 0.4 µm defined pore sizes polycarbonate filter membranes (Nuclepore brand, Whatman (now part of GE Healthcare)) using a vacuum apparatus. The filter was then incubated for 20 minutes with 5 ml of fixative (2% glutaraldehyde in 0.1 M sodium cacodylate buffer, 5 µM CaCl$_2$, pH 7.4). The filter was washed twice with cacodylate buffer, once in sterile water, and then subjected to a series of ethanol dehydrations (5 minutes each): 25% EtOH, 50% EtOH, 70% EtOH, 90% EtOH, and three times with 100% EtOH. The sample was critical point dried, and then attached to an aluminum stud with carbon paste and sputter coated with gold (twice at 30 or 45 seconds). The specimens were observed at 5.00 kV using a LEO 1550VP FEG microscope (Carl Zeiss SMT).

**Transmission electron microscopy.** Subcultures of *S. Typhimurium* were diluted 1:50 into a 2 ml total volume containing 5 µg/ml of Sal4, or TEPC-15. The bacteria-antibody mixtures were incubated at room temperature for 15 minutes or 1 hour, then were spun down to form a pellet and fixed with 2% glutaraldehyde in cacodylate buffer (pH 7.2), washed twice in buffer, then 1% osmium in cacodylate buffer (pH 7.2). The
cells were washed in double-distilled water, dehydrated in an acetone series, and embedded in Epon-Araldite. Semithin (0.20–0.25 micron) sections were cut using a Diatome diamond knife on a Reichert Ultracut E ultramicrotome. The sections were stained with uranyl acetate for 20 min and lead for 5 min and viewed at 80 kV with a Zeiss 910 transmission electron microscope (Carl Zeiss SMT). Images were taken with a SIA AAC CCD camera.

**Cryo electron microscopy.** *S. Typhimurium* were subcultured to mid-log phase in 2 ml of filtered LB on drum rotor at 37°C for ~2.5 hours. The density was adjusted to an OD_{600} of 0.70 (~10^9 CFU/ml). The bacteria were centrifuged (6,000 rcf for 5 minutes) and resuspended in filtered LB, then diluted 1:5 into PBS, pH 7.4. Sal4 (5 µg/ml) or an equivalent volume of PBS was added to the *S. Typhimurium* and incubated for 15 minutes, then spotted onto carbon-coated copper grids, plunge-frozen in liquid ethane, and imaged at -176°C using a JEM-4000FX electron microscope equipped with a Gatan GIF 2002 energy filter.

**Analysis of Ipa secretion.** Analysis of Ipa proteins was done by a modified assay using Congo red, as described in Bahrani et al (Bahrani et al., 1997). *S. flexneri* strain M90T was incubated overnight in LB broth at 37°C, and then sub-cultured 1:50 for 2-3 hours in LB, and the optical density adjusted to OD_{600nm} ~ 0.7. Aliquots (1 ml) of bacteria were then washed twice in PBS to remove LPS and proteins from the supernatant. Bacteria were resuspended in 1 ml of PBS containing IgAC5, anti-O antisera, CCCP, or isotype control antibodies TEPC-15, Sal4, or 23D7. These assays...
were performed at an OD$_{600}$ of 0.7, not a 1:10 dilution as in all other assays, based upon detection limits. For clarity, antibody concentrations are given in the text as the equivalent µg/ml for a 1:10 dilution, for comparison with all other assays. Bacteria-antibody mixtures were incubated for 15 minutes at 37°C, and then treated with Congo red (6 µg/ml final concentration) for 10 minutes at 37°C. Following centrifugation (5 minutes at 8,000 rcf), bacterial pellets were suspended in 20-100 µl of 1M Tris-HCl (pH 7.4) and an equal volume of Laemmli sample buffer containing β-mercaptoethanol (5%, vol/vol), boiled for 5 minutes, and diluted 1:10 prior to SDS-PAGE. Bacterial supernatants were passed through a 0.2 µm filter (Pall Life Science, Ann Arbor, MI). Of the (750 µl to 1ml) flow through, 750 µl was concentrated using 750 µl ice cold 50% trichloroacetic acid (TCA) for 10 minutes, subjected to centrifugation (10 min at 12,000 rcf), washed with acetone, and then suspended in 20 µl 1M Tris-HCl (pH 7.4). The concentrated proteins were mixed with an equal volume of Laemmli sample buffer containing β-mercaptoethanol (5%, vol/vol), boiled for 5 min, and then size-fractionated on a pre-cast 12% Tris-HCl PAGE (Bio-Rad, Hercules, CA), alongside pre-stained broad-range molecular weight standards (Bio-Rad). For Western blot analysis, polyacrylamide gels were equilibrated in Bjerrum and Schafer-Neilson transfer buffer for 30 minutes before being transferred to 0.45-µm pore size nitrocellulose (Bio-Rad) in a Trans-blot semi-dry transfer cell (Bio-Rad). The nitrocellulose membranes were blocked in PBS-Tween-20 (0.1%) with 2% goat serum for 1 hour or overnight at 4°C, before being probed with mouse monoclonal antibodies against IpaB, IpaC, or IpaD. Primary antibodies were detected using horseradish peroxidase-labeled goat anti-mouse IgG
secondary antibodies (Southern Biotech, Birmingham, Alabama), in conjunction with ECL Western blotting substrate (Pierce).

Hemolysis. Hemolysis assays were adopted from Field et al (Salmonella) and Mounier et al (Shigella) (Field et al., 2008; Mounier et al., 1999). Defibrinated whole sheep blood was diluted 1:25 into PBS and washed 3-4 times in PBS (pH7.4) at 1,000 rcf for 5 minutes at 4°C, and resuspend in PBS to equal the starting volume. Salmonella strains ATCC14028 and SJF12 were incubated overnight in LB at 37°C, and then subcultured for approximately 2 hours in fresh LB, and the optical density adjusted to OD$_{600nm}$ ~ 0.70. Aliquots of bacteria (1 ml) were then washed in PBS and resuspended in an equal volume of PBS. Aliquots of bacterial cultures were incubated for 15 minutes at room temperature with Sal4, or 23D7. The bacteria (50 µl) were then added to wells of a 96 well plate containing an equal volume of the washed sheep blood, and then incubated for 30 minutes at 37°C. PBS (200 µl) was added to each well, and the plate centrifuged for 5 minutes at 1,000 rcf at 4°C, to pellet blood cells and bacteria. The supernatants (200 µl) were removed and placed into a 96 well optical bottom microtiter plates (Costar). The absorbance at 550 nm was recorded on a VersaMax spectrophotometer (Molecular Devices). The absorbance of the wild type control was set at 100% for the purposes of the assay.

Measurement of electrical potential. The dye 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide, or JC-1 (Invitrogen) is sensitive to the electrical potential of the bacteria (Becker et al., 2005). With an intact electrical potential
the dye shifts from a monomer (Ex 514 Em 529) to a J-aggregate (Ex 585 Em 590).
Cultures of wild type S. Typhimurium, or S. flexneri strain SJF31 were subcultured 1:50 for 2-3 hours in LB, and the optical density adjusted to OD$_{600\text{nm}}$ ~ 0.7. The tolC::kan strain of S. flexneri was used to prevent active dye efflux, as JC-1 has a MW of 532. To load the dye, 1 ml aliquots were centrifuged (5 minutes at 6,000 x rcf) and resuspended in PBS, then centrifuged and responded in 1 ml of permeabilization buffer (10 mM Tris, 1 mM EDTA, 10 mM glucose, pH 7.5) (Becker et al., 2005). The JC-1 dye was added at a final concentration 10 µg/ml. The tubes were mixed by inversion, and incubated for 5 minutes at room temperature. To pellet cells and excess JC-1, the tubes were centrifuged at 6k rcf for 5 minutes. Removal of excess dye is different for S. Typhimurium and S. flexneri. JC-1 is hydrophobic and when centrifuged as above will form a layer on top of the S. Typhimurium pellet, which can be removed by gentle pipetting a small amount of supernatant, causing the dye aggregate to break free of the bacterial pellet and be removed with the supernatant. With S. flexneri the dye and bacterial pellet intermingle if centrifuged. To remove excess dye the S. flexneri tube is incubated at room temperature for 10-15 minutes, allowing for the dye to aggregate and precipitated out of solution. The relatively dye free upper half of the supernatant is removed and pelleted through centrifugation. The pellet for S. Typhimurium was resuspended in 1 ml, and S. flexneri in 0.5 ml, of M9 + 0.5% glucose, pH 7.0. The bacteria were allowed to recover 15 minutes at room temperature, then diluted 1:10 into PBS with 5 µg/ml Sal4, IgAC5, 23D7, or 100 µM CCCP in triplicate.

The electrical potential was measured by microscopy or plate reader after the indicated incubation time points at room temperature. Agarose slides (1%) were prepared
and 10 µl of bacteria spotted onto a slide. The droplet was mounted with a coverslip (22 x 50 mm), and 100+ bacteria per treatment visualized at room temperature using a Axioscope II plus microscope (Zeiss). Measurements were taken with FITC and Texas Red filter sets: green channel (Ex 455-495, beam splitter 500, Em 505-555) and red channel (Ex 550-580, splitter 585, Em 590-650). For fluorescence by plate reader, 200 µl of bacteria (10^7-10^8 CFU/ml) were added per well of 96 well optical bottom black polystyrene microtiter plates (Costar). A Synergy HT (BioTek, Winooski, VT) fluorescent plate reader was used to measure fluorescence using single excitation (485/20), and dual emission (528/20, 590/35).

**Measurement of intracellular pH.** The dye 2′, 7′-bis-(carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF) is a fluorescent indicator used to measure intracellular pH over a range of pH 6.2-9.5 (Invitrogen). Midlog phase subcultures of *S. flexneri* strain SJF31 was grown to an OD600 of ~0.70. To load the dye, 1 ml aliquots of bacteria were centrifuged (5 minutes at 6,000 x rcf) and resuspended in PBS, then centrifuged and resuspended in 1 ml of permeabilization buffer (10 mM Tris, 1 mM EDTA, 10 mM glucose, pH 6.5) (Becker et al., 2005). To each tube, 2 µl of 5 mg/ ml BCECF stock solution (final concentration 10 µg/ml) was added, mixed by pipetting, and incubated for 5 minutes at room temperature. Bacteria were collected by centrifugation (6,000 rcf for 5 minutes) and the resulting pellets were resuspended in 1 ml M9 + 0.5% glucose, pH 7.0, twice to remove dye aggregates. The bacteria were allowed to recover for 15 minutes at room temperature before being diluted 1:10 (vol/vol) in triplicate into optical bottom black polystyrene microtiter plates (Costar) containing phosphate buffers at pH 7.4 and
9.6, and anti-O antiserum (5 µg/ml), 23D7 (5 µg/ml), 10 µM Nigericin, or 100 µM CCCP, 200 µl final volume. After incubation at room temperature for 15 minutes the fluorescence was measured using a Synergy HT fluorometer (BioTek, Winooski, VT). Fluorescence was assessed at a single excitation (485/20) and dual emission (528/20, 645/40). The 530/645 ratio was determined (Balkay et al., 1997).

**Analysis of ATP levels.** Mid-log phase subcultures of *S*. Typhimurium and *S*. flexneri were grown to an OD600 of ~0.70. To measure total cellular levels, bacteria were diluted 1:10 into well of microtiter plates containing M9 + 0.5% glucose or 0.5% succinate with Sal4 (5 µg/ml), IgAC5 (10 µg/ml), 23D7 (5-10 µg/ml) or CCCP (100 µM) in triplicate. Cultures were incubated at 37°C and 50 µl aliquots removed at 0, 10, 20, 30, 40 minutes. To measure ATP levels in supernatants, bacteria were diluted 1:10 into 1ml PBS with Sal4 (5 µg/ml), IgAC5 (10 µg/ml), 23D7 (5-10 µg/ml) or CHCl3 to lyse bacteria. Bacteria-antibody mixtures were incubated for 15 minutes and then subjected to centrifugation (5 minutes at 8,000 rcf), and the supernatants passed through a 0.2 µm filter (Pall Life Science). In both assays, 50 µl of bacteria or bacterial supernatant was added to 50 µl of PBS in white polystyrene 96 well plates (Costar). To each well 100 µl of BacTiter-Glo (Promega, Madison, WI) was added and mixed by pipetting. Luminescence was measured using a SpectraMax L luminometer (Molecular Devices), using the photon counting mode. Luminescence was normalized to that of the 23D7 control.
**Loss of OM components.** S. Typhimurium strains were grown to mid-log phase in LB with aeration, adjusted with fresh LB to equivalent optical densities (OD600 nm ~ 0.7), washed in PBS twice, and then diluted 1:10 in PBS or TE (Tris 50 mM, EDTA 110 mM). S. Typhimurium were treated with either Sal4 (5 µg/ml), or 23D7 (5 µg/ml) for 15 (or 30) minutes. After treatment, samples were centrifuged for 5 minutes at 8,000 rcf. After centrifugation, supernatant was removed and placed in new tube. Supernatants were filtered through a 0.2 µm HT Tuffryn membrane, Acrodic syringe filter (PALL Life Science). To determine the percent of components in the supernatant compared to the total cellular levels, an aliquot of bacteria were fixed with 2% paraformaldehyde. The supernatants were subjected to dot blot analysis and densitometry analysis. The supernatants were serially diluted and 3 µl were spotted onto nitrocellulose membranes (0.45 µm pore size; Bio-Rad) and allowed to air dry at room temperature. Some samples required concentration of the supernatant for detection, and were serially diluted in a 100 µl volume and pulled down onto the nitrocellulose using a BioRad dot blot apparatus. For O-antigen and flagellin detection, the nitrocellulose membranes were then blocked in PBS containing 0.5% Tween-20 (PBS-T) and 2% goat serum overnight at 4°C. For OmpC detection the block was 2% rabbit serum. The blots were washed with PBS-T (0.1%) prior to probing. O-antigen was detected using rabbit polyclonal antisera against Salmonella O-antigens (Group B Factors 1, 4, 5, 12) at a 1:1,000 dilution (~1 µg/ml) in PBS-T for 1 hour at room temperature. Flagellin was detected with rabbit polyclonal antisera against Salmonella H-antigens (single factor 2 and factor i) at 1:1000, and OmpC with a monoclonal mouse anti-OmpC antibody CM 12.1 at 1:1,000. The membranes were then washed with PBS-T to remove unbound antibody, treated with the HRP-conjugated
goat anti-rabbit IgG antibodies at 1 µg/ml (anti-LPS and anti-H), or HRP-conjugated goat anti-mouse IgG antibodies (OmpC), and developed with the ECL Western Blotting Substrate (Pierce). The membranes were exposed to X-ray film (IBF, Brazil) then a digital image taken with a ChemiDoc (BioRad). Densitometry analysis was done using Quantity One software (BioRad).

**Endotoxin assay.** LPS endotoxin was assayed by the Limulus amebocyte lysate (LAL) technique (GenScript, Piscataway, NJ). Supernatant was collected as described above (loss of OM components). Samples were diluted and assayed as per the manufacture’s directions.

**Fluorescence Permeability Assays.** Fluorescence assays were conducted with ethidium bromide (EtBr) or sodium fluorescein. The EtBr assays were preformed similar to those described previously (Murata et al., 2007). Mid-log phase cultures of *S.* Typhimurium strains carrying the *tolC::kan* mutation, SJF14 (*tolC::kan*) and SJF32 (*tolC::kan* oafA::Tn10d), were used to prevent active dye efflux. Bacterial strains were diluted 1:10 into sterile water in black 96-well plates with optical bottoms with Sal4 or 23D7 (5 µg/ml) in triplicate, and incubated for 15 minutes. EtBr was added at 10 µg/ml and readings were immediately taken at Ex. 530 and Em. 645 with a Synergy HT fluorometer (BioTek, Winooski, VT) over 10 minutes. In the sodium fluorescein assays, mid-log phase cultures of *S.* Typhimurium strain SJF14 (*tolC::kan*) was incubated with 10 mM sodium fluorescein in a pH 5.5 phosphate buffer, to reduced fluorescein’s fluorescence. The bacteria-dye mix was immediately diluted 1:10 into pH 7.4 PBS in
black 96-well plates with optical bottoms, and incubated for 15 minutes with Sal4 or PBS. Readings were taken at 15 minutes after antibody exposure at Ex. 490 and Em. 514 with a Synergy HT fluorometer over 10 minutes. Percent increase was determined by the increases over the 23D7 control.

**Alkaline phosphatase Leakage.** Mid-log phase subcultures of *S. Typhimurium* and *S. flexneri* were grown to an OD600 of ~0.70. Bacteria were washed twice in PBS, then diluted 1:10 in PBS. Bacteria were treated for 15 minutes with Sal4 (5µg/mL), 23D7 (5µg/mL), or CHCl₃. The samples were centrifuged for 5 minutes at 8,000 rcf. After centrifugation, the supernatants were filtered using 0.2 µm HT Tuffryn membrane Acrodic syringe filter (Pall Life Science). Supernatant, 100 µl, of each treatment was transferred to a NUNC Maxisorp 96-well plate (Costar) in triplicate. The presence of alkaline phosphatase was measured utilizing para-nitro phenyl phosphate (PNPP) in a glycine buffer (2 mg/mL). Overnight incubation (16-20 hours) was carried out to allow color change. Absorbance was recorded via spectrophotometry at 405 nm using a SpectraMax plate reader (Molecular Devices).
Chapter 3

Inhibition of *Salmonella enterica* serovar Typhimurium Motility and Entry into Epithelial Cells by a Protective Anti-lipopolysaccharide Monoclonal Immunoglobulin A Antibody

Adapted from:


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INTRODUCTION

*Salmonella enterica* serovar Typhimurium is an invasive, pathogenic, gram-negative bacterium. In humans these bacteria cause acute gastroenteritis, whereas in mice *S. Typhimurium* cells proliferate in the intestinal mucosa and then spread systemically to the liver and spleen, eliciting a disease that resembles typhoid fever (Haraga et al., 2008; Mackaness et al., 1966). Several key attributes of *S. Typhimurium* underlie its capacity to successfully colonize and invade the intestinal epithelium. The foremost is lipopolysaccharide (LPS), the major constituent of the outer leaflet of the bacterial outer membrane. In particular, the O-antigen component of LPS aligns laterally to form a protective coat surrounding the bacterium that confers resistance against antimicrobial agents present in intestinal secretions (Dann and Eckmann, 2007; Eckmann and Kagnoff, 2001; Nikaido, 2003). *S. Typhimurium* is also highly motile due to the presence of flagella, which act in concert to propel the bacterium through liquid and viscous
environments (Berg, 2003; Ilino, 1974). This motility is postulated to enable the bacterium to penetrate the thick mucus coat that covers the intestinal mucosa, as well as to promote contact with epithelial cell surfaces (Jones et al., 1994; Schmitt et al., 2001).

Finally, *S. Typhimurium* cells express a type 3 secretion system (T3SS) encoded by *Salmonella* pathogenicity island 1 (SPI-1) which allows the bacteria to specifically invade intestinal epithelial cells (Cossart and Sansonetti, 2004; Galan and Wolf-Watz, 2006; Haraga et al., 2008). Once *S. Typhimurium* cells have breached the epithelial barrier (at least in the mouse), the bacteria disseminate systemically and reside primarily within macrophages.

In the intestinal tract, secretory immunoglobulin A (SIgA) antibodies directed against the O antigen of *S. Typhimurium* are sufficient to prevent mucosal infection (Chowers et al., 2007; Iankov et al., 2004; Michetti et al., 1992). This was first demonstrated experimentally by Kraehenbuhl and Neutra and Michetti and colleagues who produced and characterized a collection of B-cell hybridomas isolated from the Peyer’s patches of mice immunized with an attenuated strain of *S. Typhimurium* (Kraehenbuhl and Neutra, 1992; Michetti et al., 1992). From this screen, Michetti and colleagues identified Sal4, an anti-O-antigen-specific, dimeric monoclonal IgA antibody (IgA) that when delivered into the intestinal lumen by normal receptor-mediated transepithelial transport was sufficient to protect mice against a lethal oral challenge with *S. Typhimurium* (Michetti et al., 1992). Using an in vitro model system, it was subsequently demonstrated that Sal4 (~5 µg/ml) prevented *S. Typhimurium* from invading polarized epithelial cell monolayers (Michetti et al., 1994). Sal4 did not protect
mice against a systemic challenge with *S. Typhimurium*, revealing that the monoclonal antibody’s mechanism of protection was mucosal specific (Michetti et al., 1992).

It is generally assumed that secretory antibodies function by “immune exclusion,” a term which refers to the ability of polyvalent IgA to promote bacterial agglutination, entrapment in mucus, and clearance via peristalsis (Brandtzaeg, 2007; Phalipon et al., 2002). While immune exclusion may account for some of the protection conferred by Sal4 *in vivo*, it cannot explain the capacity of Sal4 to prevent the invasion of polarized and non-polarized epithelial cell monolayers by *S. Typhimurium* *in vitro*. The epithelial cell lines used in these previous studies do not produce detectable amounts of mucus, nor are they able to mediate mechanical clearance (i.e., peristalsis) (Michetti et al., 1994). Agglutination is also unlikely to explain Sal4-mediated immunity, as others have shown that cross-linking of *S. Typhimurium* or *Salmonella enterica* serovar Enteritidis cells with antiflagellin (anti-H) antibodies has no effect on their ability to invade epithelial cells in vitro (Hohmann et al., 1978; Iankov et al., 2002b).

Therefore, I postulated that Sal4 has additional “effector” function(s) which account for its capacity to inhibit *S. Typhimurium* invasion of epithelial cells. In this study, I undertook an examination of the effects of Sal4 on bacterial processes known to be involved in invasion of the intestinal mucosa. I put forth evidence demonstrating that Sal4, at concentrations previously shown to prevent bacterial entry into epithelial cells, is a potent inhibitor of both *S. Typhimurium* flagellum-based motility and SPI-1-mediated entry into host cells. These data suggest a possible mechanism to explain Sal4’s protective capacity in vitro and in vivo and challenge our previous assumptions about how secretory antibodies interfere with microbial pathogenesis at mucosal surfaces.
RESULTS

Sal4 is neither bacteriostatic nor bacteriocidal.

Michetti and colleagues previously reported that incubation of S. Typhimurium with Sal4 for 2 hr at 37°C had no direct impact on bacterial viability (Michetti et al., 1992). Although these data indicate Sal4 is not bacteriocidal, it was not determined whether Sal4 is bacteriostatic. Therefore, I performed viability assays in which strain JS107 was cultured in LB containing Sal4 at concentrations ranging from 0.15-20 µg/ml. I assessed viability by measuring CFUs at hourly time points in multiple experiments.

As expected, treatment of JS107 with Sal4 promoted agglutination that was first evident between 1.25 and 2 hours of incubation, and increased over time. Agglutination caused bacteria to form clumps at the bottom of the culture flasks, and thereby interfered with our ability to monitor growth by optical density. Despite agglutination of JS107, there was no significant difference in growth as determined by CFUs, when compared to JS93, an isogenic derivative of JS107 which lacks the Sal4 epitope. This was observed in rich medium (LB), while in minimal media or PBS the same has been observed for time up to an hour. From these results, I conclude that Sal4 is neither bacteriostatic or bacteriocidal for S. Typhimurium.

Sal4 arrests flagellum-based motility of S. Typhimurium, independent of agglutination.

The mucus overlying the intestinal epithelium is one of the first barriers encountered by S. Typhimurium during the infection process and is a site where SIgA antibodies have been proposed to entrap enteric pathogens (Phalipon and Corthesy, 2003). I used a standard soft agar motility assay as a model system in which to examine
the effects of Sal4 on the ability of *S.* Typhimurium to migrate through a semi-viscous medium (Adler, 1966). Stab inoculation of wild-type *S.* Typhimurium strain JS107 into 0.3% LB agar containing a control IgA antibody, TEPC-15, resulted in measurable migration (~8 cm) of the bacteria away from the initial site of inoculation over a period of 8 h (Fig. 3.1). The addition of Sal4 (5 µg/ml) to the medium resulted in a ~60% reduction in bacterial migration (Fig. 3.1). This effect on motility was specific, as revealed by the fact that the migration of *S.* Typhimurium strain JS93 was unaffected by Sal4 in this assay (data not shown). Strain JS93 is an isogenic derivative of JS107 which, due to a null mutation in the *oafA* gene, lacks the O5 epitope recognized by Sal4 (Slauch et al., 1995).

While the results of the soft agar motility assays described above suggested that Sal4 was capable of impairing *S.* Typhimurium cells’ ability to swim, I were unable to discern whether this was a direct effect on flagellum-based motility or simply the result of antibody-induced agglutination based on bacterial concentration. To distinguish between these two possibilities, I used video light microscopy to visualize and quantitate the motility of individual bacteria in the absence and presence of Sal4 under nonagglutinating conditions. By video light microscopy, I observed that wild-type *S.* Typhimurium strain JS107 demonstrated normal swimming behavior, which consisted of “runs” (directional forward movement for ~1 s) and “tumbles” (~0.1-s period of random reorientation) (Berg, 2003). In a typical overnight culture, approximately 75% of the bacteria were motile, while subcultures typically had greater motility.
Figure 3.1 Measurement of bacterial migration in agar motility assays.

Strain JS107 was stab inoculated into 0.3% LB agar containing TEPC-15 (5 µg/ml) or Sal4 (5 µg/ml) and incubated at 37°C for 8 hours. The diameter (cm) of the migration was measured at hourly intervals. The results shown are the average of two plates.
Exposure of S. Typhimurium strain JS107 to TEPC-15 (1 to 15 µg/ml) had no measurable effect on bacterial motility or swimming behavior. Neither did isotype controls 2D6, IgAC5, or 23D7 (data not shown). In contrast, within minutes of the addition of Sal4 (1 to 5 µg/ml), S. Typhimurium stopped swimming (Fig. 3.2). Sal4-mediated motility arrest was both time and dose dependent. Sal4-treated bacteria appeared “paralyzed” and were phenotypically indistinguishable from a S. Typhimurium nonmotile mutant (data not shown). These bacteria were, however, subject to Brownian motion, indicating that they were not somehow tethered by Sal4 to the surface of the slide or coverslip, any more than the controls. Finally, the antibody’s effects were antigen specific, because the motility of JS93, which lacks the O5 epitope due to a mutation in the oafA gene, was unchanged upon treatment with Sal4 (Fig. 3.2).

To address the possibility that these effects were specific to strain JS107 (a derivative of ATCC 14028), I examined the outcome of Sal4 exposure on the parental ATCC14028 and other S. Typhimurium O5 serotypes. Sal4 arrested flagellum-based motility of all O5 strains tested, including two common laboratory strains, SL1344 and LT2, as well as five clinical isolates which were obtained from the Bacteriology Laboratory at the Wadsworth Center (data not shown).

Interestingly, a S. Typhimurium cheA mutant which swims constitutively due to a mutation in the chemotaxis signaling pathway (Jones et al., 1992) was halted upon exposure to Sal4, in both ATCC14028 and SL1344 backgrounds, revealing that Sal4’s effects occur independent of chemotaxis.
Figure 3.2

Sal4 arrests motility of *S. Typhimurium* in a dose- and time-dependent manner. Wild-type *S. Typhimurium* (strain JS107) or an *oafA* mutant (strain JS93) was mixed with Sal4 at indicated concentrations, spotted on glass microscope slides, and imaged by video light microscopy at the indicated time points. Motility was expressed as percent of control: (% of motile bacteria in experimental sample / % motile bacteria in control samples) x 100. Control samples were treated with TEPC-15 (10 µg/ml) for 15 min, as described in the Materials and Methods. Each value represents the average (with standard error) of 4-6 independent video recording. Asterisks indicate a statistically significant (P<0.01) reduction in motility, as compared to TEPC-15 treated bacteria.
To investigate whether motility arrest is a general response of *S. Typhimurium* bacteria to anti-LPS antibodies, bacteria were treated with commercially available polyclonal rabbit anti-O antiserum (group B factors 1, 4, 12, and 27 or factors 1, 4, 5, and 12), which is used routinely in clinical laboratories for serotyping. As shown in Table 3.1, *S. Typhimurium* strain JS107 stopped swimming within minutes of being treated with rabbit anti-O antibodies. No difference was seen between antiserum types. These data reveal two items of interest: first, that motility arrest can be caused by IgG as well as IgA; second, that motility arrest can occur with antibodies against epitopes other than the O5 antigen.

Anti-O antiserum was as effective as anti-H (i.e., antiflagellum) antiserum in inhibition of motility (Table 3.1). It should be noted that, even at the light-microscopic level, the mechanisms by which these antibody preparations immobilized *S. Typhimurium* could be seen to differ. Bacteria exposed to anti-H antiserum underwent a “twitching phase” prior to motility arrest. This twitching phase likely represents movement in which flagellum rotation is partially impaired due to flagellum cross-linking and/or bundling (Berg and Anderson, 1973). In contrast, bacteria treated with Sal4 or anti-O antisera became almost instantaneously immobilized in the absence of any detectable twitching or aberrant swimming behavior. These observations suggest that the mechanisms by which anti-H and anti-O antibodies alter *S. Typhimurium* motility are in fact distinct.

The fact that Sal4 is polymeric in nature (i.e., primarily a dimer) seemed of little consequence with respect to its ability to interfere with *S. Typhimurium* motility, as anti-
Table 3.1

**Effect of anti-LPS and anti-flagellum antibodies on S. Typhimurium motility**

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>TEPC-15</th>
<th>Sahl</th>
<th>Anti-O</th>
<th>Anti-H</th>
<th>F(ab')2</th>
<th>Fab</th>
<th>Fab plus IgG</th>
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<tr>
<td>1-2</td>
<td>+++</td>
<td>+</td>
<td>+</td>
<td>++/+</td>
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*a* Bacteria (~1 x 10⁸ per ml) were mixed with antibodies (this was designated time zero) and immediately spotted (10 µl) onto a glass microscope slide, mounted with a coverslip, and visualized by light microscopy. Antibodies were used at a final concentration of 5 µg/ml, except for TEPC-15, which was at 10 µg/ml. Motility was assessed by light microscopy and scored as follows: **+++**, more than 60% of the bacteria were motile; **++**, between 30 and 60% of the bacteria were motile; **+**, less than 30% of the bacteria were motile; **-**, none of the bacteria were motile.

*b* Anti-H antiserum reacts with both phase 1 and 2 flagellins.

*c* Bacteria were exposed to Fab fragments (5 µg/ml) for 2 min and then mixed with rabbit anti-goat IgG (H+L) immediately prior to being spotted onto glass microscope slides.
LPS IgG antibodies (which consist exclusively of monomers) were equally capable at arresting bacterial swimming. However, both IgG and IgA are bivalent molecules capable of binding to two adjacent O-antigen side chains and thereby are potentially cross-linking LPS molecules. To determine the importance of LPS cross-linking in motility arrest, I performed bacterial motility assays in the presence of monovalent antigen binding fragments (Fab fragments). Fab fragments were generated from rabbit polyclonal anti-LPS antisera rather than Sal4, because murine IgA Fab fragments are unstable due to the lack of an interchain (H+L) disulfide bond (Michetti et al., 1994). At 5 µg/ml, Fab fragments had a slight effect on bacterial motility, whereas IgG antibodies at the same concentration completely stopped bacterial movement (Table 3.1). However, when anti-LPS Fab fragments (5 µg/ml) were incubated with S. Typhimurium and then artificially cross-linked by the addition of goat antirabbit polyclonal antibodies, bacterial motility was arrested almost instantaneously (Table 3.1). Monovalent Fab fragments were capable of fully inducing arrest, but only at concentrations of ~15 µg/ml. As expected, anti-LPS F(ab)_2 fragments, which retain bivalent binding activity but lack the IgG Fc region, were as effective as whole IgG antibodies in arresting motility (Table 3.1). I conclude that cross-linking enhances, but is not essential for, the ability of anti-LPS antibodies to arrest S. Typhimurium motility.

I wished to examine the possibility that the mechanism by which Sal4 arrests the motility of S. Typhimurium involves physical (“steric”) interference in flagella rotation (i.e., the Fc region of Sal4 may extend beyond the LPS and physically impede flagella rotation). This was accomplished by testing the effect of Sal4 on the motility of a S. Typhimurium Δrfc strain. Whereas LPS in wild-type S. Typhimurium cells consists of 20
to 35 O-antigen repeats and is ~20 to 30 nm in length, the LPS of the Δrfc strain has only a single O-antigen unit and extends ~6 nm from the outer membrane. As a dimeric IgA, Sal4 would be expected to be ~28 nm from one end to the other and would not project sufficiently far from the cell surface that it could effectively interfere either with the flagella filament or the hook (Boehm et al., 1999). Indeed, the motility of the Δrfc strain was impaired by the addition of Sal4 or anti-LPS Fab fragments (data not shown). These and other data (see Discussion) suggest that it is highly unlikely that Sal4, when bound to LPS on the surface of S. Typhimurium cells, physically interferes with flagellum rotation.

Sal4 inhibits S. Typhimurium invasion of epithelial cells, even when the requirement for motility is bypassed through centrifugation.

Motility is required for S. Typhimurium cells to gain entry into epithelial cells in vitro, as evidenced by the fact that nonmotile mutants are 20 to 50 times less invasive than are wild-type controls (Jones et al., 1992). Therefore, Sal4’s effect on S. Typhimurium’s flagellum-based motility might account for the previously reported capability of Sal4 to inhibit bacterial invasion of polarized epithelial cell monolayers (Michetti et al., 1994). This possibility is testable, as the requirement for motility in the invasion process can be overcome through the use of centrifugation to artificially promote bacterium-host cell contacts (Jones et al., 1992). If Sal4 acts solely by interfering with the motility of S. Typhimurium cells, then I would expect that centrifugation of Sal4-treated bacteria onto polarized epithelial cell monolayers would restore invasion to levels observed for untreated bacteria. On the other hand, if Sal4 interferes with another
step in the entry process, then I would expect that Sal4-treated bacteria would remain noninvasive, even when contact with host cells is enhanced by centrifugation.

I used a competitive in vitro invasion assay (see Materials and Methods) to distinguish between these two possibilities. Using this method, I confirmed that Sal4 reduced the ability of \( S. \) Typhimurium bacteria to invade polarized epithelial cell monolayers by more than 10-fold (Fig. 3.3A). Identical results were obtained whether invasion assays were performed under bacteria-agglutinating conditions (data not shown), as done previously by Michetti and colleagues (Michetti et al., 1994), or under nonagglutinating conditions (Fig. 3.3A). I then introduced centrifugation into the invasion assay protocol. Under these conditions, Sal4-treated bacteria attached to epithelial cells as well as control bacteria (Fig. 3.4). However, in spite of the addition of centrifugation, Sal4-treated \( S. \) Typhimurium remained noninvasive (Fig. 3.3A). To ensure that the centrifugation protocol was sufficient to restore the invasiveness of a nonmotile mutant of \( S. \) Typhimurium, as originally reported by Jones and colleagues (Jones et al., 1992), I performed invasion assays using an \( motB \) mutant. The \( motB \) strain of \( S. \) Typhimurium assembles flagella normally on the cell surface, but these flagella are unable to rotate due to a defect in the motor protein. As expected, the \( motB \) mutant was highly attenuated for entry into polarized epithelial cell monolayers, and this defect could be overcome by centrifugation (Fig. 3.3B). From the results of these experiments, I conclude that Sal4, in addition to inhibiting flagellum-based motility, affects a step downstream of attachment in the SPI-1-dependent entry of \( S. \) Typhimurium bacteria into epithelial cells.
Figure 3.3 Sal4 blocks S.Typhimurium invasion of epithelial cells, even when the requirement for motility is bypassed through centrifugation. (A) Wild-type (strain JS107) and oafA mutant (strain JS93) of S.Typhimurium were mixed 1:1, incubated for 15 min with 5 µg/ml TEPC-15 (shaded bars) or Sal4 (black bars), before being applied to MDCK II cell monolayers in 96-well microtiter plates, and subjected (or not) to brief centrifugation, as described in Materials and Methods, so as to promote (or not promote) bacterium-epithelial cell contact. The results shown are the average values (with standard error) from three independent experiments, each done in triplicate. Asterisks indicate a statistically significant ($P < 0.05$) reduction in invasion in the presence of Sal4, as compared to the TEPC-15 control.

(B) Centrifugation enhances uptake of a non-motile mutant of S. Typhimurium. Wild-type and a non-motile motB strain of S.Typhimurium were mixed 1:1 before being applied to MDCK II cell monolayers in 96 well microtiter plates, and subjected (or not)
to brief centrifugation. The results shown are the average values (with standard error) from three independent experiments, each done in triplicate. Asterisks indicate bacterial invasion in the presence of centrifugation was significantly ($P \leq 0.05$) greater than in the absence of centrifugation.
Figure 3.4 Sal4 does not impede S. Typhimurium attachment to epithelial cells. Wild type or ΔSPI1 strains of S. Typhimurium were mixed with the oafA mutant at a 1:1 ratio, incubated without antibody (white bars) or with Sal4 (5 μg/ml; black bars) for 15 min, applied to HeLa cells seeded in 96 well microtiter plates, and then subjected to centrifugation (10 min at 4°C) to promote bacteria-epithelial adherence. Immediately thereafter, the HeLa cells were washed to remove unbound bacteria, and then lysed with 1% Triton-X 100. The number of bacteria in the lysates (CFU) was determined by serial dilution onto LB. The competitive index (CI) reflects the attachment of wild type or the ΔSPI1 strains to HeLa cells relative to the oafA mutant. Sal4 marginally enhanced attachment of both wild type (P=0.06) and the ΔSPI1 (P=0.02) S. Typhimurium strains to HeLa cells, possibly due to antibody-mediated agglutination that may have occurred during centrifugation.
While these data strongly suggested that agglutination by Sal4 is not a determinant in the blockage of bacterial entry, I could not exclude the possibility that some antibody-mediated cross-linking occurred on the surfaces of epithelial cells immediately following centrifugation. To address this concern, I performed invasion assays in the presence of Fab fragments derived from polyclonal anti-LPS IgG antiserum, as described above. I found that monovalent Fab fragments (14 µg/ml) blocked \( S. \) Typhimurium entry into epithelial cells as effectively as did Sal4 (5 µg/ml) or anti-LPS F(ab)2 (5 µg/ml) (Fig. 3.5). The extent of invasion observed following antibody treatment was similar to that observed for an \( \Delta \)SPI-1 strain, which lacks the SPI-1 T3SS and effector proteins (Ellermeier et al., 2005). These data demonstrate conclusively that agglutination is not integral to Sal4’s capability to block \( S. \) Typhimurium entry into epithelial cells.

Sal4 interferes with SPI-1-dependent, but not SPI-1-independent, entry of \( S. \) Typhimurium into host cells.

\( S. \) Typhimurium entry into intestinal epithelial cells is strictly dependent on the SPI-1 T3SS and its effector proteins (Galan and Wolf-Watz, 2006). In contrast, bacterial uptake by phagocytic cells, such as macrophages, is fundamentally different; it occurs by host cell-mediated phagocytosis and does not require SPI-1. I wished to examine whether Sal4 blocks SPI-1-independent, as well as SP-1-dependent, uptake of \( S. \) Typhimurium into host cells. This was accomplished by means of a J774 macrophage invasion assay (Buchmeier and Heffron, 1989). Whereas Sal4 blocked \( S. \) Typhimurium invasion of epithelial cells (Fig. 3.3), Sal4 did not interfere with bacterial entry into J774 cells.
fact, I observed that under agglutinating conditions, Sal4 slightly enhanced bacterial entry into J774 cells (Fig. 3.6), possibly because the macrophages were able to engulf bacteria-antibody aggregates. It should be noted that J774 cells do not express the recently described Fcα/μ receptor (Shibuya et al., 2000). From these data, I concluded that Sal4 inhibits SPI-1-dependent, but not -independent, entry of S. Typhimurium bacteria into host cells.
Figure 3.5 Anti-LPS antibodies inhibit invasion of *S. Typhimurium* into epithelial cells, independent of bacterial agglutination. *S. Typhimurium* strain JS107 was incubated with TEPC-15 (5 µg/ml), Sal4 (5 µg/ml), polyclonal anti-LPS F(ab)2 (5 µg/ml) or Fabs (14 µg/ml) for 15 min, and then applied to HeLa cells seeded in 96 well microtiter plates. The non-invasive ΔSPI1 strain was used in parallel as a control. Due to the reactivity of polyclonal anti-O antibodies with the *oafA* strain of *S. Typhimurium*, I were unable to perform competitive invasion assays in this experiment. Therefore, invasion is expressed as % of initial inoculum. The data shown are the average values (with standard error of the mean) from a single representative experiment done in triplicate. Asterisks indicate a statistically significant (P< 0.05) reduction in invasion in the presence of Sal4, as compared to the TEPC-15 control.
Figure 3.6 S. Typhimurium uptake by murine macrophages in the presence of Sal4.

A 1:1 mixture of wild type and oafA strains of S. Typhimurium were incubated with Sal4 (5 µg/ml), TEPC-15 (5 µg/ml), or PBS, and then applied to J774 cells seeded in 96 well microtiter plates. The competitive index (CI), which reflects the ratio of wild type S. Typhimurium to the oafA strain that were taken up into J774 cells, was determined as described in Materials and Methods. The results shown are from a single representative experiment done in quadruplicate. The asterisk indicates that Sal4 enhanced (P≤0.05), uptake of S. Typhimurium into macrophages, as compared to TEPC-15 treated bacteria, possibly due to the fact that Sal4 was used under agglutinating conditions in this assay.
DISCUSSION

Despite the recognized importance of anti-LPS SIgA antibodies in preventing the colonization and invasion of epithelial cells by enteric pathogens (Apter et al., 1993b; Brandtzaeg, 2007; Chowers et al., 2007; Iankov et al., 2002a; Michetti et al., 1992; Michetti et al., 1994; Phalipon et al., 2002; Phalipon et al., 1995; Winner et al., 1991), the underlying mechanisms by which these antibodies function in mucosal immunity remains unclear. In this study, I have demonstrated that Sal4, an anti-LPS monoclonal IgA that was previously shown to protect mice against oral challenge with *S. Typhimurium* bacteria, is a potent inhibitor of *S. Typhimurium* flagellum-based motility. Sal4’s inhibitory effects were rapid (<15 min) and occurred independently of bacterial agglutination. The concentration of Sal4 (3 to 5 µg/ml) required to arrest motility is identical to what Michetti and colleagues previously reported as being necessary to block *S. Typhimurium* invasion of intestinal epithelial cells (Michetti et al., 1994). In addition to its effect on motility, I also propose that Sal4 interferes with SPI-1-mediated type three secretion. This conclusion is based primarily on our observation that Sal4-treated *S. Typhimurium* cells were unable to invade epithelial cells in vitro, even when the requirement for motility in the invasion process was obviated by centrifugation of antibody treated bacteria directly onto host cells. While the underlying mechanism by which Sal4 interferes with these two microbial processes (i.e., flagellum-based motility and type three secretion) remains to be elucidated, the data presented in this study reveal a previously unrecognized capacity of SIgA to potentially “disarm” enteric pathogens in mucosal secretions and thereby prevent colonization and invasion of the intestinal epithelium.
The capacity of anti-LPS IgA and IgG antibodies to interfere with the flagellum-based motility of S. Typhimurium is an observation that, to our knowledge, has not been previously described in the literature and which may potentially have important implications for understanding microbial physiology, as well as mucosal immunity (as discussed below). While anti-LPS antibodies have been used for decades in clinical and diagnostic laboratories for serotyping members of the Salmonella, the end point of these assays is typically agglutination, which is measured macroscopically. In this study, the effect of Sal4 on S. Typhimurium motility was only revealed by light microscopy and when the ratio of antibody-to bacteria was adjusted so as to minimize agglutination. However, an effect of anti-LPS antibodies on flagellar motility of an enteric pathogen is not completely unprecedented. It has been recognized for more than 20 years that anti-LPS antiserum interferes with the motility of V. cholerae (Fuerst and Perry, 1988; Gustafsson and Holme, 1985). V. cholerae differs from S. Typhimurium in that it has a single, polar flagellum that is sheathed with LPS. It has generally been assumed, in the case of V. cholerae, that anti-LPS antibodies interfere with motility by coating the flagellum and impeding rotation by physical drag or cross-linking (Fuerst and Perry, 1988; Gustafsson and Holme, 1985). This cannot be the case for S. Typhimurium bacteria, as their flagella (4 to 6 per cell) are not sheathed and are not recognized by anti-LPS antibodies.

A known mechanism for loss of motility can occur by antibody mediated cross-linking of the flagella. It has been demonstrated that flagella can tolerate monovalent antibody binding (equivalent to four times the mass of the flagella) to the filament without detectable loss of motility (Greenbury and Moore, 1966). Immobilization of the
filaments and arrest motility of 50% of a culture of \textit{S. Typhimurium} requires $\sim 2 \times 10^2$ antibodies per bacterium (Greenbury and Moore, 1966). This implies that for direct interaction with the flagella numerous cross-links by the antibodies is needed. I propose the $\sim 10^4$ antibodies binding to the LPS have a low chance of binding around the flagella in sufficient numbers to have enough indirect interactions with the hook or filament to result in loss of motility.

How does Sal4 (and anti-LPS antibodies in general) interfere with \textit{S. Typhimurium} flagellum-based motility? Theoretically, it is unlikely that Sal4, when bound to the O antigen, sterically interferes with flagellum rotation. \textit{S. Typhimurium} flagella each consist of a basal body spanning the inner and outer membranes, a hook, and a helical filament (Berg, 2003; Ilino, 1974). The flagellar hook is a highly flexible structure $\sim 55$ nm in length, while the filament is up to $10 \mu$m long and can assume different polymorphic forms in response to elastic strain (Berg, 2003; Turner et al., 2000). As a dimeric immunoglobulin, Sal4 is nominally 28 nm from one end to the other and would not be expected to project sufficiently far from the cell surface that it could effectively interfere with either the hook or filament (Boehm et al., 1999; Woof and Kerr, 2006). Fab fragments, which also affected motility (albeit at relatively high concentrations), are $\sim 7$ nm in length. This theoretical argument is supported by the fact that Sal4 impaired the motility of an $\Delta rfc$ strain of \textit{S. Typhimurium}, whose severely truncated O-antigen extends less than 6 nm from the outer membrane.

I estimated the number of antibodies that were required per bacterium to cause these results. I estimated that at 5 $\mu$g per ml of Sal4, a concentration sufficient to cause rapid arrest in motility, $\sim 1\%$ of the LPS molecules were occupied by IgA. This number
was derived as follows. Sal4 has a molecular mass of ~315 kDa, consisting of two IgA monomers (150 kDa each) plus J chain (15 kDa). Therefore, 1 µg of Sal4 is roughly equivalent to $1.9 \times 10^{12}$ IgA molecules (Harlow and Lane, 1988). Motility assays were done in 10 µl volumes, which at a Sal4 concentration of 1 µg/ml is equal to $1.9 \times 10^{10}$ total IgA molecules. There was $1 \times 10^7$ CFU of S. Typhimurium used per assay. Assuming there are $3.5 \times 10^6$ LPS molecules per bacterium (Nikaido, 1996), then there are approximately $3.5 \times 10^{13}$ LPS molecules in the assay sample. From this I estimated that ≤1% of the LPS molecules were occupied by IgA at a concentration of 5 µg/ml. However, this could be an overestimate considering that each LPS molecule has multiple (16-35) O-antigen repeats which may be accessible to Sal4 (Nikaido, 1996). It could also be an underestimate, as each IgA has four antigen binding sites, which could result in 2 to 4% of the LPS molecules bound by Sal4. It seems unlikely that Sal4 bound to only a small fraction of LPS molecules on the cell surface could impede the rotation of the flagella, especially when one considers that a single flagellum generates enough torque to rotate an entire bacterium (Berg, 2003; Turner et al., 2000).

I found that monovalent Fab fragments were less effective than IgGs at arresting motility of S. Typhimurium, as evidenced by the fact that higher concentrations of Fabs (i.e., 10 µg/ml) were required to stop bacterial movement to the same degree as IgGs as a lower concentration (i.e., 5 µg/ml). On an equimolar basis, taking into account the differences in molecular masses between Fabs and IgGs (50 kDa vs. 150 kDa), I estimate that Fabs were approximately six times less effective than IgGs. One interpretation of this data is that cross-linking of LPS induced by bivalent IgGs is involved in mediating motility arrest. In support of this possibility, I observed that artificially cross-linking
Fabs with anti-Fab antibodies caused an immediate arrest in *S. Typhimurium* motility. While I favor this interpretation of the data, I cannot exclude the possibility that the difference in effectiveness between Fabs and IgGs pertains to antibody avidity, that number of antigen binding sites affects the relative affinity of overall molecule for the antigen. The bivalent nature of IgG enables a single antibody molecule to associate with two antigen binding sites, whereas a monovalent Fab can only associate with one antigen binding site. Discerning between these possibilities would require a collection of monoclonal antibodies of varying affinity and avidities for *S. Typhimurium* LPS.

Does the association of Sal4 with the O antigen trigger a signaling cascade that could account for motility arrest? While I expect that one or more so-called extracytoplasmic stress responses (ESR) in *S. Typhimurium* may be induced as a consequence of Sal4 binding to the cell surface, there is little evidence in the literature to suggest that the activation of an ESR could be the cause of flagellar dysfunction. Cpx and σ^E^ are the two major ERs in *S. Typhimurium*: Cpx is induced by high pH, misfolded periplasmic proteins, and abnormalities in the inner membrane, whereas σ^E^ is triggered by heat, ethanol, unfolded outer membrane proteins, and abnormal LPS (Alba and Gross, 2004; Rowley et al., 2006; Ruiz et al., 2006). Although there is a connection between these ERs and flagellar assembly/biosynthesis, there is no known direct link between these regulons and control of flagellum rotation (Lin et al., 2008). Therefore, while binding of Sal4 to LPS could result in downregulation at the transcriptional level of the flagellar operons, it is unlikely that the activation of either Cpx or σ^E^ is directly responsible for the antibody-mediated motility arrest observed in this study.
While it is largely speculative at this point, I propose that the proton motive force (PMF) may be the link between Sal4, motility arrest, and inhibition of SPI-1-mediated entry into epithelial cells. In *S. Typhimurium*, the PMF is the primary transmembrane proton electrochemical gradient across the inner membrane and is determined by both the transmembrane electrical potential and proton gradient (Berg, 2003). Flagellum based motility is entirely dependent on the PMF, to the degree that flagellar motor speed varies linearly with the PMF gradient (Gabel and Berg, 2003). In the presence of an intact PMF, the flagella are constantly rotating in a counterclockwise or clockwise manner, such that at any point in time *S. Typhimurium* cells are either running or tumbling, respectively (Berg, 2003). “Paralysis” of *S. Typhimurium* cells (as observed following Sal4 treatment) occurs only upon dissipation of the PMF (Berg, 2003). There is also increasing evidence that the export of effector proteins through T3SSs, such as SPI-1, requires an intact PMF (Galan, 2008; Wilharm et al., 2004). While it is unknown at this time how Sal4, through its interaction with LPS, might affect bacterial bioenergetics, I postulate that the antibody may induce physical and/or mechanical stress on the bacterial envelope. It is known that physical/mechanical alterations in the curvature or tension of the bacterial envelope can perturb cytoplasmic homeostasis and result in reductions in bioenergetic potential (Booth et al., 2007). It should be underscored that Sal4 is neither bacteriostatic nor bactericidal, even in the presence of complement (Michetti et al., 1992). Therefore, any effects on *S. Typhimurium* physiology induced following antibody association with the outer leaflet of the outer membrane are certainly transient. This is in contrast to bactericidal IgG and IgM antibodies directed against certain surface-exposed outer membrane proteins of
Borrelia species (Connolly and Benach, 2005; LaRocca et al., 2008) and Haemophilus influenza (Nelson et al., 1988).

The possibility that Sal4 may affect the PMF is not completely unfounded. It is well-established that factors that perturb the OM of E. coli and other Gram-negative bacteria cause a reduction in PMF, although the mechanism(s) by which this occurs remains unknown (Darwin, 2005). Another line of evidence consistent with the possibility that Sal4 may interfere with the PMF is that Sal4 also blocks S. Typhimurium invasion of epithelial cells, possibly due to the inability of S. Typhimurium to secrete effector proteins through the SPI-1 TTSS. In Yersinia entercolitica motility and type three-dependent secretion are dependent on PMF (Wilharm et al., 2004).

The results presented in this study are in accordance with Michetti and colleagues’ original conclusion that Sal4 alone can prevent the adherence and invasion of S. Typhimurium to epithelial cells in the absence of other immune or nonimmune protective mechanisms (Michetti et al., 1992; Michetti et al., 1994). While these authors noted that Sal4’s protective effects correlated with bacterial agglutination, they were careful to point out that agglutination per se was unlikely to explain the antibody’s observed activity. I now propose that Sal4’s protective effects are more likely due to the antibody’s ability to interfere with motility and SPI-1 type three secretion. Indeed, anti-LPS SIgA antibodies are known to be protective against virtually all enteric pathogens, including Shigella flexneri, Escherichia coli, and Vibrio cholerae (Apter et al., 1993b; Chowers et al., 2007; Iankov et al., 2002a; Michetti et al., 1992; Michetti et al., 1994; Phalipon et al., 2002; Phalipon et al., 1995; Winner et al., 1991), and the results of this
and other studies (Peterson et al., 2007) challenge us to rethink the long-held assumption that SIgA is primarily a physical barrier separating host and microbe.
INTRODUCTION

In Chapter 3, I demonstrated that Sal4 inhibits both Salmonella enterica serovar Typhimurium flagella-based motility, and invasion of epithelial cells, in vitro. The decrease in invasion was equivalent to that of an attenuated SPI-1 mutant, which lacks the type three secretion system (T3SS) and effector proteins necessary for invasion of host cells. Based on these findings, I hypothesize that Sal4 may cause a reduction in T3SS, which would account for the loss of invasion. The flagellar apparatus and the T3SS are structurally and functionally similar. I made a second hypothesis, that the loss of motility, and the proposed decrease of type three secretion (T3S), were both based on an interruption of the bacterial bioenergetics.

In S. Typhimurium, the chromosomally encoded SPI-1 type-3 secretion system T3SS is responsible for bacterial invasion of the epithelium (Cossart and Sansonetti, 2004). The T3SS consists of a basal body spanning the inner membrane (IM) and the outer membrane (OM), and a needle-like structure that protrudes from the OM (Blocker et al., 2003). Contact of the needle with the membranes of host cells results in the formation of a translocation pore in the host membrane, through which an array of effector proteins are injected into the host cell cytoplasm. This results in cytoskeletal rearrangements and macropinocytosis of the bacteria (Cossart and Sansonetti, 2004; Galan and Wolf-Watz, 2006).
The *S. Typhimurium* translocase complex (SipB, SipC, and SipD) forms a pore in the host cell membrane. Pore formation is dependent on the presence of cholesterol in the host cell membrane, with which SipB and SipC interact (Cossart and Sansonetti, 2004). The pore can remain in the host cell membrane, even after macropinocytosis of the bacteria. Pore formation can be used as an indicator of T3S, as in the hemolysis assay using sheep blood, where the leakage of hemoglobin is measured (Field et al., 2008).

A second model of a protective anti-LPS antibody and a bacterial species is the antibody IgAC5 against the O-antigen of *Shigella flexneri*. The antigen recognized by IgAC5 is in the O-antigen repeat of the LPS, the subunits of which consist of a tri-rhamnose (rha)-N-acetyl glucosamine (N-ag) tetrasaccharide backbone, with a glucose side chain. IgAC5 was produced by oral immunization of mice with *S. flexneri* serotype 5a (Phalipon et al., 2002; Phalipon et al., 1995). IgAC5 was shown to provide protection to challenged mice (Phalipon et al., 1995), and has also been shown to be sufficient to protect rabbits against oral challenge with *S. flexneri* (Boullier et al., 2009). Although IgAC5 alone is sufficient to confer mucosal immunity to *S. flexneri*, the mechanism by which this occurs remains unknown. IgAC5 was shown to be neither bacteriocidal nor bacteriostatic, at least *in vitro* (Phalipon et al., 1995).

Invasion of epithelial cells by *S. flexneri* also involves a T3SS, which is encoded on a 220 kb virulence plasmid, pWR100 (Cossart and Sansonetti, 2004; Sansonetti et al., 1986). It is estimated that there are approximately 20 T3SS per cell (West et al., 2005). The needle of the T3S extends ~60 nm beyond the OM (West et al., 2005). The needle is almost twice the length of the lipopolysaccharide (LPS) layer (35 nm), and increase in the O-antigen length is associated with decreased invasion (West et al., 2005). Upon contact
with epithelial cells, effector proteins are injected into the host cell cytoplasm, resulting in cytoskeletal rearrangements and macropinocytosis of *S. flexneri*, similar to the effects observed upon interaction of *S. Typhimurium* with host cells (Cossart and Sansonetti, 2004; Galan and Wolf-Watz, 2006). The T3SS is critical in the invasion process, as strains of *S. flexneri* lacking pWR100 are non-invasive (Sansonetti et al., 1982; Sansonetti et al., 1986). Unlike *S. Typhimurium*, the T3SS of *S. flexneri* can be induced, free from cell contact, by exposure of the bacteria to the dye Congo red (Bahrani et al., 1997; Parsot et al., 1995).

The *S. flexneri* T3SS secreted effector proteins, the so-called invasion plasmid antigens, or Ipas, have multiple roles in the invasion process (Ogawa et al., 2008; Schroeder and Hilbi, 2008). Initially, the T3SS injects IpaB and IpaC into epithelial cells forming a translocation pore in the host cell membrane, in an IpaD regulated manner (Picking et al., 2005). IpaB binds to CD44 on the host cell membranes (Cossart and Sansonetti, 2004). Translocon pore formation is followed by secretion of IpaA and IpgD, IpgE, IpgF into the host cytoplasm, which are involved in actin rearrangements and formation of the macropinocytic pocket (Cossart and Sansonetti, 2004). IpaA, IpaB, IpaC, and IpaD are homologues of SipA, SipB, SipC, and SipD from Salmonella (Hermant et al., 1995). Only a fraction of the total Ipas are required for invasion, as a mutant carrying a polar insertion in *icsB* had reduced Ipa protein expression, but retained wild type levels of invasion (Mounier et al., 2009).

Both the flagellar apparatus and the T3SS are structurally and functionally similar. Motility and T3S are both driven by the same energy source, the proton motive force (PMF). The PMF (Δp) is a localized transmembrane proton electrochemical
gradient that occurs across the IM, and consists of the proton gradient ($\Delta p\text{H}$) and the electrochemical potential ($\Delta \Psi$). PMF is defined as $\Delta \Psi - 2.3RT \Delta p\text{H}/F$, where $R =$ gas constant, $T =$ temperature, $F =$ Faraday’s constant (Kadner, 1996). The PMF is typically -180 mV in aerobically growing cells (Kadner, 1996). In *E. coli* and *S. Typhimurium* there are measurable physiologic effects associated with loss of PMF, including a decrease in $\Delta \Psi$ and $\Delta p\text{H}$, the inability to grow on succinate as sole carbon source, and a decrease in ATP levels (Kinoshita et al., 1984). Proton ionophores such as carbonyl cyanide m-chlorophenylhydrazone (CCCP) that dissipate the PMF cause an immediate arrest in motility (Berg, 2003) and disable T3SS (Wilharm et al., 2004). ATP also plays a role in T3S through the separation of the effector protein from the chaperone prior to secretion (Akeda and Galan, 2005; Minamino and Namba, 2008).

I demonstrated in Chapter 3 that Sal4 inhibited *S. Typhimurium* SPI-1 mediated invasion of epithelial cells, independent of agglutination. Here, I present data which indicates that Sal4, and IgAC5, are rapid and potent inhibitors of T3S, a bacterial process required for invasion of intestinal epithelial cells. Although I have not discerned the exact mechanism by which this occurs, the results described here suggest that the reduction of T3S is the consequence of an antibody-mediated disruption in bioenergetics, possibly caused by physical and/or mechanical stress due to antibody binding to the LPS.
RESULTS

Neither Sal4 nor IgAC5 interferes with bacterial attachment to epithelial cells.

In the previous chapter, I demonstrated that Sal4 does not interfere with S. Typhimurium attachment to epithelial cells. This indicated that the protective effect of this monoclonal IgA must occur at a step downstream in the invasion process (Forbes et al., 2008). To determine if IgAC5 blocked attachment of S. flexneri to epithelial cells, I compared the ability of S. flexneri strain M90T to adhere to MDCK cells in the absence and presence of IgAC5. M90T was incubated with IgAC5, or a control monoclonal IgA, for 15 minutes, then applied to MDCK cell monolayers grown in 96-well microtiter plates. To promote bacteria-host cell contact, the microtiter plates were briefly centrifuged, as done previously (Forbes et al., 2008). The MDCK cells were washed to remove unbound bacteria, and the remaining cells were treated with Triton-X to lyse the epithelial cells, but not the bacterial cells. The cell lysates were then plated on LB agar and CFUs were enumerated. When centrifugation was included in the assay, there was no difference in attachment of S. flexneri treated with IgAC5 compared to the isotype control (Fig 4.1A). To exclude the possibility that the centrifugation was overriding an effect by IgAC5, I repeated these experiments without centrifugation. There was no difference in bacterial attachment, regardless of the presence of IgAC5 (Fig 4.1B). These assays were repeated with HeLa cells and similar results observed (data not shown). Therefore, similar to my experiments with Sal4 and S. Typhimurium, IgAC5 did not interfere with S. flexneri attachment to host cells.

Additionally, it should be noted that the avirulent strain of S. flexneri, BS176, adhered to MDCK cell monolayers as well as the wild type strain. These data indicate
that the genes on the virulence plasmid, which includes the T3SS, are not necessary for attachment to epithelial cells. To investigate if the bacteria need to be energetically active to adhere to host cells, the strain M90T was treated with the proton ionophore CCCP. A concentration of 100 µM was used, as this dose of CCCP is sufficient to completely dissipate the PMF (Minamino and Namba, 2008; Paul et al., 2008). CCCP treatment did not alter adhesion, as similar results were obtained for untreated control cells (Fig 4.1B). This finding demonstrated that bacterial energetics were not required for adhesion.

Sal4 inhibits T3S in S. Typhimurium.

I next addressed if Sal4’s protection of epithelial cells (Michetti et al., 1994), previously shown to be involved in a decrease in invasion, in Chapter 3 (Forbes et al., 2008), may be caused by a decrease in SPI-1 mediated T3S. In S. Typhimurium, T3S can be measured by formation of a translocation pore in the cellular membrane of erythocytes. The subsequently released hemoglobin in the supernatant can be measured by spectrophotometry (Field et al., 2008; Picking et al., 2005). The wild type S. Typhimurium strain ATCC14028, or the isogenic oafA mutant strain SJF12 were used. The oafA strain lacks the Sal4 epitope due to a mutation in the gene encoding the enzyme required for acetylation of the O-antigen on the abequose residue (Slauch et al., 1995). The bacteria were incubated for 15 minutes with Sal4, or the isotype control antibody 23D7, then added to 50% defribriated sheep whole blood for 30 minutes.
Figure 4.1 IgAC5 does not block *S. flexneri* adhesion to epithelial cells.

*S. flexneri* strain M90T was incubated with IgAC5 (5-10 µg/ml) for 15 min before being applied to MDCK II cells seeded in 96 well microtiter plates, and then centrifuged at 4°C for 10 minutes to promote bacteria-epithelial adherence (A), or without centrifugation (B). The MDCK II cells were lysed with 1% Triton-X 100 and the bacterial numbers were determined by the serial dilution of lysates onto LB. MAbs 23D7 and TEPC-15 were used as isotype controls. The proton ionophore, CCCP (100 µM), was used to determine the role of PMF in adhesion. The strain BS176 was included as a noninvasive control. The results shown are the average values (with standard error) from an experiment done in triplicate.
The level of hemolysis for the control condition was considered 100%, and all other data obtained was normalized to this value. Erythrocytes exposed to the Sal4-treated wild type strain released ~ 75% less hemoglobin compared to bacteria treated with a control IgA (Fig. 4.2). In contrast, Sal4 had no effect on the ability of the oafA strain to promote hemolysis (Fig. 4.2). These results suggest that Sal4 prevented the formation of translocation pores in the erythrocyte membranes, blocking the initial steps of SPI-1 T3S necessary for the translocation pore formation.

**IgAC5 inhibits *S. flexneri* T3S of Ipa effector proteins.**

Since Sal4 treatment reduced SPI-1 mediated T3S in *S. Typhimurium*, I next addressed if IgAC5 exerted similar effects on *S. flexneri*. The *S. flexneri* T3SS can be induced gratuitously, in the absence of host cells, by the addition of the dye, Congo red, to the bacterial cultures (Bahrani et al., 1997; Parsot et al., 1995). Congo red triggers the T3SS-dependent delivery of the invasion-associated effectors proteins (i.e., IpaB and IpaC) into the supernatant, which can be measured by Western blot analysis (Bahrani et al., 1997; Parsot et al., 1995).

To test the possibility that IgAC5 decreased the activity of the *S. flexneri* T3SS, wild type strain M90T was incubated for 15 minutes, in the presence or absence of IgAC5, prior to addition of the Congo Red for 15 minutes at 37°C. Western blot analysis confirmed that exposure of strain M90T to Congo red resulted in the secretion of IpaB, IpaC, (Fig. 4.3) and IpaD (data not shown). Treatment of *S. flexneri* with IgAC5 (2-10 µg/ml), prior to Congo red exposure, reduced IpaB secretion, as only a residual amount
Figure 4.2 Sal4 prevents Salmonella induced red blood cell hemolysis.

The wild type (ATCC14028) or isogenic derivative strain SJF12 (oafA::Tn10d-Tc) of S. Typhimurium was incubated for 15 min with Sal4 (5 µg/ml) before being added to 50% sheep whole blood (defibribrated) in a 96 well plate for 30 minutes at 37°C. Two volumes of PBS were then added to each well, and the plate was subjected to centrifugation for 10 minutes at 4°C to pellet blood cells and bacteria. The supernatants were removed and the absorbance at OD$_{550}$ nm measured. MAb 23D7 was used an isotype control. Hemoglobin leakage through the translocation pores is expressed as percent of the control. The results shown are the average values (with standard error) from an experiment done in triplicate.
of IpaB was detected in culture supernatants, and most of the IpaB was retained in the bacterial pellet (Fig. 4.3A, B). Treatment of *S. flexneri* with an isotype control antibody, TEPC-15, had a negligible effect on IpaB secretion (Fig. 4.3A, B, D). Similar results were observed with other isotype control monoclonal antibodies (MAbs), Sal4 (Fig. 4.3C), 2D6, and 23D7 (data not shown). As a negative control for the T3S experiments, *S. flexneri* were treated with the proton ionophore, CCCP. There was no (or limited) detection of Ipa secretion from bacteria treated with CCCP (Fig. 4.3A, B, D-G).

IgAC5’s inhibited T3S activity in a dose dependent manner (Fig. 4.3C, D). Antibody concentrations of 2-5 µg/ml were the minimum required for the reduction of T3S, as at 0.5 µg/ml and 1 µg/ml IgAC5, there was no visible difference in IpaB present in culture supernatants, compared to the Sal4 control (Fig. 4.3C). IgAC5 concentrations of greater than 5 µg/ml reduced both IpaC (Fig. 4.3D) and IpaD (data not shown) secretion.

To investigate whether the loss of secretion was a general response of *S. flexneri* to anti-LPS antibodies, strain M90T was treated with rabbit polyclonal anti-O antiserum (Group 1-6), which consists predominantly of IgG antibodies. Anti-O antiserum was as effective as IgAC5 in blocking secretion IpaB and IpaC (Fig. 4.3D, G). IpaC secretion at a dose of 2.5 µg/ml was only faintly detectable, and was no longer observed at 5 µg/ml (Fig. 4.3D). Since anti-LPS IgG antibodies were as effective as IgAC5 in reducing T3S, these findings demonstrate that the polymeric nature of IgAC5, primarily a dimer, was not important in imparting this activity. I estimate that on molar basis, adjusted for the fact that IgA is more than twice the mass of IgG (315 kDa vs. 150 kDa), that IgAC5 and the anti-O antiserum were equally effective.
Figure 4.3 IgAC5 inhibits secretion of Ipa proteins by *S. flexneri*.

(A-B) Mid-log phase cultures of *S. flexneri* M90T were exposed to the following treatments, as described in Materials and Methods; no treatment (lane 1); Congo red (lane 2); TEPC-15 (5 µg/ml) and Congo red (lane 3); IgAC5 (2 µg/ml) and Congo red (lane 4); IgAC5 (5 µg/ml) and Congo red (lane 5); 100 µM CCCP and Congo Red (lane 6).

Following these treatments, the bacterial culture supernatants (A) or cell pellets (panel B) were subjected to Western blot analysis and immunoblotted with MAbs directed against IpaB proteins. (C) Dose-dependent inhibition of IpaB secretion by IgAC5. Cultures of *S. flexneri* M90T were incubated with the indicated concentrations of IgAC5, or Sal4 as a isotype control, for 15 min prior to the addition of Congo red. IpaB levels were assessed in the culture supernatants by Western blot, as described above. (D) Ipa secretion is inhibited by polyclonal O-antigen antibodies. Cultures of *S. flexneri* M90T were incubated with the indicated concentrations of IgAC5, TEPC-15 as a negative isotype control, rabbit anti-O antisera, or CCCP, for 15 min prior to the addition of Congo red. IpaC levels were assessed as described above. (E-F) Time dependence of IgAC5 on Ipa secretion is transient. Cultures of *S. flexneri* M90T were incubated with IgAC5 (10 µg/ml), anti-O antisera (10 µg/ml), or CCCP (100 µM), for 45 min (E) or 90 min (F) prior to the addition of Congo red. Culture supernatants were collected and subjected to immunoblotting with an anti-IpaC MAb. CCCP was the negative control. (G) Dose-dependent inhibition of IpaB secretion by CCCP. Bacteria were exposed to CCCP at 10-fold dilutions for 15 min, and then treated with Congo red. Culture supernatants were collected and subjected to immunoblotting with anti-IpaB MAb.
To determine the duration of the antibody-induced disruption of T3S, *S. flexneri* was treated with IgAC5 (10 µg/ml) for 45 and 90 minutes prior to the addition of Congo red. At both time points, IpaC secretion was largely normal ([Fig. 4.3E, F](#)), and resembled the secretion observed from cultures treated with an isotype control antibody (data not shown). IpaC secretion was reduced when *S. flexneri* was treated with rabbit polyclonal anti-O antiserum for 45 minutes ([Fig. 4.3E](#)), but had returned to normal levels 90 minutes after treatment ([Fig. 4.3F](#)). Strain M90T treated with CCCP had little IpaC present in the culture supernatants at 45 minutes ([Fig. 4.3E](#)), and even less after 90 minutes ([Fig. 4.3F](#)). These results suggested that the effects of IgAC5 and anti-O antiserum on the T3S of *S. flexneri* were transient.

**Reduction in PMF correlates with loss of T3S.**

Bacteria were treated with the proton ionophore CCCP, which is known to dissipate the bacterial PMF (Wilharm et al., 2004), to determine at what concentration T3S was affected. The concentration of CCCP used as a control in the majority of the assays was 100 µM, as 10 µM-50 µM is usually sufficient to abolish PMF (Minamino and Namba, 2008; Paul et al., 2008). I examined Ipa secretion following treatment of *S. flexneri* with a range of CCCP concentrations, including those known to dissipate PMF in *E. coli* and *S. Typhimurium* (Minamino and Namba, 2008; Paul et al., 2008). As the concentration of CCCP increased, T3S decreased in a dose dependent manner ([Fig. 4.3G](#)). Concentrations of CCCP between 0.01 to 0.1 µM had no visible effect on T3S. At 1 µM T3S was visibly reduced, and at 10 µM or 100 µM completely abolished. These
data indicated that a decrease in T3S could occur with diminished PMF, and did not require complete ablation of the PMF.

**Association of Sal4 and IgAC5 with the bacterial OM is associated with a reduction in bacterial electrical potential.**

I hypothesized that the reduction in T3S observed following treatment of *S. Typhimurium* and *S. flexneri* with Sal4 and IgAC5, respectively, may be due to a reduction in the bacterial PMF. To test this hypothesis, JC-1, an electrosensitive dye, was used as an indicator of electrical potential ($\Delta \Psi$). JC-1 has been used in Gram-negative bacteria previously, including *S. Typhimurium* (Becker et al., 2005; Jovanovic et al., 2006). JC-1 forms J-aggregates in cells with an intact membrane potential, but is a monomer in cells lacking an electrochemical gradient (Smiley et al., 1991). As an aggregate, the dye emits at 590 nm, fluorescing red, whereas as a monomer, the dye emits at 529 nm, fluorescing green.

In these experiments, *S. Typhimurium* was loaded with JC-1 and then incubated with Sal4 for 15 minutes. The ratio of red (intact potential) to green (depleted potential) bacteria was determined by fluorescence microscopy, and the untreated control was set arbitrarily to 100%. Sal4 treatment of wild type *S. Typhimurium* for 15 minutes caused a ~40% decrease in the number of bacteria with an intact electrical potential ($\Delta \Psi$) (Fig. 4.4A). EtOH (10%), a concentration determined to inhibit growth and reduce viability, was used as a control that provided partial loss of the electrical potential in the bacterial population (Fig. 4.4A). This reduction was similar to that observed when the bacteria were treated with Sal4. Treatment of *S. Typhimurium* with CCCP (100 µM) completely
depleted the electrical potential, as there were no bacteria with red fluorescent in these samples (Fig. 4.4A). These data suggested that the association of Sal4 with the O-antigen of S. Typhimurium results in a reduction in the bacterial electrical potential.

To determine if IgAC5 had a similar effect on the S. flexneri electrical potential, similar studies with JC-1 were preformed. For these experiments, I first constructed a strain of S. flexneri that contained a deletion of the tolC gene, herein referred to as SJF31. This strain is an isogenic derivative of the wild type strain M90T. The tolC gene encodes an outer membrane protein (OMP) involved in the efflux of small molecules (Murata et al., 2007). As JC-1 has a MW of 532, it was potentially a target for TolC export, as is EtBr (Murata et al., 2007). Indeed, I found that the retention time of JC-1 in S. Typhimurium was significantly enhanced in a tolC mutant (a gift from Hiroshi Nikaido), as compared to the wild type control (data not shown).

Furthermore, I developed a microtiter plate-based method to assess JC-1 emission in a population of cells, as a substitute for the microscopy based method described above. This method allows for more samples to be simultaneously examined, as well as time course studies that were not possible by microscopy. To validate this methodology, strain SJF31 was loaded with JC-1, then dispensed into black polystyrene 96 well plates with optical bottoms, and incubated with a range of concentrations of CCCP, for 15 minutes. Fluorescence was measured using dual excitation (485/20, 530/25) and dual emission spectra (528/20, and 590/35) with a BioTek Synergy HT. The 530/590 ratio of the untreated control was set arbitrarily to 100%, and the difference from the control
Figure 4.4 Sal4 and IgAC5 decrease the electrical membrane potential of 
*S. Typhimurium* and *S. flexneri*, respectively.

(A) Mid-log phase cultures of *S. Typhimurium* were loaded with the dye JC-1 and then 
treated with Sal4 (5 µg/ml), ethanol (10%) or CCCP (100 µM) for 15 minutes. The 
samples were then visualized immediately by fluorescence microscopy, as described in 
Materials and Methods. The number of red (intact potential) and green (depleted 
potential) fluorescent bacteria in each sample was determined. The values obtained from 
the untreated control were arbitrarily set to 100%. Each column is the average value (with 
standard error) obtained from a minimum of three experiments. The number of cells in 
each treatment were: control n=1005; Sal4, n=867; EtOH, n=899 EtOH; CCCP, n=77.

(B) A mid-log phase culture of *S. flexneri* strain SJF31 was loaded with the dye JC-1, 
and then loaded into black polystyrene 96 well plates with optical bottoms, before 
treatment with the indicated concentrations of CCCP for 15 min. The fluorescence of the 
wells was then measured using a fluorimeter at dual excitation (485/20, 530/25) and dual 
emission (528/20, and 590/35). The 530/590 ratio was determined as in the standard 
usage (see Materials and Methods). The fluorescent ratio of untreated control cells was 
set arbitrarily to 100%, and the difference from the control expressed as a decrease. The 
results are the average values (with standard error) from an experiment done in triplicate.

(C) *S. flexneri* strain SJF31 was loaded with JC-1, treated with IgAC5 (5 or 10 µg/ml) or 
CCCP (0.1-100 µM) for 15 min, and then analyzed at indicated time points (2, 15 and 30 
min) using a fluorimeter, as previously described.

Significance, as determined using the Student’s *t*-test, is indicated by asterisks: * P < 
0.05, ** P < 0.01, *** P < 0.001.
expressed as a decrease, as described in Materials and Methods. The fluorescent ratio obtained for increasing concentrations of CCCP from 0.01 to 100 µM showed a dose dependent decrease in ΔΨ (Fig. 4.4B). Even at the lowest concentration tested, 0.01 µM, there was a significant decrease compared to the control (Fig. 4.4B). CCCP at 10 µM caused an 80% reduction, and 100 µM ablated the electrical potential (Fig. 4.4B). This experiment demonstrated that CCCP caused a concentration dependent decrease in the number of cells with an intact electrical potential, and validated use of the plate-based method to assess electrical potential in a population of cells.

The effect of IgAC5 on the electrical potential of S. flexneri was then determined using this microtiter plate-based method. Strain SJF31 was loaded with JC-1, then treated with IgAC5 (5 or 10 ug/ml), and fluorescence was monitored over a period of 30 minutes, with measurements at 2, 15, and 30 minutes. At 2 minutes, the bacteria treated with IgAC5 were significantly reduced in ΔΨ, compared to untreated control samples; at IgAC5 concentrations of 5 µg/ml and 10 µg/ml, the ΔΨ values were 6% and 30% lower, respectively (Fig. 4.4C). However, there was no further reduction in the ΔΨ in samples treated with 5 ug/ml of IgAC5, whereas there was a slight reduction over time in samples treated with 10 ug/ml of IgAC5 at 20 and 30 minutes. As observed in Fig. 5B, treatment of the bacteria with CCCP (0.1-100 µM) caused a dose-responsive reduction in PMF. Samples treated with 1 µM and 10 µM of CCCP had ΔΨ values decreased 9% and 25%, respectively, compared to the control; while at 100 µM of CCCP, samples were reduced to 58% lower than the control at ~2 minutes (Fig. 4.4C). These results demonstrated that the IgAC5 induced decrease in ΔΨ was rapid, occurring within 2 minutes of antibody exposure, and was a sustained decrease in ΔΨ. Additionally, there was a strong similarity
between the 10 µg/ml IgAC5 and 10 µM CCCP at all time points. At 2 minutes, the 70% decrease of the 10 µg/ml IgAC5 sample was similar to the 75% decrease of the 10 µM CCCP treatment. Likewise, at 15 minutes, the 58% decrease of the 10 µg/ml IgAC5 sample was similar to the 63% decrease of the 10 µM CCCP treatment (Fig. 4.4C). This was consistent with the results from Fig. 4.4B, that 10 µM CCCP decreased electrical potential by ~80% at 15 minutes. These results demonstrated a similarity in IgAC5 and CCCP induced decrease in ΔΨ.

The reduction in PMF induced by treatment of the bacteria with 10 µg/ml of IgAC5 was comparable to that observed following treatment of the cells with 10 µM CCCP, over the course of the experiment (Fig 4.4C). This concentration of CCCP completely inhibits T3S (Fig. 4.3). These data demonstrated that Sal4 and IgAC5 exerted similar effects on the electrical potential in S. Typhimurium and S. flexneri, respectively. These findings suggest a causal link between the association of these monoclonal antibodies with the bacterial O-antigens and a reduction in T3S.

**Anti-LPS antiserum causes a reduction in ΔpH.**

To determine if anti-LPS antibodies caused a reduction in the ΔpH component of the PMF, as there was a reduction in the ΔΨ, the dye BCECF (2′, 7′-bis-(carboxyethyl)-5-(and-6)-carboxyfluorescein), a fluorescent indicator used to measure intracellular pH over a range of pH 6.2-9.5, was used in this study. The ratio of fluorescence emission intensities was calculated using the maximum of 535 nm and minimum of 620 nm, generated by an excitation wavelength of 488 nm (Balkay et al., 1997). A pH titrated standard curve was generated using nigericin to equilibrate the pH of the cytoplasm with
that of the buffer. Nigericin is a polyether ionophore that catalyzes the electroneutral exchange of the alkali metal K\(^+\) for H\(^+\) by antiport exchange (Guffanti et al., 1979), which disrupts the membrane potential (Eytan et al., 1990; Rottenberg and Scarpa, 1974), and facilitates the diffusion of ions across membranes (Ahmed and Booth, 1983; Guffanti et al., 1979). The pH of the *E. coli* cytoplasm is estimated to range between 7.4 and 7.8 (Kadner, 1996; Kinoshita et al., 1984). Compared to a bacterial cell with an intact membrane, a permeabilized cell would have a decreased fluorescence in a low pH buffer, or an increased fluorescence in a high pH buffer.

The *S. flexneri* strain, SJF31, was treated with polyclonal anti-O antiserum, CCCP, or nigericin in a phosphate buffer, at pH 7.4 or pH 9.6, containing 100 mM KCl. At pH 7.4 all treatments caused an increased fluorescence (Fig. 4.5). While these results were unexpected, they suggest that the acidic condition used to load the dye had lowered the cytoplasmic pH. At pH 9.6, cells treated with nigericin, CCCP or anti-O antiserum had increased fluorescence, as compared to the untreated controls, and these levels were greater than those observed at pH 7.4 (Fig. 4.5). As my IgAC5 supply was limited, this assay was developed using anti-O antiserum, which was commercially available. These results suggest that exposure of *S. flexneri* to polyclonal anti-O antiserum affected the permeability of the cell envelope, thereby impacting both the \(\Delta p\)H component, as well as the \(\Delta \Psi\) component, of the PMF.
Figure 4.5 Anti-O antiserum decreased the ΔpH of *S. flexneri*.

*S. flexneri* strain M90T was loaded with the dye BCECF then diluted 1:10 into pH 7.4 or 9.6 phosphate buffer in optical bottom black polystyrene 96 well plates. M90T was incubated with anti-O antiserum (10 μg/ml), nigericin (10 mM), or CCCP (100 μM), for 15 minutes. The fluorescence was measured using a fluorimeter with an excitation wavelength of 485/20 and dual emissions at 528/20, and 645/40. The ratio of emission values, 530/645, are representative of internal pH. The results shown are the average values (with standard error) from an experiment done in triplicate.
Sal4 and IgAC5 decrease ATP production.

ATP plays a role in T3S by the separation of the effector protein from the chaperone prior to secretion (Akeda and Galan, 2005). Therefore, a reduction in the cellular ATP pool would be expected to result in a decrease in T3S. As a drop in the ΔΨ component of PMF was observed following both Sal4 and IgAC5 treatment (Fig. 4.4 A, C), I postulated that there may be a concomitant reduction in ATP levels. Media containing glucose or succinate can be used to determine the way energy is produced in the cell. In minimal medium containing glucose, the bacteria can perform glycolysis to produce ATP by substrate level phosphorylation, in the absence of PMF. In contrast, in minimal medium containing succinate, the bacteria produce lower levels of ATP in the absence of PMF. This occurs by utilizing succinate as the sole carbon source, which requires the TCA cycle and electron transport chain.

To test this hypothesis, S. Typhimurium or S. flexneri were washed and resuspended in minimal media containing glucose or succinate, with or without antibodies, and incubated for 15 minutes. Following incubation, bacteria were transferred to white polystyrene 96 well plate, and 100 µl of BacTiter-Glo was added. ATP levels were determined by luminescence on a SpectraMax L luminometer, and measured using the photon counting mode. Luminescence was normalized to that of the 23D7 antibody control. S. Typhimurium treated with Sal4, in minimal medium containing glucose, had the same level of ATP as the control at 2 minutes, and ~30% lower levels of ATP than the control at minutes 10 to 30. At 40 minutes post exposure there was no difference from control levels of ATP. The CCCP control had similarly reduced ATP levels, ~30% at minutes 2 to 30, and was restored to control levels at 40 minutes (Fig.
4.6A). In minimal medium containing succinate, the difference in ATP levels for Sal4 and CCCP, compared to the control, was similar to minimal media containing glucose. The CCCP treated sample in succinate differed in that ATP levels remained reduced at 40 minutes (Fig. 4.6B).

*S. flexneri* treated with IgAC5, in minimal medium containing glucose, had the same level of ATP as the control at 2 minutes, and ~10% lower levels of ATP at minutes 10-30, but returned to control levels at 40 minutes. The CCCP control reduced ATP levels by ~50% at minutes 2 to 40 (Fig. 4.6C). In minimal medium containing succinate, the difference in ATP levels of IgAC5 treated samples, compared to the control levels, was 20-30% at minutes 10 to 40, while CCCP samples had a reduction of >30% at all time points (Fig. 4.6D).

The duration of the disruption in ATP levels in *S. flexneri* is consistent with the period of decrease in ΔΨ, and the loss of T3S and recovery. The ΔΨ levels are reduced at minutes 2 to 30 (Fig. 4.4C), preceding the reduction in ATP at 10 to 30 minutes after IgAC5 treatment (Fig. 4.6C). The *S. flexneri* T3S is decreased at minutes 15 to 45 (Fig. 4.3). The ΔΨ levels were not determined after 30 minutes, and ATP levels were recovered by 40 minutes (Fig. 4.6C), except in medium containing succinate (Fig. 4.6D). Likewise, T3S was the same as the control at 60 min. (Fig. 4.3). Therefore, ΔΨ, ATP, and T3S were all affected and show recovery in a similar time frame. While these results showed a correlation between bioenergetics and T3S, it was not necessarily causation.
Figure 4.6 ATP levels in *S. Typhimurium* and *S. flexneri* decline following treatment with Sal4 and IgAC5.

(A-B) Sal4 was added to *S. Typhimurium* and ATP was measured by luminescence (see Materials and Methods) over the course of 40 minutes. *S. Typhimurium* were treated with Sal4 (5 µg/ml), isotype control antibody 23D7 (5 µg/ml), or CCCP (100 µM) in minimal media with glucose (A) or succinate (B) as the carbon source.

(C-D) IgAC5 was added to *S. flexneri* strain M90T and ATP was measured by luminescence as above. *S. flexneri* were treated with IgAC5 (10 µg/ml), 23D7 (5 µg/ml), or CCCP (100 µM) in minimal media with glucose (C) or succinate (D) as the carbon source.

Significance by t-test is indicated by asterisks: * P < 0.05, ** P < 0.001.
The effect of antibodies on invasion of epithelial cells *in vitro.*

As both Sal4 and IgAC5 inhibited T3S (Fig. 4.2, 4.3), thus decreasing the ability of the bacteria to inject effector proteins into the host cell, which are necessary for invasion, this should result in decreased invasion. While it has been shown that Sal4 treated *S. Typhimurium* are attenuated for invasion of epithelial cells *in vitro* (Forbes et al., 2008), the role of antibodies on Shigella invasion of epithelial *in vitro* is less clear, and the role of IgAC5 on invasion *in vitro* has not previously been described. To determine the role of IgA in *S. flexneri* invasion of epithelial *in vitro,* I preformed standard gentamycin protection assays (Michetti et al., 1994). Treatment of *S. Typhimurium* (Fig. 4.7A) or *S. flexneri* (Fig. 4.7B) with CCCP was utilized as a control. *S. flexneri* strain M90T was treated with 5 µg/ml of IgAC5, under standard conditions to minimize agglutination. There was no difference in the invasion of M90T, compared to the control, and invasion was significantly higher than the non-invasive control strain, BS176 (Fig. 4.7C).

I next examined the ability of *S. flexneri* strain M90T to invade epithelial cells under a range of antibody concentrations, corresponding to increasing agglutination, as observed by microscopy. At 5 µg/ml of IgAC5, with standard conditions for minimizing agglutination, there was no difference in invasion of M90T relative to the untreated control, but there was significantly higher invasion than the avirulent control strain, BS176 (Fig. 4.7C). An increase in the antibody concentration to 10 µg/ml or 25 µg/ml was associated with an increase in invasion, by 2 and 4 fold, respectively. Invasion peaked at 25 µg/ml, and then decreased at 50 µg/ml. The IgAC5 concentration of 50
Figure 4.7.

**Figure 4.7.** CCCP inhibits *S. Typhimurium* and *S. flexneri* invasion, while IgAC5 did not inhibit *S. flexneri* invasion of epithelial cells.

**(A)** CCCP treated wild type *S. Typhimurium* were incubated for 15 minutes, centrifuged and resuspended in fresh PBS, then applied to HeLa cells seeded in 96 well microtiter plates. The non-invasive ΔSPI-1 strain was as a control. Due to the toxicity of the CCCP, the bacteria had to be washed prior to addition to the HeLa cells. Invasion is expressed as % of initial inoculums.

**(B)** *S. flexneri* were treated as the *S. Typhimurium* in (A). The non-invasive BS176 was used as a control. Invasion is expressed as % of initial inoculums.
(C) Increasing levels of IgAC5 were used to promote agglutination of *S. flexneri*. The non-invasive BS176 was used in parallel as a control. All strains diluted 1:10, as described in Material and Methods.

(D) IgA, SIgA, and IgG were antibody isotypes were used to assess invasion of *S. flexneri*. The non-invasive BS176 was used in parallel as a control. All strains diluted 1:100 to prevent agglutination, as described in material and methods.

The results shown are the average values (with standard error) from experiments each done in triplicate. Statistically significant reduction in invasion was determined using the Student’s *t*-test, is indicated by asterisks: * P ≤ 0.05, ** P ≤ 0.01, *** P ≤ 0.001.
µg/ml had double the number of invasive bacteria than the control, and invasion was comparable to that of 10 µg/ml. Thus, small amounts of agglutination likely resulted in the increased chance of invasion of a small groups of bacteria, dependent on one bacteria making contact with the epithelial cells, and injecting effector proteins.

As there was no protection against *S. flexneri* invasion of epithelial cells with IgAC5, a dimeric IgA, under both subagglutinating conditions or agglutinating conditions, I examined the ability of secretory IgA (SIgA), or anti-O antiserum (predominantly IgG) to confer protection. To test this hypothesis, IgAC5, with or without secretory component, was used in invasion assays. The ability of *S. flexneri* strain M90T treated with of 5 µg/ml of IgAC5, SIgAC5, anti-O antiserum, or isotype control antibody 23D7, to invade epithelial cells was not different compared untreated M90T, and was significantly higher than the avirulent control strain, BS176 (Fig. 4.7D). These results indicated that the structural differences of the antibodies, IgA, SIgA, or IgG, did not result in protection *in vitro*. As IgAC5 is a protective antibody *in vivo*, yet does not possess corresponding protection in *in vitro* invasion assays, Shigella-HeLa interactions are not a good model system, as it does not mirror *in vivo* protection.

**CCCP causes a loss of motility in S. Typhimurium.**

As Sal4 reduces PMF and ATP production, similar to CCCP, I hypothesized that motility would also be affected in a similar manner. CCCP treated *S. Typhimurium* rapidly lost motility in 100 µM CCCP, a concentration which depletes the PMF. *S.Typhimurium* had decreased motility in 10 µM CCCP, which decreases the PMF.
(Table 4.1). These data demonstrated that motility was sensitive to the decrease in PMF, and not just the complete depletion of the PMF.

IgAC5 does not interfere with growth or viability.

IgA is not known to be bacteriocidal or bacteriostatic (Michetti et al., 1992; Michetti et al., 1994; Phalipon et al., 1995). Sal4 is not bacteriocidal nor bacteriostatic when *S. Typhimurium* is grown in rich medium (Michetti et al., 1992), which I have confirmed previously (Forbes et al., 2008). Also, IgAC5 is neither bacteriocidal nor bacteriostatic, which I confirmed under our assay conditions. Treatment of M90T with 10 µg/ml IgAC5 results in no significant difference in viability in PBS as determined by colony forming units over 45 minutes, or growth measured by optical density every 30 minutes for 3 hours in LB, compared to an isotype control (data not shown). From these results I conclude that IgAC5 is not bacteriocidal nor bacteriostatic against *S. flexneri*. This implied that the IgA mediated decrease in T3S is associated neither with a loss of viable bacteria, nor a decreased growth rate. It also implied that the PMF is decreased, not eliminated, as in the case of treatment with CCCP, which reduces PMF, ATP and viability.
Bacteria (~1 x 10⁸ per ml) were mixed with CCCP (this was designated time zero) and immediately spotted (10 µl) onto a glass microscope slide, mounted with a coverslip, and visualized by light microscopy. Motility was assessed and scored as follows: ++++, more than 60% of the bacteria were motile; ++, between 30 and 60% of the bacteria were motile; +, less than 30% of the bacteria were motile; -, none of the bacteria were motile.

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DISCUSSION

Despite the importance of SIgA as a first line of defense on mucosal surfaces, the fundamental mechanisms by which these antibodies interfere with bacterial colonization and invasion of epithelial cells are not well understood. In this study, I have demonstrated that two protective anti-O antigen specific monoclonal IgAs, Sal4 and IgAC5, interfere with the activity of the T3SS in *S. Typhimurium* and *S. flexneri*, respectively, that are required for bacterial entry into epithelial cells. I propose that the underlying cause of the observed decrease in T3S is due to antibody-induced reduction in PMF. This conclusion is based on the observation that the decrease in PMF coincides with the decrease in T3S, and that PMF is necessary to drive T3S (Galan, 2008; Wilharm et al., 2004).

First, I demonstrated that Sal4 interferes with the formation of *S. Typhimurium* SPI-1 T3SS-dependent translocation pores in erythrocytes, suggesting the antibody blocks the initial stages of T3S. The conclusion is in agreement with my observation in Chapter 3, that Sal4 treatment was sufficient to reduce *S. Typhimurium* invasion into epithelial cells to the level of a SPI-1 mutant, which lacks the SPI-1 encoded T3SS and effector proteins. Sal4 is neither bacteriocidal nor bacteriostatic, which demonstrates that the observed IgA-mediated decrease in T3S is not the result of a loss of viability, or a decrease in growth rate. If lysis had occurred, there should have been evidence of ATP or effector proteins in the assay supernatants, but this was not observed, indicating that the IM was not permeable to ATP or proteins following Sal4 treatment. This is not to say that the IM may have become permeable to protons (see below).
Next, I presented evidence that IgAC5 is a potent inhibitor of T3S in *S. flexneri*. Secretion of IpaB, IpaC, and IpaD by *S. flexneri* in a cell free secretion assay was reduced upon the treatment of the bacteria with IgAC5 (5-10 µg/ml) for 15 minutes. IgAC5’s inhibitory activity occurred in a dose-dependent manner, as antibody concentrations of 2-5 µg/ml only minimally reduced IpaB secretion into culture supernatants, while concentrations of 5 to 10 µg/ml reduced IpaB, IpaC, and IpaD to virtually undetectable levels. IgAC5 is neither bacteriocidal nor bacteriostatic, which demonstrates that the observed IgA-mediated decrease in T3S is not the result of a loss of viability, or a decrease in growth rate. If lysis had occurred, there should have been an increase in effector proteins in the supernatant; yet I observed a decrease in effector protein concentrations. The inhibition of T3S was not IgA specific, as polyclonal anti-O antiserum against *S. flexneri* was as effective as IgAC5 in blocking secretion of IpaB and IpaC. These data also reveal that the polymeric nature of IgAC5 (*i.e.*, primarily a dimer) was of little importance in imparting this activity, as polyclonal monomeric IgG antibodies had the same effect on secretion as IgAC5. Finally, the affect of IgAC5 on *S. flexneri* T3S was transient, as normal levels of IpaC secretion were observed at 45-90 minutes post IgAC5 treatment.

While IgAC5 caused a significant reduction in Ipa secretion as determined by in the cell free secretion assay, the antibody did not block *S. flexneri* invasion of epithelial cell *in vitro*. The same results were obtained following treatment of the bacterial with polyclonal anti-O antiserum. This is in contrast to what was observed with Sal4 and *S. Typhimurium*, in which Sal4 was sufficient to block bacterial invasion of epithelial cells (Chapter 3). I propose that this apparent inconsistency is due to the fact that *S. flexneri*
produces high concentrations of Ipa effector proteins. While the IgAC5 treatment dramatically reduced T3S, the cells may still secrete sufficient levels of effector proteins to promote invasion epithelial cells in vitro. This would agree with a report by Mournier et. al. who demonstrated that a strain of S. flexneri carrying a polar insert in icsB produced significantly fewer Ipas than a wild type strain, yet invaded epithelial cells at normal levels (Mounier et al., 2009). Moreover, it is well established in the literature that S. flexneri invasion assays in vitro do not reflect invasion in vivo (Hale and Bonventre, 1979). In contrast, treatment of S. flexneri with CCCP reduced Ipa secretion and epithelial cell invasion. CCCP has multiple effects on the cell physiology; therefore CCCP’s effects on invasion may not be due solely to a reduction in Ipa secretion.

Chowers et. al. recently reported that treatment of S. flexneri with anti-O antiserum decreased bacterial adhesion and invasion of epithelial cells in vitro (Chowers et al., 2007). My findings contradict this report, as I found that neither IgAC5 nor anti-O antiserum decreased the ability of S. flexneri to adhere to or invade epithelial cells. I attribute these differences to the fact that Chowers et. al. utilized much higher concentration of antiserum than I did. These high concentrations would be expected to results in the formation of large antibody-bacterial immune complexes. Large aggregates, >5 µm in diameter, would not be up taken by macropinocytosis (Sun et al., 2003).

In this chapter, I observed a correlation between the reduction in ΔΨ, and the simultaneous loss of motility, T3S, and ATP levels in S. Typhimurium, following Sal4 treatment. Similar effects were observed when S. flexneri was treated with IgAC5. I propose that the PMF may be the link between Sal4 binding to the LPS, and the observed
reductions in T3S, motility, and ATP levels. Flagella-based motility in *S. Typhimurium* is driven entirely by the PMF (Berg, 2003; Gabel and Berg, 2003; Paul et al., 2008). As such, motility is an extremely sensitive indicator of cellular PMF. The PMF is determined by both the transmembrane electrical potential (ΔΨ) and proton gradient (ΔpH) (Kadner, 1996). In this chapter, I detected a decrease in the ΔΨ. However, it is known that there is a membrane potential across both the IM (typically -180 mV), and OM (-38 mV) (Kadner, 1996; Nikaido, 1996; Nikaido, 2003). Therefore, I cannot rule out that part of the change I detected in ΔΨ using the dye JC-1 is associated with a perturbed OM potential. JC-1 fluorescence does not discriminate between the two membrane potentials. Another caveat of this study is that a decrease in ΔΨ may not necessarily result in a decrease in the PMF, as an increase in ΔpH can compensate for changes in ΔΨ (Kadner, 1996). Unfortunately, without a practical method to simultaneously measure both ΔΨ and ΔpH, the effect of the antibodies on PMF cannot be determined.

However, additional evidence supports a model in which the PMF across the IM is disturbed in *S. Typhimurium* and *S. flexneri*, following antibody treatment. An antibody mediated decrease in the ΔpH was detected. This indicated the disruption of the IM PMF, as there is thought to be only the ΔΨ across the OM, as the OM is permeable to protons (Nikaido, 1996; Nikaido, 2003). Furthermore, the transient decrease in ATP levels indicates a defect at the IM; though this could be a decrease in PMF, a disruption in the TCA cycle, or the electron transport chain. Since the secretion of effector proteins through T3SS requires an intact PMF (Galan, 2008; Minamino and Namba, 2008; Wilharm et al., 2004), the IgA mediated decrease in PMF resulting in the loss of T3S and invasion, is an attractive model.
Following antibody treatment, *S. Typhimurium* and *S. flexneri*, in minimal media containing glucose have transiently lower levels of ATP (30-40 min). In minimal media containing succinate, Sal4 treated *S. Typhimurium* also had transiently lower levels of ATP. In contrast, IgAC5 treated *S. flexneri* were not observed to regain control levels of ATP during the 40 minute experiment. The duration of the disruption in ATP levels is consistent with the period of decreased $\Delta \Psi$ and the loss of T3S, as well as the recovery of both ATP levels and T3S.

It has been shown that a PMF is not needed for the growth of *E. coli* if glucose is present at pH 7.5 (Kinoshita et al., 1984), as glycolysis is utilized to provide ATP by substrate level phosphorylation. Utilization of succinate as the sole carbon source requires the TCA cycle, and electron transport chain, which is $\Delta p\text{H}$ dependent. While decreased ATP levels maybe due to a decreased PMF, it could also be due to the disruption in the TCA cycle or electron transport chain. All these mechanisms have in common that they occur inside or across the IM.

To determine the mechanism of the decrease in ATP levels, and if it is important in the inhibition of T3S and motility, would require experiments to test the involvement of the TCA cycle or the electron transport chain. Arsenic inhibits the TCA cycle, and cyanide or azide could be used to inhibit the electron transport chain. Both methods would decrease ATP production, and could be used to test for motility and secretion/invasion. Inhibition of host cells used in invasion assays can be avoided by washing the bacteria prior to addition to the epithelial cells, as was done with CCCP in these experiments. Additionally, a plasmid containing the bacterial *lux* operon from *Vibrio fischeri* (pUCD615), can be utilized to measure the effect of antibody on the TCA
cycle, as the lux operon uses intermediates from the TCA cycle to produce photons (Rogowsky et al., 1987). Measurement of O\textsubscript{2} levels can also elucidate the state of the TCA cycle and the electron transport chain. Additionally, a plasmid encoding proteorhodopsin could be used to determine the integrity of the IM to protons (a pBAD vector encoding proteorhodopsin) (Walter et al., 2007). These experiments would also help elucidate why Sal4 inhibits growth in media containing succinate. The CCCP which I have used in these assays depletes both components of the PMF. Nigericin and valinomycin, which disturb ΔpH and ΔΨ, respectively, could be utilized as controls, to test for motility and secretion/invasion.

While it is unknown how Sal4 and IgAC5, through interactions with LPS might impact bacterial bioenergetics, I postulate that the antibodies may induce physical and/or mechanical stress on the bacterial envelope. There are a number of possible mechanisms by which changes in the outer leaflet of the OM could result in dissipation of the PMF or bioenergetics, including mechanosenstive channels, and improper insertion of proteins into the membranes. In E. coli and Salmonella, mechanosensitive channels (MSC) are situated in the bacterial IM and are gated in response to changes in tension and/or curvature of the cell envelope (Booth et al., 2007; Martinac, 2004). Another mechanism is improper insertion of proteins into the membranes (IM and OM), which can result in damage to the membranes, triggering of extracytoplasmic stress responses (ESR), and loss of PMF (Darwin, 2005). There are other mechanisms by which physical changes in the outer leaflet of the OM could result in disruption of the bioenergetics or induce or
disrupt signaling. Physical disruption of the membrane can result in the loss of
chemoreceptors function, despite the receptors being in the IM (Vaknin and Berg, 2006).

In conclusion, these data indicate Sal4 binding to the LPS of S. Typhimurium, or
IgAC5 to the LPS of S. flexneri, results in a reduction of T3S, PMF, and ATP. The
reduction of T3S and PMF are consistent with the previously observed results of Sal4
based loss of invasion and motility in S. Typhimurium (Chapter 3) (Forbes et al., 2008).
Less is known about protective effects of IgAC5, but this data may indicate in part why
this antibody is protective in vivo (Phalipon et al., 1994). These findings reveal a
previously unappreciated capacity of protective, anti-LPS monoclonal IgA to directly
interfere with microbial processes necessary for S. Typhimurium and S. flexneri to
colonize and invade the intestinal epithelium. Finally, these data further support my
previous assertion (Chapter 3) that anti-LPS IgA can have direct effects on microbial
physiology.
Chapter 5

Sal4 Induces Distortion and Damage to the Outer Membrane

INTRODUCTION

In Chapter 3, the anti-LPS monoclonal IgA, Sal4 was shown to inhibit S. Typhimurium flagella-based motility, and invasion of epithelial cells in vitro. In Chapter 4, I demonstrated the decreased invasion to be caused by a reduction in the secretion of effector proteins by the type three secretion system (T3SS). Furthermore, in Chapter 4, I described a decrease in the electrical potential, which is part of the proton motive force (PMF) that powers both motility and the T3SS, following Sal4 treatment.

Based on these findings, I hypothesize that Sal4 disrupts the outer membrane (OM) structure and function. Despite the fact that Sal4 is protective (Michetti et al., 1992), little is known about how this antibody interacts with the LPS, beyond the ability to cause agglutination. The direct interaction of Sal4 with the LPS of S. Typhimurium may have direct consequences on OM structure or function. This is significant in that changes in OM structure could result in sensitizing the bacteria to other host innate defenses, and/or alter virulence.

S. Typhimurium is a Gram-negative bacterium, and has a phospholipid inner membrane (IM) and an asymmetrical OM, which is comprised of a phospholipid inner leaflet and a LPS outer leaflet (Kadner, 1996; Nikaido, 1996). Between the IM and OM is the periplasmic space, a region that is separate from from cytoplasm and the outside environment. In addition to enzymatic reactions, the periplasm contains a thin structural
layer of peptidoglycan (Meroueh et al., 2006). In addition to lipids, both the IM and OM contain integral proteins and membrane bound proteins. There are estimated to be approximately $3.5 \times 10^6$ LPS molecules per bacterium, occupying an area of $4.9 \, \mu m^2$ of the $6.7 \, \mu m^2$ OM surface (Nikaido, 1996). The remaining ~25% of the area of the outer leaflet is occupied by proteins (Nikaido, 1996), both the outer membrane proteins (OMPs), and the IM and OM spanning proteins, like the flagella and T3SS.

The LPS is composed of the membrane-anchored lipid A, the carbohydrate LPS core and carbohydrate O-antigen repeats. In *S. Typhimurium* strain ATCC14028, the O-antigen consists of mannose, rhamnose, galactose, and abequose, in the form of: $\{ \rightarrow 2\}[[\alpha D\text{-Abe}(1\rightarrow 3)]\alpha D\text{-Man}(1\rightarrow 4)\alpha L\text{-Rha}(1\rightarrow 3)\alpha D\text{-Gal}(1\rightarrow \} \text{ (Raetz, 1996). This}$ carbohydrate structure forms the O4, O5, and O12 epitopes. In strains that lack *oafA*, there is no O5 epitope (Michetti et al., 1994). The standard number of O-antigen repeats is 35-40 (Raetz, 1996). The estimated length of the LPS is 22-70 nm (Kastowsky et al., 1992). In *S. Typhimurium* strain ATCC14028, the wild type LPS is ~20 to 30 nm in length, as determined experimentally in this chapter. The core of the LPS extends less than 5 nm from the OM, and can be divided into the inner and outer core, which are relatively rigid compared to the O-antigen repeats. The O-antigen repeats are thought to be flexible structures and to coil (Kastowsky et al., 1992). Truncation of the core region results in the destabilization of the OM, due to an increased permeability, and by lack of normal OMP composition. Strains that lack the O-antigen polymerase, *rfc*, express only one O-antigen repeat (Nikaido, 1996). A strains that lacks the O-antigen, but has a complete LPS core, is a *rfaL* mutant, while a *galE* rough mutant lacks part of the LPS
core (Nikaido, 1996). The Ra-LPS (core only) is 4.4 nm in length (Kastowsky et al., 1992), which makes the \textit{rfc, rfaL, and galE} LPS less than 6 nm in length.

The O-antigen repeats of LPS blocks entry of many large molecular weight (MW) and hydrophobic molecules (Nikaido, 2003; Nikaido and Vaara, 1985). The length of the O-antigen repeats prevents complement deposition, thereby blocking complement mediated lysis (Holzer et al., 2009). The hydrophilic nature of the LPS inhibits the entry of large hydrophobic molecules, such as some antibiotics that work preferentially against Gram-positive bacteria, due to their lack of an LPS (Vaara, 1992).

The lipid A molecules are composed of two head groups and 6 acyl chains. One head group has 2 acyl chains, while the other has 4 acyl chains. The lipid with 4 acyl chains is linked to two KDO (3-deoxy-D-\textit{manno}-actulosonic acid) residues, which link to the LPS core (Nikaido, 1996). The negative charges from phosphate groups, on the outside of each molecule, which are masked by the positive charges of divalent cations, primarily \text{Mg}^{2+} and \text{Ca}^{2+}, which also function to link adjacent LPS molecules. The lipid A molecules can be modified by the PhoPQ two component system, which alters two of the acyl chains and masks the negative charges of the phosphate groups (Miller et al., 2005).

The lipid and lipid A portion of the membrane provides a barrier against hydrophilic and small molecular weight molecules (Nikaido, 2003). The interactions between the lipid A molecules of the LPS form a defined lattice structure (Nikaido, 2003). The lipid portion of the membrane is a barrier, slowing the diffusion of small molecules, such as ethidium bromide (Murata et al., 2007; Nikaido, 2003). The diffusion of small molecules is affected by LPS length (rough mutants) and lipid structure (affected
by PhoPQ) (Murata et al., 2007; Nikaido, 2003). The OM was originally thought to be impermeable to some compounds. However, it was recently demonstrated that the TolC protein is utilized to export compounds that diffuse across the membranes, and the disruption of tolC reversed this effect (Murata et al., 2007).

Gram-negative bacteria are known to release membrane vesicles. Vesicles can be formed at a rate of up to 20-30% of the OM per hour (Loeb, 1974). Despite the loss of membrane, there is no decrease in membrane integrity (McBroom, 2005). The percent of the total cellular material in vesicles is 0.2-0.5%, or 1-5% of the OM (McBroom, 2005; Schooling and Beveridge, 2006). Gram-negative bacteria are also known to “shed” LPS, and other components of the OM in response to membrane damaging agents, which include polycationic peptides, aminoglycosides, and Tris/Tris-EDTA (Vaara, 1992; Vaara and Vaara, 1983). Each of these chemical agents function through different mechanisms, and therefore affects the membrane differently. Treatment with Tris-EDTA can cause ~50% of OM to be released within minutes (Leive, 1965). The physical damage that results in OM shedding can induce blebs, which are similar to vesicles but induced by membrane damage. In some instances of OM shedding, such as by chemically induced blebs, there is an accompanying decrease in the OM barrier function and permeability (Vaara, 1992; Vaara and Vaara, 1983).

In this chapter, I demonstrate that Sal4 has a direct impact on the OM of S. Typhimurium. Independent of agglutination, Sal4 mediates OM distortion, of which crosslinking of the LPS by the antibody is an important mechanism. Moreover, Sal4 induces the loss of LPS from the OM, and alters the OM permeability.
RESULTS
Sal4 induces ultrastructural changes in the OM of S. Typhimurium.

I hypothesized that Sal4 binding to LPS induces alterations in the ultrastructure of the OM. I utilized scanning electron microscopy (SEM) to visualize the effects of anti-O-antigen specific IgA and IgG on the OM of S. Typhimurium (Fig. 5.1). The OM of S. Typhimurium, 15 minutes post exposure with the isotype control antibody, TEPC-15, appeared uniformly ridged and grooved (Fig. 5.1A, B), and flagella were regularly observed emanating from the bacteria. In contrast, the OM of S. Typhimurium treated with Sal4 (Fig. 5.1B, C), appeared smooth and lacked defined grooves or ridges. The surface was punctuated with regularly spaced “blebs”, approximately 20-50 nm in diameter, while some of the larger “blebs” were 85 and 110 nm in diameter. Sal4, and anti-O antiserum, treated bacteria rarely had distinguishable flagella. No flagella were identified in over 50% of the experiments, but in the two experiments flagella were identified, it was present on 7.5% and 12% of the bacterial cells. In one experiment, the flagella that were seen appeared to be entangled around the bacterium. Treatment of S. Typhimurium with polyclonal anti-O antiserum resulted in morphology similar to treatment with Sal4 (Fig. 5.1E). As a control, S. Typhimurium was treated with polyclonal anti-flagella antiserum, with no noticeably change on the surface of the bacteria (as compared to controls), but did promote bundling of flagella, as expected (Fig. 5.1F). I concluded that IgA and IgG antibodies against the O-antigen induced structural changes in the OM.
Figure 5.1 Anti-O antibodies induce changes in the surface topology

*S. Typhimurium.* *S.* Typhimurium were visualized by SEM following 15 minutes of treatment with (A) TEPC-15, an isotypec control antibody; (B) enlargement of the boxed region in A; (C) Sal4; (D) enlargement of the boxed region in C; (E) polyclonal anti-O antiserum; (F) polyclonal anti-flagella antiserum. Monoclonal antibodies were added at a final concentration of 5 µg/ml, whereas polyclonal antibodies were used at 1:200 dilution. Arrows indicate flagella, arrowheads indicate membrane ‘blebs’. Scale bars: 0.5 µm (A, C, E, F), 0.1 µm (B, D).
Similar effects on the OM were observed with the closely related pathogenic enteric bacterial species *Shigella flexneri*. In contrast to *S. Typhimurium*, *S. flexneri* lacks flagella and is non-motile. *S. flexneri* was treated with IgAC5, a protective monoclonal IgA against the O-polysaccharide side chain (Phalipon et al., 1995). IgAC5 treated *S. flexneri* had an altered OM (Fig. 5.2B), as compared to the untreated control (Fig. 5.2A), which was similar to the Sal4 treated *S. Typhimurium* (Fig. 5.1C). Thus, the antibody associated structural changes in the OM, were not limited to *S. Typhimurium*, as *S. flexneri* possessed the same morphology.

Since Sal4 caused drastic changes in OM topology after 15 minutes of incubation with *S. Typhimurium* (Fig. 5.1), earlier time points were examined, in an effort to visualize the earliest changes that occur in the bacterial OM. *S. Typhimurium* was treated with Sal4, or the isotype control TEPC-15. Samples were collected and processed for SEM at 2, 5, 10, and 15 minutes after antibody exposure. The OM of *S. Typhimurium*, treated with TEPC-15, appeared uniformly ridged and grooved, and flagella were observed, at all time points. The OM of *S. Typhimurium*, treated with Sal4, appeared the same as the control at both 2 and 5 minutes post Sal4 exposure (Fig 5.3A, B). At 10 minutes post Sal4 exposure, the membrane was visibly different, appearing to have less prominent ridges and some “blebs” (Fig. 5.3C), and at 15 minutes, the bacteria had assumed the altered morphology observed in Figure 5.1C, and D (Fig. 5.3D). Thus, Sal4 induced structural changes in the OM of *S. Typhimurium*, proceeding from a uniformly ridged and grooved appearance, to a smoothing of the ridges. Finally, there was a smooth appearance with “blebs”, within 15 minutes.
Figure 5.2 IgAC5 antibodies induce changes in the surface topology *S. flexneri*.

*S. flexneri* were analyzed by SEM following 15 minutes of treatment with (A) PBS and (B) IgAC5 (5 µg/ml). IgAC5 is a monoclonal polymeric IgA specific for the O-antigen of *S. flexneri* serotype 5a. Scale bars: 0.5 µm.
Figure 5.3 The surface topology of Sal4 treated S. Typhimurium changes over time.

S. Typhimurium were visualized by SEM at 0, 5, 10 and 15 minutes after Sal4 treatment at 5 µg/ml. (A) Immediately following Sal4 treatment; (B) 5 minutes post Sal4 exposure; (C) 10 minutes post Sal4 exposure; (D) 15 minutes post exposure. Scale bars: 200 nm.
I next determined if Sal4 was inducing physical changes in the OM of
*S. Typhimurium* directly, or if the bacteria were actively modifying their surface in
response to antibody treatment. I examined the effect of Sal4 on the OM of bacteria that
had been chemically fixed prior to antibody exposure, with the remaining fixative
neutralized with glycine, prior to Sal4 addition. These bacteria were then fixed and
processed for SEM. This method differs from the previous assays, in which
*S. Typhimurium* was first treated with Sal4, and then subjected to fixation. Since
glutaraldehyde fixation only affects proteins, the carbohydrate LPS would not be
affected. The OM of control *S. Typhimurium* in PBS appeared the same as the fixed
bacteria; the OM appearance was ridged and grooved (Fig. 5.4A, C). Some flagella were
observed, but not at a high frequency in this experiment. In contrast, the OMs of
*S. Typhimurium* treated with Sal4, or fixed then treated with Sal4, appeared smooth and
lacked defined grooves or ridges, and were punctuated with regularly spaced “blebs”
(Fig. 5.4B, D). These data suggest that the Sal4 antibody induced structural changes in
the OM of *S. Typhimurium*, which was independent and irrespective of whether the
bacteria were alive or dead.

I next sought to determine if antibody mediated agglutination produced similar
morphological changes in the OM. The longest antibody treatment in the experiments
described above was 15 minutes post Sal4 exposure, and at conditions with minimal
agglutination. To examine agglutination, *S. Typhimurium* was treated with Sal4 at 8.5
µg/ml for 1 hour. In control cultures treated with TEPC-15 (8.5 µg/ml), the bacteria were
generally found individually, or occasionally in contact with one or more other bacteria
(Fig. 5.5A). The OMs were clearly defined between adjacent bacteria, and there was
Figure 5.4

Figure 5.4 Changes in the surface topology $S$. Typhimurium occurred independent of viability. $S$. Typhimurium were visualized by SEM following 15 minutes in (A) PBS or (B) Sal4. Alternately, $S$. Typhimurium were fixed in glutaraldehyde, then treated for 15 minutes with: (C) PBS or (D) Sal4. In both cases, following PBS or Sal4 treatment, samples were fixed in gluteraldehyde, dehydrated by EtOH series, critical point dried, and sputter coated with gold. Sal4 was added at a final concentration of 5 µg/ml, gluteraldehyde was used at 2%. Scale bars: 200 nm.
no evidence of any close cell-cell contact. Flagella were visible as long, thin strands emanating from the bacterial OM. At high magnification (75,000 x) the OM appeared to have a ridged and grooved structure (Fig. 5.5B). In contrast, the Sal4 treated bacteria were primarily found in clumps, which ranged greatly in size (Fig. 5.5C). Flagella were rarely observed in Sal4 treated cultures. The deformations of the OM of the individual bacterium that had been treated with Sal4, differed from the morphology observed at 15 minutes of non-agglutinated bacteria. The OM had fewer, but larger, less well-defined “blebs”, and appeared mucoidy. There was also wide-spread bridging between two or more bacteria in regions of apparent cell-cell adhesion (Fig. 5.5D). These membrane deformations occurred laterally and at the poles of bacteria, and cell-cell bridging also occurred in all orientations (Fig. 5.5E, F). Based on these images, agglutination with Sal4 induced membrane deformations, which were different than the membrane alterations seen on non-agglutinated, Sal4 treated bacteria at 15 minutes.

Multiple antigen binding sites are required to induce changes in the OM.

Previous studies demonstrated that the ability to cross-link LPS molecules was important in Sal4-mediated motility arrest of S. Typhimurium (Chapter 3) (Forbes et al., 2008). Therefore, I hypothesized that the ability to cross-link, or bridging, of LPS molecules by Sal4, may also be important in inducing the observed morphological changes in the OM. Both IgG and IgA are multivalent molecules; IgG have two antigen binding sites, and dimeric IgA have four antigen binding sites. Fab fragments were used to determine if crosslinking of LPS molecules is important to the process of altering
Sal4 promotes deformations of the OM of S. Typhimurium under agglutinating conditions. S. Typhimurium strain JS107 was treated with Sal4 (8.5 µg/ml) for 1 hour, captured on 0.4 or 0.2 µm filters, fixed in glutaraldehyde, dehydrated by EtOH series,
critical point dried, sputter coated with gold, and then visualized by SEM. Control samples were treated with TEPC-15 (8.5 µg/ml). (A, B) TEPC-15 treated bacteria imaged at 12.5k and 75k magnification respectively. (C-F) Sal4 treated bacteria at low magnification (C, 1.75k magnification), and (D 12.5k magnification). (E-F) Sal4 treated bacteria at high magnification (75k magnification). Scale bars: 10 µm (C), 1 µm (A, D), 200 nm (B, E, F).
the OM topology. Fab fragments are monovalent, and are also smaller than IgG molecules, as these antibody fragments lack the Fc region, ~7 nm of a ~14 nm molecule (Woof and Kerr, 2006). Fab fragments were generated from polyclonal rabbit IgG from antiserum (O:1, 4, 5, 12) (Harlow and Lane, 1988), as mouse IgAs, like Sal4, lack the disulfide bond needed to make stable Fabs (Michetti et al., 1994). The OM of S. Typhimurium treated with Fab fragments appeared to have accentuated ridges and grooves (Fig. 5.6B). These Fab fragment treated cells resembled the control cells (Fig. 5.6A), as opposed to Sal4 treated cells. Therefore, the crosslinking of the LPS molecules is important in the alteration of the OM surface topology. While crosslinking played a role in the induced changes in the OM, however, the Fab lack the Fc region, and I could not determine if part of the difference observed may be due to the loss of the Fc region.

Sal4 induces changes in the OM of S. Typhimurium.

To determine the effects of Sal4 on the morphology of LPS in the OM of S. Typhimurium, I utilized transmission electron microscopy (TEM). TEM of embedded and sectioned S. Typhimurium allowed for imaging of cross sections, which could not be achieved by SEM. Cross sectional analysis of the OM allowed for visualization of gross architectural changes in the OM (Vaara and Vaara, 1983). S. Typhimurium was treated with Sal4 for 15 or 60 minutes, pelleted, and fixed with 2% glutaraldehyde, prior to dehydration, embedding and sectioning. The control, untreated bacteria, had a relatively smooth and clearly defined OM, as visualized against the background (Fig. 5.7A, B).
Figure 5.6 Fab fragments are ineffective at inducing changes in the surface topology of *S. Typhimurium*. *S. Typhimurium* were visualized by SEM following 15 minutes of treatment with (A) PBS or (B) Fab fragments in PBS. Fab fragments were generated from polyclonal anti-O antiserum, and was added at a final concentration of 10 µg/ml. Scale bars: 200 nm.
In contrast, there were dramatic morphological changes in the OM of bacteria treated with Sal4. At 15 minutes post exposure, there was a decrease in the smoothness of the OM, and the appearance of a less well defined area of electron density, which was lower than that of the cell body. This area formed a ~50 nm thick “fuzzy coat” (Fig. 5.7C, D). In some instances, the IM and OM appeared distorted. The effects of Sal4 were more pronounced after 1 hour, with a large increase in the less well defined area of lower electron density (Fig 5.7E, F). The coat appeared capsular-like, and there was widespread cell-cell bridging, which probably corresponded to agglutination. Both the IMs and OM of antibody-treated cells lacked definition. These data suggested that Sal4 had a profound effect on the architecture of the cell envelope.

Sal4 induces changes in the LPS of S. Typhimurium.

In collaboration with the Resource for the Visualization of Biological Complexity at the Wadsworth Center, S. Typhimurium was visualized by cryo-electron microscopy. Cryo-electron microscopy allowed for imaging at near-native state, free of fixation and sectioning artifacts, which might have been present in the SEM and TEM imaging (Izard et al., 2008; Marko et al., 2007). This state of the art technique has also provided advancement in the visualizing of membranes (Mannella, 2006; McEwen et al., 2008; Ting et al., 2007). S. Typhimurium, with or without Sal4 treatment, were spotted on grids, plunge frozen, and visualized at -134°C. The OM of untreated S. Typhimurium appeared almost uniformly smooth in both IM and OM. Flagella were regularly observed emanating from the bacterial cells. The LPS formed a ~20-25 nm layer out from the OM,
Figure 5.7 Sal4 causes distortion of the bacterial envelope. Bacteria in mid-log phase were treated with Sal4 (5 μg/ml) for 15 or 60 min, then collected by centrifugation, fixed
with glutaraldehyde, and processed for thin sectioning, prior to being visualized by TEM.

(A, B) Untreated control cells at 15 minutes (A), and an enlargement of boxed region (B).

(C, D) Sal4 treatment at 15 minutes (C), and enlargement of boxed region (D).

(E, F) Sal4 treatment at 60 minutes (E), and enlarged (F).

The outer edge of membranes is indicated by arrows, or arrowheads (enlargement of boxed regions).

Scale bars: 0.5 µm (A, C, E), 100 nm (B, D, F).
of low electron density (Fig. 5.8A, C). The OM of *S. Typhimurium*, treated with Sal4 for 15 minutes, appeared smooth, yet differed drastically in that the OM was surrounded by a ~50-60 nm thick area, extending out from the OM, and possessing an electron density greater than that observed for the LPS in the control (Fig. 5.8B, D). This increase in the region proximal to the OM was significant in comparison to the untreated bacteria, which had a visible layer of LPS closer to the OM, and was similar to that observed by TEM (Fig. 5.7). This “coat” likely corresponds to O-polysaccharide, as it was decreased in size on the OM of a Sal4 treated Δrfc strain, which has only a single O-antigen repeat (Forbes et al., 2008; Nikaido, 1996) (Fig. 5.9C, D). As a control, the oafA strain was treated with Sal4, and no difference was observed compared to the oafA control sample (Fig. 5.9A, B). Although these data supported the TEM findings, they differed in that no membrane distortion (wrinkling) was observed by cryo-electron microscopy. I concluded from these experiments that Sal4 induces alterations in the LPS of *S. Typhimurium* OM.

**Localization of Sal4 on the OM of *S. Typhimurium* by immunogold labeling.**

The ultrastructural changes observed on the surface of Sal4 treated *S. Typhimurium* may be caused by direct and uniform antibody binding to O-antigens distributed around the cell. Alternatively, a localized clustering of antibody at distinct patches on the cell surface may result in a global destabilization of the outer leaflet of the OM. To determine the antibody-LPS interactions, three attempts were made at immunogold labeling to determine the location and spatial distribution of Sal4 on the
Figure 5.8 Cryo-electron micrographs of S. Typhimurium following treatment with Sal4. A mid-log phase culture of S. Typhimurium (A, C) or Sal4 treated S. Typhimurium (5 µg/ml) (B, D). Samples were treated for 15 minutes then spotted onto carbon-coated copper grids, plunge-frozen in liquid ethane, and imaged at -176°C using a JEM-4000FX electron microscope equipped with a Gatan GIF 2002 energy filter. Stars indicate the increased envelope material. IM, inner membrane; PG, peptidoglycan layer; OM, outer membrane. Scale bars: 250 nm (A, B), 30 nm (C, D).
Figure 5.9

Figure 5.9 Cryo-electron micrographs of *S. Typhimurium* *oafA* & *rfc* strains following treatment with Sal4. Cryo electron microscopy imaging *S. Typhimurium* strains JS93 (*oafA::Tn10d-Tc*) and SJF3 (*rfc::kan*) untreated or with Sal4 (5 µg/ml). Samples were treated as described above. (A) Cryo-electron microscopy imaging of JS93 (*oafA::Tn10d-Tc*) untreated, or with Sal4 (B). *S. Typhimurium* strain SJF3 (*rfc::kan*) untreated (C), or with Sal4 (D). Stars indicate the increased envelope material. IM, inner membrane; PG, peptidoglycan layer; OM, outer membrane. Scale bars: 30 nm.
surface of \textit{S. Typhimurium}, in collaboration with the Resource for the Visualization of Biological Complexity. \textit{S. Typhimurium} was exposed to Sal4 (5-10 µg/ml) for 10-15 min, washed (or not) once to remove unbound antibody, and then probed with a biotin-labeled goat anti-mouse IgA, followed by avidin-colloidal gold (10 or 15 nm). Labeling sufficient to make a determination was not successful.

Sal4 induces the loss of OM molecules, principally LPS.

Based on the changes in the organization of OM observed by electron microscopy, Sal4 may promote the release of LPS and other components of the OM. To determine whether Sal4 caused damage to the OM, LPS and flagellin released into bacterial culture supernatants was measured by dot blot, in combination with densitometry for quantification. Dot blot and densitometry analysis were used since LPS levels were generally too low to be detected by ELISA. Assays for outer membrane protein OmpC used the aforementioned technique, while lipid A was assayed by a Limulus amebocyte lysate (LAL) test.

The O-antigen of the LPS present in bacterial culture supernatants was measured following a 30 minute exposure to Sal4 (5 µg/ml), either in PBS or in Tris-EDTA (TE). Supernatants were collected by centrifugation, and filtered to remove whole bacteria prior to the dot blot. Sal4 caused a ~5 fold increase in O-antigen release over the background levels (Fig. 5.10A), which was equivalent to that elicited by TE. TE was chosen as a control as it is known to disturb the OM, and cause the release of O-antigen into the culture supernatant (Vaara, 1992). When TE and Sal4 were combined, there was a synergist effect, as a ~20 fold increase in O-antigen detected in the supernatant. In a
Figure 5.10

A.  

B.  

C.  

Fold Increase

Fold Increase

% Cellular LPS

TE

TE

None  TEPC-15  2D6  Sal4  

None  TEPC-15  2D6  Sal4  

TE

TE
Figure 5.10 Sal4 enhances the release of the O-antigen from the OM.

(A) Mid-log phase cultures of S. Typhimurium were treated for 30 min with Tris-EDTA (50 mM Tris, 112 mM EDTA, pH 7.4), Sal4 (5 µg/ml), or Tris-EDTA + Sal4 (5 µg/ml). Samples were filtered (0.2 µm) and supernatants were spotted onto nitrocellose membranes, and probed with rabbit polyclonal anti-O antisera. Blots were analyzed by densitometry. Data are presented as fold-increase over the background.

(B) S. Typhimurium was incubated with isotype controls TEPC-15 or 2D6 (5 µg/ml), or Sal4 (5 µg/ml), or for 15 min, in a Tris-EDTA solution (50 mM Tris, 112 mM EDTA) to sensitize the OM. Samples were centrifuged, the supernatants were filtered, and spotted onto nitrocellulose for immunodetection and densitometry analysis. Fold increase is relative to a control in PBS without Tris-EDTA to represent native LPS release.

(C) The data from panel (B) present as % of the total cellular O-antigen. Total O-antigen was determined be fixing a PBS control, with no filtration before spotting.

Pannels A and B are individual representative experiments.
second assay, supernatants were collected and filtered, following a 15 minute exposure to Sal4 (5 µg/ml), in a solution of TE. As TE is known to weaken the OM, it was used to sensitize the OM, and as a control for LPS release into the supernatant. As controls, the bacteria were treated with isotype control antibodies TEPC-15, or 2D6. TE alone, and TEPC-15 or 2D6 in TE, had a ~2 fold increase in O-antigen, as compared to control, untreated cells in PBS (Fig. 5.10B). In contrast, Sal4 caused a ~7 fold increase in O-antigen, as compared to control, untreated cells in PBS (Fig. 5.10B). This increase corresponded to ~0.8% of the total O-antigen, as determined by a whole bacterial control (Fig. 5.10C). While this was a significant increase in O-antigen compared to the control, a loss of <1% would seem unlikely to alter the barrier function of the O-antigen (tested below). These data suggested that Sal4 destabilized the OM, resulting in the loss of the LPS O-antigen.

The loss of the carbohydrate O-antigen from the OM implicated, but did not prove, the loss of entire LPS molecules. To test for the presence of lipid A, or endotoxin, which is the membrane bound portion of the LPS molecule, a standard Limulus amebocyte lysate (LAL) test was utilized. S. Typhimurium was treated with Sal4 (5 µg/ml) for 15 minute in PBS, supernatants were collected by centrifugation, and filtered to remove whole bacteria. TE was not used, as the LAL assay is very sensitive, and did not require the OM to be chemically sensitized. Lipid A levels in S. Typhimurium culture supernatants were compared to those of the control bacteria, treated with isotype control antibody 23D7. The levels of lipid A were found to be 2 fold higher in Sal4 treated samples, compared to the isotype control (data not shown). This data, in conjunction with
the O-antigen data, suggested that Sal4 destabilized the OM resulting in the loss of entire LPS molecules.

Sal4 may promote the release of membrane blebs or promote increased vesicles formation, which would include LPS and outer membrane proteins (OMPs). The spherical objects in the SEM of Sal4 treated S. Typhimurium (Fig. 5.1C, D), and the increase in the O-antigen (Fig. 5.10), and lipid A, supported the idea of antibody induced membrane damage in the form of membrane blebing or increased vesicle formation. To test this hypothesis, the release of OmpC, a major OM porin, into bacterial culture supernatants was measured by dot blot and densitometry. Supernatants were collected and filtered following a 15 minute exposure to Sal4 (5 µg/ml) in PBS. As a control, bacteria were treated with 23D7. OmpC concentrations were below the level of detection, for the antibodies available, and this method of assay. Therefore, the presence of OMPs in support or opposition of the hypothesis could not be determined.

As flagella were rarely seen in electron micrographs of Sal4 treated S. Typhimurium, I tested for the presence of flagellin, the monomer subunit that composes the flagella filament. The bacteria were treated, and dot blot and densitometry quantification was used, as described above. In a population of S. Typhimurium each bacterium expresses either the subunits for phase I (FliC), or phase II (FljB) flagellin. There was a significant increase in the amount of flagellin, in Sal4 treated supernatants, when the bacteria were in a TE solution. In sharp contrast, there was no significant difference in the amount of flagellin in Sal4 treated supernatants when the experiment was repeated in PBS. These data suggested that TE, which damages the OM, when combined with Sal4 resulted in the loss of flagellin/flagella. Moreover, these data
suggested that Sal4 does not promote the release of flagellin, suggesting that Sal4 does not physically disrupt the flagellar filament.

**The integrity of the O-antigen barrier is not perturbed by Sal4.**

Sal4 induced changes in the LPS may compromise the barrier function of the OM. The decreased ability of the O-antigen to serve as a barrier would sensitize *S. Typhimurium* to agents that were known to have little or no effects on Gram-negative bacteria with full-length LPS. The integrity of the O-antigen barrier was assessed by the viability and growth of *S. Typhimurium*, treated with several types of agents, which included hydrophobic antibiotics, complement, cationic peptides (defensins), anionic detergent, and bile salts. The deleterious effects of these agents are prevented due to protective capabilities of the O-antigens of the LPS, as indicated by the difference in resistance of smooth versus rough LPS mutants (Kohashi et al., 1992; Vaara, 1992; Vaara and Vaara, 1983; Wijburg, 6 June 2006). Therefore, if the protective capabilities of the O-antigen were compromised, due to Sal4 binding, then these agents may decrease viability, at what are normally sub-lethal concentrations.

Sub-lethal concentrations of the agents used were initially based on published studies (Vaara, 1992), which I then optimized experimentally (data not shown), and Sal4 itself has no effect on the viability of *S. Typhimurium* (Forbes et al., 2008; Michetti et al., 1994). Cultures of *S. Typhimurium* were treated with Sal4, or an isotype control antibody, then exposed to the various agents for 60 minutes, and the bacterial viability was determined by CFUs. The synergistic interaction of Sal4, with one of the agents, was defined as a reduction in viability of at least 10 fold compared to the control. Sal4, in
combination with any of the listed agents, has minimal effect on bacterial viability (Table 5.1). However, there was one exception, a decrease in viability was observed with saturated levels of bile salt, a solution at 20% by weight (Table 5.1), but this concentration was not reflective of physiological levels. These finding indicated that Sal4 did not alter the barrier function of the O-antigen, as determined by viability.

In addition to testing for bacteriocidal interactions with Sal4, the bacteriostatic abilities of these agents in combination with Sal4 treatment, was determined. The Sal4 induced disturbance of the barrier function may not be great enough to result in bacterial death, but may be sufficient to hinder growth. S. Typhimurium in LB broth was treated with Sal4, or an isotype control antibody, then exposed to the various agents, in a 96-well microtiter plates. Bacterial growth was measured by optical density, as determined by spectrophotometry at 600 nm every 30 minutes for 4 hours. None of the agents tested had a significant difference in growth, in combination with Sal4, as defined by at least a 10 fold difference compared to the LB control (Table 5.1).

The O-antigen repeats of the LPS protected S. Typhimurium, even with Sal4 treatment, against all agents tested. Viability of S. Typhimurium was also assessed in the presence of TE, and other chemical controls that cause the release of LPS from the OM, for synergistic bacteriocidal affects. Similar to the previous experiment, a 10 fold difference compared to the control was considered significant. Based on this criterion, no synergistic effect was observed for TE and other agents (Table 5.1, and see Appendix 2). Therefore, even though the OM was disturbed, as observed by electron microscopy, and LPS molecules are shed as a result of Sal4 binding, the amount of LPS loss did not effect the O-antigen barrier function of the OM.
Table 5.1

**Effect of Sal4 on the O-antigen barrier function**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Growth</th>
<th>Viability</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Hydrophobic antibiotics</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Erythromycin</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Novabioxin</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><strong>Complement</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Classical Method</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Alternative Method</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><strong>Defensin</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HNP-1</td>
<td>NT</td>
<td>+</td>
</tr>
<tr>
<td>Crypt-4</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Lactoferrin</td>
<td>+ ¹</td>
<td>+</td>
</tr>
<tr>
<td><strong>Detergent</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bile Salt</td>
<td>+</td>
<td>+ ²</td>
</tr>
<tr>
<td>SDS</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><strong>Chemical control</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TE</td>
<td>NT</td>
<td>+</td>
</tr>
<tr>
<td><strong>Media/pH</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LB</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>M9 glucose</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>M9 succinate</td>
<td>- ³</td>
<td>+</td>
</tr>
<tr>
<td>LB pH 5.8 - 8.0</td>
<td>+ ⁴</td>
<td>+ ⁴</td>
</tr>
</tbody>
</table>

+ = growth or viability was not affected.

- = growth or viability was decreased, minimally 10 fold.

¹ Lactoferrin has cationic residues at the N terminus

² Bile salt (desoxycholate) occurs at 10 mg/ml, unphysiological levels

³ Decreased by 10 fold

⁴ pH 5.8, 6.4, 7.0, 7.6, and 8.0.
Sal4 alters carbon source utilization.

The EM images (Fig. 5.1, 5.2-9), and the increased LPS in the supernatant (Fig. 5.10), indicated that Sal4 binding to the O-antigen resulted in the OM disturbance; I utilized two different approaches in examining the membrane integrity. The viability of S. Typhimurium was examined in rich media (LB broth) at pH 5.8, 6.4, 7.0, 7.6, and 8.0. Over this pH range, the bacteria grew normally in liquid culture. If the OM function was disrupted, or the IM permeability was effected, there could be a decrease in growth or viability. Growth and viability were determined by plating dilutions at 15 and 60 minutes post-Sal4 exposure. There was no significant difference of the Sal4 treated bacteria versus the control at any pH tested (Table 5.1). Since this experiment was performed in rich media, Sal4 treated S. Typhimurium growth and viability in minimal media was also tested, with an alternate carbon source for their cellular energy production. M9 media, with glucose or succinate, was used to examine growth in a media where the substrate could or could not be used anaerobically, respectively. In M9 media with glucose, no difference in growth or viability was observed, as with the rich media (Table 5.1). In M9 media with succinate as a carbon source, there was also no difference in viability, at least in the short duration of the viability test. In contrast, there was a significant effect of Sal4 on bacterial growth in M9 media with succinate, as compared to the control (Table 5.1). Sal4 treatment did not alter the barrier function of the O-antigen, as observed by viability, but there was a growth defect in media with a carbon source that could not be utilized under anaerobic conditions.
Sal4 compromises the lipid barrier of the OM.

Based on the observed changes in the OM morphology (Fig. 5.1, 5.2-9), the increased loss of LPS (Fig. 5.10), and no detectable change in the ability of Sal4 to alter the barrier function of the O-antigen, Sal4 may increase the permeability of the OM, at the level of the lipid membrane. To test this hypothesis, the capacity of small fluorescent dyes to cross the membrane was examined (Murata et al., 2007). In order to accurately measure the influx of dyes across the membranes, it was necessary to use a strain carrying a tolC mutation (Murata et al., 2007). TolC is a transmembrane protein that has the capability to efflux chemicals, including ethidium bromide (EtBr). The ability of the fluorescent dye and intercalating agent, EtBr, to cross the bacterial membranes was tested in S. Typhimurium strains SJF14 (tolC::kan) and SJF32 (tolC::kan oafA::Tn10d). SJF14 and SJF32 were treated with Sal4 for 15 minutes in black 96-well microtiter plates with optical bottoms, prior to EtBr addition. The resulting fluorescence was recorded using a fluorescent plate reader. Sal4 treated bacteria were ~30% more permeable to EtBr than bacteria treated with the isotype control antibody 23D7 (Fig. 5.11A). In contrast, the oafA strain, SJF32, which lacks the Sal4 epitope, was not affected. A second dye, sodium fluorescein, was tested in a similar manner. Sodium fluorescein was added to SJF14, in a pH5.5 phosphate buffer, to reduced fluorescein’s fluorescence. The bacteria-dye mixture was immediately diluted 1:10 into pH 7.4 PBS, in black 96-well microtiter plates with optical bottoms, with or without Sal4. The rationale was to dilute the bacteria, and prevent stress from low pH, while maintaining a suboptimal pH for fluorescein fluorescence, to reduce the background fluorescence from non internalized dye.
Figure 5.11

(A) Mid-log phase cultures of *S. Typhimurium* strains SJF14 (*tolC*::kan), and SJF32 (*tolC*::kan *oafA*::Tn10d), were incubated for 15 minutes in black 96-well plates with optical bottoms, with Sal4 or the isotype control antibody 23D7. After incubation, EtBr was added and readings immediately taken with a fluorometer. Strains carrying the
*tolC::kan* mutation were used to prevent active dye efflux. Percent increase was determined by the increased fluorescence over the 23D7 control.

**(B)** Mid-log phase cultures of *S. Typhimurium* strain SJF14 (*tolC::kan*), was incubated with sodium fluorescein in a pH 5.5 phosphate buffer, to reduced fluorescein’s fluorescence. The bacteria-dye mixture was immediately diluted 1:10 into pH 7.4 PBS in black 96-well plates with optical bottoms, and incubated for 15 minutes with Sal4 or PBS. Readings were taken at 15 minutes after Ab exposure with a fluorometer. Percent increase was determined by the increased fluorescence over the PBS control.
Fluorescein is pH sensitive to acidic conditions, and non-fluorescent at pH 5.0 (Wu et al., 2000). Dye that crossed the membranes into the cytoplasm, pH 7.4-7.6 (Kinoshita et al., 1984), would fluoresce more intensely than extracellular dye. The addition of Sal4 increased the fluorescence of SJF14 by ~10%, over the PBS control at 15 minutes post Sal4 exposure (Fig. 5.11B). Experiments utilizing the dye, Nile red (Murata et. al., 2007) were inconclusive. These experiments suggested that the OM is compromised at the level of the lipid membrane.

**Sal4 disruption of OM induces alkaline phosphatase release from the periplasm.**

To further elucidate the effect of Sal4 on the permeability and integrity of the lipid portion of the bacterial OM, the presence of the periplasmic protein alkaline phosphatase, following antibody treatment was assayed. The *E. coli* alkaline phosphatase is a dimer, with a molecular weight of ~85 kD, and under normal circumstances is not found in the supernatant (Schlesinger and Olsen, 1968). *S. Typhimurium* was treated for 15 minutes with Sal4, control antibody 23D7, or chloroform (CHCl₃). Chloroform disrupts lipid membranes, and was utilized as method to lyse the bacterial cells. Supernatants were filtered, and the presence of alkaline phosphatase was measured utilizing para-nitro-phenyl-phosphate (PNPP) in a glycine buffer, and absorbance was recorded via spectrophotometry. The supernatants of Sal4 treated *S. Typhimurium* contained a ~3 fold greater levels of alkaline phosphatase than the control (Fig. 5.12A). Additionally, *S. flexneri* was assayed, as the anti-LPS antibody, IgAC5, had altered the OM topology (Fig. 5.2). The supernatants of *S. flexneri* treated with IgAC5 contained similarly elevated levels of alkaline phosphatase (Fig. 5.12C). Moreover, in the same
supernatants, there is no measureable ATP, from either S. Typhimurium (Fig. 5.12B) or
S. flexneri (Fig. 5.12D), regardless of antibody treatment. As ATP is only found in the
cytoplasm, the lack of ATP in the supernatant indicated that the IM is not compromised,
and not to small molecules. The results of this experiment demonstrated that Sal4
increased the permeability of the OM, but not the IM. In combination, the fluorescent
dye and periplasmic leakage data suggested that the lipid membrane integrity was
impaired as a result of Sal4 binding.
Figure 5.12

Supernatants were taken from bacteria treated with the isotype control 23D7, Sal4 or IgAC5, and CHCl3 and filtered. Alkaline phosphatase activity in the supernatants was used as a measure of OM integrity, and was measured in 96-well plates with the addition of PNPP in glycine buffer. The absorbance readings were measured at 405 nm for both S.Typhimurium (A) and S. flexneri (C). ATP levels in the supernatant were measured in white 96-well plates to test for damage to the IM, in S.Typhimurium (B) and S.flexneri (D), in a luminescence assay, by photon counting. CHCl3 causes lysis of bacteria and was used as a control. Antibody concentrations were 5 µg/ml.

The results shown are the average values (with standard error) from experiments each done in triplicate. Statistically significance was determined using the Student’s t-test, and is indicated by asterisks: * P < 0.05, ** P ≤ 0.001.
DISCUSSION

In this study, I have demonstrated that Sal4, an anti-LPS monoclonal IgA that was previously shown to protect mice against oral challenge with \textit{S. Typhimurium}, is able to cause alterations in the bacterial OM. Sal4 binding to the LPS of \textit{S. Typhimurium} resulted in alterations of the OM morphology, loss of LPS, and compromised the barrier function and membrane permeability. While experiments showed that the O-antigen barrier function remained unchanged, I conclude from the results obtained from the fluorescence and alkaline phosphatase experiments that Sal4 increased OM permeability by affecting the lipid A portion of the LPS molecule. These data collectively suggest that Sal4’s primary mode of action is in distorting the O-Ag and destabilizing the outer leaflet of the OM.

\textit{Sal4 induces membrane distortion.}

Electron microscopy revealed that Sal4 treatment induced bleb like structures, and regions of electron density surrounding the bacteria. The multivalent nature of Sal4 appears to be important to this process, as Fab fragments did not result in the same morphology. In Sal4 treated \textit{S. Typhimurium} in cryo-electron microscopy images, there is an increase in the length from the OM, and electron density, of material surrounding the bacteria. While the initial alterations in the OM are induced by Sal4, independent of bacterial viability, the amorphous capsular material observed after 60 minutes of Sal4 exposure, may be material secreted by the bacteria.

Sal4 may penetrate the LPS layer, and possibly alters O-antigen confirmation, from a coil to a linear formation. The O-polysaccharide of \textit{S. Typhimurium} consists of
20-35 O-antigen repeat units and is proposed to assume a helical structure (Kastowsky et al., 1992). In a coiled conformation, a single tetrasaccharide repeat unit is estimated to be ~0.8 nm in length (Kastowsky et al., 1992). This is in rough agreement with electron micrographs, which indicate the thickness of the LPS layer is ~20-25 nm. However, the O-antigen is highly flexible and can assume multiple conformations. In an elongated or linear conformation, the tetrasaccharide repeat unit would be more than twice as long (~1.5 nm) extended and would correspond to a coat approximately 50-60 nm thick (Kastowsky et al., 1992). This is the thickness of the fuzzy-coat that surrounds Sal4-treated cells, suggesting that Sal4 promotes the uncoiling of O-antigen. There is evidence to support this hypothesis, as Cygler and colleagues solved the crystal structure of aFab fragment complexed with the dodecasaccharide derived from Salmonella (Cygler et al., 1991). When the solution structures of the O-antigen repeats and of a Fab fragment bound to the O-antigen are compared, it is apparent that the oligosaccharide undergoes a protein-induced conformational shift around one of the glycosidic linkages (Bundle et al., 1994a; Bundle et al., 1994b; Glaudemans et al., 1990).

An alternative explanation is that Sal4 may only bind to the outside of the LPS layer, in which case the distance from the OM would also appear as 50-60 nm, as IgA is ~30 nm in length (Woof and Mestecky, 2005). In this scenario, there would be two regions of electron density, one for the LPS and one for the antibody. However, I observed an increase in length and electron density over the control LPS. A theory that explains both of these observations is that the Sal4 penetrates the LPS layer, and possibly alters O-antigen conformation. This theory accounts for both the increased length by O-antigen, and the fairly uniform electron density throughout the length of the region.
Sal4 induces release of LPS.

I observed that Sal4 induced a loss of LPS from the OM of S. Typhimurium. Sal4 caused a 7 to 20 fold increase in O-antigen released, which corresponds to less than 1% of the total LPS. Additionally, Sal4 treatment resulted in a 2 fold increase of lipid A. These data suggest Sal4 destabilizes the OM, resulting in the loss of whole LPS molecules, not just O-antigen. Gram-negative bacteria are known to “shed” LPS, and other components of the OM, in response to membrane damaging agents, including polycationic peptides, aminoglycosides, and Tris-EDTA (Vaara, 1992; Vaara and Vaara, 1983). Treatment with Tris-EDTA, for example, can cause ~50% of OM to be released within minutes (Leive, 1965). Physical stress can also induce OM shedding. E. coli is known to shed approximately 30% of its OM within 2 minutes of bacteriophage T4 attachment to LPS (Loeb, 1974; Loeb and Kilner, 1978). In some instances (i.e., polycations) OM shedding is accompanied by a compromise in the OM barrier function (Vaara, 1992; Vaara and Vaara, 1983). Therefore, it is not surprising that an antibody that binds the LPS may result in the loss of some LPS molecules from the OM.

Sal4 and the induction of blebs.

Since I detected the release of LPS into the culture supernatants, I needed to consider whether the LPS release occurs in the form of membrane vesicles (MVs), or as blebs. E. coli and Salmonella naturally release membrane vesicles, which do not affect membrane integrity or barrier function (McBroom and Kuehn, 2007). MVs (up to ~100 nm in diameter) can be visualized by TEM (McBroom et al., 2006; Renelli et al., 2004), which resemble the material shown in the SEM images of Sal4 or antiserum treated
bacteria. These differ from blebs, which are caused by membrane damage, and affect the membrane integrity. Blebs occur following treatment of *E. coli* and *S. Typhimurium* with membrane disorganizing agents such as Tris-EDTA and polymyxin B, that are known to sensitize the bacteria to anionic detergents (Vaara and Vaara, 1983) and bile salts (Nikaido, 2003; Vaara, 1992). Several lines of evidence support bleb formation, though I can not rule out loss of individual LPS molecules. SEM images of the Sal4 treated *S. Typhimurium* had bleb like structures on the OM. Other supporting evidence of blebs are the increase in lipid A, the membrane bound portion of the LPS, in Sal4 treated supernatants, and the increase in OM permeability. There may be an increase in blebs, from Sal4 induced OM damage, as opposed to an increase in vesicle production, as vesicle formation is not known to affect membrane permeability. The presence of OMPs in support or opposition of the hypothesis could not be determined. Additionally, the spherical objects in the SEM images of the Sal4 treated *S. Typhimurium* occurred even in dead bacteria.

I did not see bleb or vesicle-like structures in TEM or cryo EM micrographs. There are several reasons why this may be the case, based on the differences between these techniques and SEM. For the SEM preparations, a larger volume of cells is used than in the other techniques, which is pulled down onto a filter, concentrating the bacteria and supernatant. Additionally, the visualization in SEM is more three dimensional, and has a greater depth of field, than TEM or cryo-EM, as these techniques are sectioned or focused on a narrow plain, respectively. Any blebs present on the surface of the bacteria would not be obvious, unless viewed through the thickest section of the bleb. The bleb-like material, likewise, would not be seen in the supernatant, as it is not concentrated into
a single plane, aside from wash steps TEM and cryo-EM techniques employ. Follow-up experiments employing density centrifugation and TEM would be required to determine if there is an increase in bleb formation.

**Sal4 does not compromise the barrier function of O-antigen.**

Sal4 does not compromise the barrier function of O-antigen, as assessed by the viability and growth of *S. Typhimurium* treated with several types of agents, including and included hydrophobic antibiotics, complement, cationic peptides (defensins), anionic detergent, and bile salts. The agents used were at sublethal concentrations. Sal4 is neither bacteriostatic nor bacteriocidal (Michetti et al., 1992), but may sensitize *S. Typhimurium* to the effect of these agents. The effects of these agents are prevented due to protective capabilities of the O-antigens of the LPS, as indicated by the decreased resistance of rough LPS mutants, lacking the O-antigen, compared to the wild type smooth LPS (Vaara, 1992; Vaara and Vaara, 1983; Wijburg, 6 June 2006). However, a decrease in growth or viability was not observed these agents, supporting the idea that barrier function provided by the O-antigens, remains intact despite alteration and the loss of some of the LPS. The criterion to define change was rather stringent, with only a 10 fold difference or more considered to be significant. If these experiments were to be followed up, multiple replicates of a lower fold change might be considered significant.

In the presence of Sal4, there was an observed drop in the growth of *S. Typhimurium*, in media containing succinate, but not glucose. These data may indicate a decrease in ATP production, due to membrane damage. In *S. Typhimurium*, the loss of PMF is associated with the inability to grow on succinate as sole carbon source, and a
decrease in ATP levels (Kinoshita et al., 1984). Salmonella generates ATP primarily through the Krebs cycle and the electron transport chain, which is dependent on ample oxygen and a pH gradient across the inner membrane. Salmonella is a facultative anaerobe, and therefore is capable of conducting substrate level phosphorylation. The significance of using these sugars is that ATP can be generated from glucose, regardless of oxygen or electron transport, while succinate only yields ATP if both electron transport (with a pH gradient and oxygen) is intact.

Sal4 compromises the barrier function of the lipid A portion of the LPS.

Sal4 treatment increased the permeability of the OM of S. Typhimurium to fluorescent probes and periplasmic enzymes, indicating a disruption of the lipid membrane integrity. Sal4 increased the membrane permeability of S. Typhimurium to the fluorescent probe EtBr. Additionally, there was a ~3 fold increase in the amount of alkaline phosphatase in the supernatant of Sal4 treated S. Typhimurium than in the control. Similar results were obtained for IgAC5 treated S. flexneri. In addition, by assaying the same supernatants there is no measurable release of ATP from either S.Typhimurium or S. flexneri, an indication that the IM is not compromised. The results of this experiment demonstrate that Sal4 increases the permeability of the OM, but not the IM. In combination, the fluorescent and periplasmic leakage data reveal that the membrane integrity is impaired as a result of Sal4 treatment. These experiments could be further pursued by assaying for β-lactamase and superoxide dismutase.
There is an apparent contradiction between the loss of periplasmic alkaline phosphatase, yet no detectable decrease in the O-antigen barrier function. Alkaline phosphatase is a dimeric protein with a molecular weight of ~90 kDa, while antibiotics in the growth and viability assays were much smaller, 600-800 Daltons, and were unable to elicit any effects upon the bacterium. There are three explanations for why this seemingly contradiction is possible. Firstly, the antibiotics are hydrophobic and non-polar. Their non-polar nature does not favor interaction with the charges present in the lipid portion of the LPS, and the surrounding divalent cations. Alkaline phosphatase is hydrophilic, and this property would make it easier for this protein to interact and transverse a membrane of charged LPS molecules. Second, the antibiotics must transverse not only outer membrane to elicit their effects, but must also bypass the peptidoglycan layer, the IM, and inside the cytoplasm, antibiotics can bind to their targets and initiate disturbance of cellular activities. However, alkaline phosphatase only needs to transverse the OM, since it is in the periplasm. Third, in order for alkaline phosphatase escape to be detected, an enzymatic reaction needed to take place. This enzymatic activity amplified the signal detected for the assay. When compared to the viability and growth assays, not only did these compounds have to transverse the membranes, but required to do so at levels high enough to observe a significant change in growth or viability. Therefore, there is not a contradiction between the alkaline phosphatase data and the growth and viability data.
The presence or absence of flagella post Sal4 treatment.

It remains unclear if *S. Typhimurium* have intact flagella in the presence of Sal4. In SEM images, I noted that Sal4 treated bacteria primarily lacked flagella, and this is predominately the case in TEM and cyo-EM images. In a few images, flagella could be seen wrapped around the *S. Typhimurium*, which could obscure the visualization of the flagella, or be a mechanism by which the flagella are damaged. I also observed no significant increase in amount of flagellin in Sal4 treated supernatants, in PBS. That Sal4 mediated damage to the OM resulted in damage to the flagella and loss of flagellin would have been consistent with these findings. There are no known reports, or mechanisms, of Gram-negative bacteria releasing or losing their flagella in response to physical or environmental stress, except for the breakage of filaments or low pH (Berg, 2003, Turner, 2000; Berg and Turner, 1993). My data suggest that Sal4 does not promote the release of flagellin, indicating, yet not proving, that Sal4 does not physically disrupt the flagellar filament. If the flagellar filament were to break off from a bacterium, it would be a large structure, which may not pass readily through a filter. Subsequent experiments would involve determining if flagella are lost from the bacteria following antibody treatment. Flagella could be visualized by SEM using immunogold labeling with 20 nm gold, or by fluorescence of the labeled flagellar filaments (Turner et al., 2000), including in motility assays. Additionally, the integrity of the flagella can be tested with a plasmid encoding proteorhodopsin, to force flagellum rotation, provided the IM is intact (Walter et al., 2007).
Proposed Mechanism of LPS loss.

I propose the following model as the mechanism by which LPS is lost from the OM of *S. Typhimurium*, following treatment with Sal4. The LPS covers ~75% of the OM, and the O-antigen is a flexible structure, while the lipid portion is an ordered lattice structure (Nikaido, 1996; Nikaido, 2003). The multivalent antibodies bind to the O-antigen, binding multiple LPS molecules (2-4 per IgA). I propose the cross-linking of LPS results in clustering of the LPS on the OM, as observed by SEM. As the LPS clustering increases, the bleb like structures appear on the OM, as well as the smooth regions. There are likely LPS molecules in these smooth regions, as the membrane integrity assays indicate no alteration of the O-antigen barrier. The rearrangements of the OM topology could result in decreased membrane fluidity, in the local region of the cross-linked LPS. The region of the cross-linked LPS would distort the native OM structure. Interruption of the native lattice structure of the LPS molecules in relation to each other, could interrupt the lateral stabilizing interactions of the divalent cations (Nikaido, 2003). A combination of physical distortion and loss of stabilizing interactions may therefore result in the physical extraction of LPS from the membrane, as in the case of aminoglycosides (Vaara, 1992). If LPS is lost from the outer leaflet of the OM, phospholipids would be flipped up from the inner leaflet of the OM to fill these gaps (Vaara, 1992). Alternatively, the antibody could intercalate into the O-antigen, affecting the membrane integrity of the crosslinked regions by physical displacement of the head groups of lipid A. The displacement of the lipid A head groups would expose the lipid A acyl chains, resulting in increased membrane permeability, similar to the mechanism of polymyxin (Vaara, 1992).
These experiments reveal that SIgAs, and more specifically Sal4, have effects on S. Typhimurium that have previously been unrecognized. Sal4 alters the OM morphology, causing loss of LPS from the membrane, increasing the OM permeability to fluorescent probes and alkaline phosphatase escape from the periplasmic space. These data suggest that destabilization of the lipid A portion of LPS is the prime factor in eliciting the increased permeability. These results lead to the overall conclusion that Sal4, and other similar SIgAs, not only are vital to preventing invasion of the intestinal epithelium, but are also capable of making the OM of S. Typhimurium permeable to small molecules. These studies challenge the paradigm that secretory antibodies are simply a physical barrier separating host and microbe.
Chapter 6

DISCUSSION

Part I. Summary of main findings and conclusion.

Secretory IgA (SIgA) antibodies, directed against the serotype-specific O-antigen of lipopolysaccharide (LPS), are the primary determinants of mucosal immunity to enteric bacterial pathogens, including *Salmonella enterica* serovar Typhimurium and *Shigella flexneri*. While the importance of SIgA in preventing enteric infections has been recognized for years (Apter et al., 1993b; Brandtzaeg, 2007; Chowers et al., 2007; Iankov et al., 2004; Iankov et al., 2002b; Michetti et al., 1992; Michetti et al., 1994; Phalipon et al., 2002; Phalipon et al., 1995; Phalipon et al., 1994; Winner et al., 1991), the underlying mechanisms by which these antibodies prevent bacterial colonization and invasion of the mucosal epithelium remains poorly understood.

In this dissertation, I used two monoclonal anti-LPS IgA antibodies, Sal4 and IgAC5, against *S. Typhimurium* and *S. flexneri*, respectively, as a means to address this question. Sal4, an anti-O-antigen specific, dimeric monoclonal IgA was sufficient to protect mice against a lethal oral challenge with *S. Typhimurium* (Michetti et al., 1992), and prevented *S. Typhimurium* from invading polarized epithelial cell monolayers *in vitro* (Michetti et al., 1994). Sal4 recognizes the O5 epitope within the bacterial LPS, which is defined by acetylation of the abequose residue at the 2’ position (Kim and Slauch, 1999). Similarly, the anti-O-antigen specific mouse monoclonal IgA, IgAC5, was sufficient to protect mice against intranasal challenge with *S. flexneri* serotype 5a (Phalipon et al., 1995). The antigen recognized by IgAC5 is in the O-antigen repeat,
which consists of a tri-rhamnose (rha)-N-acetyl glucosamine (N-ag) tetrasaccharide backbone, with a glucose side chain (West et al., 2005). Although these monoclonal IgAs are protective in relevant animal models, it is unclear how they interfere with pathogenesis. Neither Sal4 nor IgAC5 was reported to be bacteriocidal nor bacteriostatic (Michetti et al., 1994), which I confirmed in this study. Moreover, I showed that neither monoclonal IgA affected the ability of the bacteria to adhere to epithelial cells. Based on these data, I postulated that these antibodies must interfere with another step(s) in the invasion of intestinal epithelial cells.

In Chapter 3, I demonstrated that Sal4 is a potent inhibitor of S. Typhimurium flagella-based motility. Sal4’s effects on bacterial motility occurred rapidly (<15 min), and were independent of agglutination. The concentration of Sal4 required to arrest motility (3 to 5 µg/ml) was identical to the concentration reported by Michetti and colleagues, that was required to block S. Typhimurium invasion of intestinal epithelial cells (Michetti et al., 1994). I also demonstrated that Sal4 decreased S. Typhimurium SPI-1 mediated invasion of epithelial cells. Like motility arrest, Sal4’s effects on invasion occurred within 15 minutes, and were independent of agglutination. Invasion was still inhibited when I used centrifugation to bypass the motility defect, revealing that Sal4 blocks both motility and SPI-1 mediated invasion of epithelial cells.

Several lines of evidence suggest that it is unlikely that Sal4, when bound to the O-antigen, sterically interferes with flagellum rotation. First, the flagellar hook is a highly flexible structure, ~55 nm in length (Berg, 2003; Turner et al., 2000). As a dimeric immunoglobulin, Sal4 is nominally 28 nm from one end to the other, and would not be expected to project sufficiently far from the cell surface to interfere with either the
flagellar hook or filament (Boehm et al., 1999; Woof and Kerr, 2006). Second, Sal4 impairs the motility of a Δrfc strain of S. Typhimurium, which has a severely truncated O-antigen (one O-antigen repeat), that extends less than 6 nm from the outer membrane (OM). The use of the F(ab)_2 fragments had a similar rationale, as they were much smaller than Sal4, lacking the Fc region.

The decreased invasion observed in Chapter 3 lead me to hypothesize that Sal4 may interfere with the function of the S. Typhimurium SPI-1 T3SS. In support of this hypothesis, I demonstrated in Chapter 4, that Sal4 blocked the formation of T3S-dependent translocation pores in erythrocytes. As the translocation pore formation is the first step in the invasion process (Cossart and Sansonetti, 2004), these findings demonstrated impaired T3S. I also presented evidence that IgAC5 is a potent inhibitor of T3S in S. flexneri. Addition of IgAC5, at concentrations of 5-10 μg/ml, for 15 minutes reduced S. flexneri secretion of IpaB, IpaC, and IpaD in a cell-free secretion assay, induced by Congo red. This assay was a direct measurement of effector protein secretion.

In both S. Typhimurium and S. flexneri, I also observed a decrease in the bacteria’s proton motive force (PMF), and total ATP levels, after exposure to Sal4 or IgAC5, respectively. Approximately 40% of the S. Typhimurium cells were depleted of electrical potential (ΔΨ) following Sal4 treatment, as measured using the fluorescent dye JC-1. One caveat of these studies is that JC-1 indicates only intact potential, or depleted potential, and does not reveal gradations in between. Furthermore, some cells may have had decreased ΔΨ, which would not have been detected, due to the threshold sensitivity of the dye. The observed decrease in ΔΨ following Sal4 treatment occurred within 15
minutes, the same time frame as the arrest of motility and invasion. In *S. flexneri*, the JC-1 fluorescence indicated that ~40% of the *S. flexneri* were depleted of ΔΨ, following IgAC5 treatment. The reduction in ΔΨ was concurrent with the loss in T3S activity. The ATP levels of both *S. Typhimurium* and *S. flexneri* were also decreased in this same time range, to between 70 and 90% of the control. These data suggest that the reduction of T3S induced in *S. Typhimurium* and *S. flexneri*, in response to anti-O IgA, is a consequence of antibody-mediated disruption in cellular bioenergetics across the IM, possibly caused by physical and/or mechanical stress on the OM.

In Chapter 5, I investigated the effects of Sal4 and IgAC5 on the integrity of the OM of *S. Typhimurium* and *S. flexneri*, respectively. I hypothesized that antibody-induced membrane disruption could account for the observed decrease in motility, T3S, ΔΨ, and ATP levels. SEM imaging revealed major changes to the surface topology of *S. Typhimurium* and *S. flexneri*, within minutes of antibody exposure. These initial results were confirmed and extended by TEM and cryo-EM. Whereas the surfaces of untreated cells were uniformly smooth, bacteria treated with Sal4, for as little as 15 minutes, were surrounded by a ~50 nm thick coat which likely corresponds to O-antigen. At 1 hour post exposure, this coat thickened and the surfaces of Sal4-treated bacteria appeared mucoidy. Dot-blot analysis revealed that Sal4 treatment caused a ~5 fold increase in O-antigen release into culture supernatants, as compared to untreated control cells. This increase is roughly equivalent to that elicited by Tris-EDTA (TE) treatment, which is known to cause the release of LPS from the OM (Leive, 1965; Vaara, 1992; Vaara and Vaara, 1983). Indeed, the combination of TE + Sal4 resulted in a >7 fold increase in the amount of O-antigen found in the supernatant. It should be noted that the
increase in O-antigen corresponded to less than 1% of the total O-antigen, as determined by a whole bacterial control. Sal4-treated S. Typhimurium also had 2 fold higher levels of lipid A, relative to the control. These data suggest that Sal4 destabilizes the OM, which results in the loss of whole LPS molecules. In support of this, I found antibody-treated bacteria were ~30% more permeable to the dye ethidium than were control cells, and ~3 fold more permeable to alkaline phosphatase. Based on these data, I propose that Sal4, and IgAC5, destabilize the outer leaflet of the OM of S. Typhimurium, and *S. flexneri*, respectively, and thereby compromise the integrity of the bacterial envelope, resulting in an arrest in both flagella-based motility and T3S.

Collectively, the data presented in these studies reveal a previously unrecognized capacity of SIgA to potentially “disarm” enteric pathogens in mucosal secretions, thereby preventing their ability to colonize and invade the intestinal epithelium. Moreover, this represents the first demonstration of a monoclonal IgA having a direct effect on microbial physiology. These results challenge the current view in the field of mucosal immunology, that SIgA is primarily a physical barrier separating host and microbe by immune exclusion.

Taken together, as shown in Figure 6.1, I propose that Sal4 (and IgAC5) binding to the O-antigen (1) causes distortion of the OM, (2) loss of LPS, (3) an increase in OM permeability, (4) a decrease in the PMF and ATP levels, (5) and decreased motility and invasion. While it is unknown exactly how Sal4, through its interaction with LPS, affects bacterial bioenergetics, motility and T3S, I postulate that the antibody may induce physical and/or mechanical stress on the bacterial envelope. The ability to crosslink
Figure 6.1 Proposed model at the cellular level.

(A) The native LPS on the OM of Typhimurium is 20-25 nm in length. Motility is based on the rotation of the flagella. The T3SS is to function in the secretion of effector proteins, mediating host cell invasion.
(B) IgA binds to the O-antigen of the LPS, and causes crosslinking of the LPS. The IgA distorts the LPS structure, resulting in the loss of LPS molecules, and an increase in OM permeability. The “fuzzy coat” that surrounds the OM, is likely composed of LPS and IgA. Flagella based motility and T3S are inhibited, coinciding with a decrease in the PMF and ATP levels.
multiple LPS molecules is an important attribute of Sal4. Sal4, which is multivalent, alters the OM structure, and reduces motility and T3S. This in contrast to monovalent Fab, which caused little change in the OM, and only affected motility and invasion at 10 fold higher concentrations than used for Sal4. The importance of crosslinking is also supported by divalent F(ab)_2, which have very similar affects to that of Sal4. It is likely that a critical threshold exists, based on the level of crosslinking induced OM distortion. Thresholds are common for signaling and energetic events (Berg, 2003; Berg and Turner, 1993). I had estimated that at 5 µg per ml of Sal4, a concentration sufficient to cause alteration of the OM, and arrest motility, ~ 1% of the LPS molecules were occupied by IgA molecules.

There are a number of possible mechanisms by which changes in the outer leaflet of the OM could result in dissipation of the PMF/bioenergetics, including mechanosenstive channels, improper insertion of proteins into the membranes, energy coupled transporters such as TolC, or the T3SS and flagella themselves. It has been documented that physical/mechanical changes in the curvature or tension of the bacterial envelope can perturb cytoplasmic homeostasis, and result in reductions in bioenergetic potential (Booth et al., 2007).

Mechanosensitive channels (MSC) are involved in physical-signal transduction that occurs across all biological membranes, and functions to convert mechanical forces into electrical and/or chemical signals (Martinac, 2004). In *E. coli* and *Salmonella*, MSCs are situated in the bacterial inner membrane and are gated in response to changes in tension and/or curvature of the cell envelope (Booth et al., 2007). The channels are also activated in response to changes in growth phase (Stokes et al., 2003) and phage
infection (Arisaka et al., 2003). *Salmonella* has three major MSCs: MscS, MscK, and MscL (Booth et al., 2007; Corry and Martinac, 2008). If these channels are involved in proton gating in response to Sal4, then I would expect mutants to be more resistant to the effects of antibody exposure. Alternatively, the efflux of cytoplasmic $K^+$ could be monitored by using a $K^+$/valinomycin sensitive electrode.

Improper insertion of proteins into the membranes (inner or outer) can result in damage to the membranes, thereby triggering an extracytoplasmic stress response (ESR), and loss of PMF (Darwin, 2005). This is particularly true with the proteins known as secretins. Secretins form pores in membranes, and overproduction or mis-insertion can lead to the death of the bacteria. Plasmids over-expressing proteins that selectively trigger one of the three main ESR pathways were provided by Andrew Darwin (NYU School of Medicine). I have started testing the over expression of these proteins, and comparing them to Sal4 treatment, to evaluate signaling pathways, by qPCR (see below).

Physical stress and curvature alterations in the OM could be physically transmitted from the OM to the IM by membrane spanning structures, such as the energy dependent transporter TolC, and/or the T3SS and flagella (Murata et al., 2007). Preliminary data for strains with deletions in transmembrane structures demonstrate that the flagellar (*flhD*) and T3SS (SPI-1) knockout strains did not have a change in $\Delta \Psi$ after Sal4 treatment by the JC-1 assay, and the ATP levels were nearly unaffected. In contrast, strains lacking TolC had the same reductions in $\Delta \Psi$ and ATP levels as the wild type. The literature indicates that binding and distortion of the OM can affect the IM (Booth et al., 2007; Vaknin and Berg, 2006). These studies seem to indicate that structures that span the membranes can transmit distortion from the OM to the IM.
Part II. Overall significance and model.

As shown in Figure 6.2, I propose the following model of IgA mediated protection. In the naïve host, S. Typhimurium enter the host intestinal lumen (1), and express virulence genes required for invasion of the intestinal epithelium (2). In an immune host, SIgA is secreted into the intestinal lumen. The current paradigm of SIgA function, in the field of mucosal immunology, is that protection only occurs by immune exclusion (3). The data presented in this study suggest an alternate mechanism of SIgA-mediated protection, independent of agglutination and immune exclusion. Specifically, I propose the association of SIgA with the O-antigen causes distortion of the OM, the loss of LPS, an increase in OM permeability, a decrease in the PMF and ATP levels, and suppression of both motility and invasion (4) and Figure 6.1. Ultimately, the antibody-bacteria immune complexes may be cleared by peristalsis. Additionally, the bacteria may respond to the SIgA by altering gene expression (5). Since anti-LPS antibodies have similar properties against two closely related bacterial species, the mechanism of IgA mediated immunity may apply to other Gram-negative gamma proteobacteria. These findings challenge the assumption that SIgA is primarily a physical barrier separating host and microbe, and demonstrates the direct impact of SIgA functions in mucosal immunity, independent of agglutination.
Figure 6.2

**Figure 6.2 Model of IgA mediated protection to *S. Typhimurium*.**

*S. Typhimurium* survives the innate defenses of the gastrointestinal tract (1), uses motility to approach the intestinal epithelium, adheres and invades the intestinal epithelial cell or M cell of the Peyer’s Patches (2). The paradigm of IgA mediated protection is immune exclusion (3), which is caused by SIgA mediated agglutination and clearance by peristalsis. My findings indicate an additional route of IgA mediated protection. IgA binds to the O-antigen, altering the OM and affecting cellular bioenergetics, resulting in the loss of motility and T3S based invasion (4). The bacteria respond to this insult by producing a capsule-like material (5).
Part III. Future directions.

Gene expression in S. Typhimurium is highly attuned to the status of the cell, including the cell envelope. Therefore, I hypothesize that Salmonella “senses” and responds to the association of antibodies with the OM. To identify the signaling systems responsible for sensing Sal4-mediated membrane damage, as well as determining the role of signaling systems and their regulated genes in bacterial adaptation to antibody exposure, I have begun using MudJ lacZ fusion strains and DNA microarrays.

My initial tests utilized lacZ fusion strains in the major regulatory systems that are activated in response to stimuli that perturb the integrity of the outer or inner membranes, including Cpx, $\sigma^E$, and the phage shock pathway (PSP) response. I tested lacZ fusions to genes regulated by each these pathways, and found no change in expression upon Sal4 exposure. As an alternative strategy, I have created a library of MudJ fusion strains (Hughes and Roth, 1988), and screened them for changes in $\beta$-galactosidase activity following Sal4 treatment. Approximately 1,000 of these strains have been tested to date. While many strains exhibited weak responses to Sal4, 23 responded strongly to antibody treatment. Of these strongly responsive strains, 6 had increased $\beta$-galactosidase assay activity, while 17 had a decreased activity (Miller, 1972). The location of the MudJ transposons in these strains will be determined by sequencing the flanking chromosomal DNA regions.

As an alternative and complementary approach to the MudJ experiments, I have also begun using DNA microarrays derived from the S. Typhimurium LT2 genome (McClelland et al., 2001), and RT-PCR analysis to identify Sal4-inducible and-repressible genes. Comparing the wild type strain to an oafA control, at 15 minutes post
Sal4 exposure, I have found 415 genes with a 1.5 fold change or greater; 244 up-regulated and 171 down-regulated. Most of the changes in gene expression are between 1.5 and 4 fold. Interestingly, there was no change in the expression of genes in the main ESR pathways, and expression of the heat shock sigma factor ($\sigma^H$) rpoH actually decreased upon Sal4 treatment. There was however, a detectable increase in the expression of genes involved in flagellar biosynthesis, chemotaxis, and LPS biosynthesis (rfa and rfb gene clusters), as well as the PhoB regulon (phoBR, pstSCAB). Up regulation of these gene clusters is interesting in the context of restoring bacterial motility and repairing membrane damage. Increased expression of the PhoB regulon is interesting as these genes have been implicated in virulence and capsule production (Baxter and Jones, 2005; von Kruger et al., 1999).

In an effort to discover an underlying mechanism for the evasion of Sal4, a small sub-group of the MudJ transposon mutant strains were also used to look for S.Typhimurium mutants that could “escape” the motility inhibiting effects of Sal4. Soft agar motility plates containing Sal4 were used to screen for escape mutants. Putative escape mutants were subjected to second round of screening. Although ~100 mutants were identified in this initial round of screening, I cannot rule out at this stage that many of these may carry insertions in the same or similar locations on the genome. Preliminary characterization of these mutants indicate that they all carry the O5 antigen and are recognized by Sal4, as determined by dot blot analysis and agglutination assays. As described above, the location of the MudJ transposons in these strains will be determined by sequencing the flanking chromosomal DNA regions. These strains will help determine the mechanism of motility loss, or adaptation/immunity to Sal4. I hypothesize that a
subset of the genes will be involved in membrane remodeling and/or adaptation to
membrane damage.

Another outstanding issue that needs to be pursued in future studies is to
determine whether other anti-LPS antibodies besides Sal4 have a similar effect on the
virulence (i.e., motility and T3S) of S. Typhimurium. To test this would require the
production of additional monoclonal IgA and IgG antibodies against distinct epitopes on
O-antigen. The capacity of these antibodies to protect against Salmonella infection could
then be examined both in vitro and in vivo using techniques described in this dissertation.
These studies would have important implications for vaccine design. It is currently
unknown if all LPS epitopes are protective, or equally protective, or if antigens, such as
the OMPs, are sufficient to generate a protective response. These are critical issues to
resolve when designing more effective vaccines against Salmonella and Shigella species,
which continue to cause morbidity and mortality in both developing and developed
countries.
## Appendix 1  Summary of the Effects of Sal4 and IgAC5

### Table A1.1  Summary of the Effects of Sal4 and IgAC5: Alterations in viability, invasion, energetics, and the OM.

<table>
<thead>
<tr>
<th>Attribute</th>
<th>S. Typhimurium</th>
<th>S. flexneri</th>
</tr>
</thead>
<tbody>
<tr>
<td>Viability</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td><strong>Invasion</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Motility</td>
<td>yes</td>
<td>nonmotile</td>
</tr>
<tr>
<td>Adherence</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>Invasion</td>
<td>yes</td>
<td>no</td>
</tr>
<tr>
<td>T3S</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td><strong>PMF</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\Delta \Psi$</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td>$\Delta \rho$H</td>
<td>NT</td>
<td>yes</td>
</tr>
<tr>
<td>ATP levels</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td><strong>OM</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OM distortion</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td>O-ag barrier function</td>
<td>no</td>
<td>NT</td>
</tr>
<tr>
<td>lipid barrier function</td>
<td>yes</td>
<td>NT</td>
</tr>
<tr>
<td>periplasmic leakage</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td>cytoplasmic leakage</td>
<td>no</td>
<td>no</td>
</tr>
</tbody>
</table>

Yes = attribute affected by antibody.

No = attribute is not affected by antibody.

NT = not tested. Nonmotile, *S. flexneri* is nonmotile natively.
Appendix 2  Membrane Distorting and binding agents

Membrane binding and distorting compounds used in SEM, motility, barrier function, viability and LPS assays. A summary table of the effect of these agents by SEM, motility, barrier function is presented in Table A2.1. The result of the agents used in the LPS loss assay on viability and with the addition of Sal4 is presented in Table A2.2. The affect of ethanol, used in the $\Delta \Psi$ and motility assays, is presented in Table A2.3. The affect of membrane binding and disordering agents used as controls for LPS loss, are given in Figure A2.1, and visualized by SEM in Figure A2.2.
Table A2.1

The effects of membrane binding compounds by SEM, motility, and barrier function.

<table>
<thead>
<tr>
<th>Membrane binding and distorting agents</th>
<th>Agent</th>
<th>Concentration</th>
<th>Blebs</th>
<th>Filopodia</th>
<th>Membrane alteration</th>
<th>Fusion</th>
<th>Motility</th>
<th>Barrier function</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Polymyxin B</td>
<td>50 µg/ml</td>
<td>Yes, large</td>
<td>Yes</td>
<td>blebs, smoothing of surface</td>
<td>No</td>
<td>No</td>
<td>affected</td>
</tr>
<tr>
<td></td>
<td>Poly-L-Lysine</td>
<td>100 µg/ml</td>
<td>No</td>
<td>Yes</td>
<td>Short ridges</td>
<td>No</td>
<td>Yes</td>
<td>normal</td>
</tr>
<tr>
<td></td>
<td>Tris-HCl</td>
<td>100 mM</td>
<td>Yes</td>
<td>No</td>
<td>Some blebs</td>
<td>No</td>
<td>No</td>
<td>normal</td>
</tr>
<tr>
<td></td>
<td>EDTA</td>
<td>10 mM</td>
<td>Yes, small</td>
<td>No</td>
<td>Many small blebs</td>
<td>No</td>
<td>Yes</td>
<td>affected</td>
</tr>
<tr>
<td></td>
<td>CorA</td>
<td>40 µg/ml</td>
<td>Yes, small</td>
<td>No</td>
<td></td>
<td>No</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td></td>
<td>P22 H5</td>
<td>83 PFU</td>
<td>No</td>
<td></td>
<td></td>
<td>No</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table A2.2 S. Typhimurium viability with the chemical agents used as controls in the LPS shedding assays.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Viability</th>
<th>+ Sal4</th>
</tr>
</thead>
<tbody>
<tr>
<td>TE s</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>TE o</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Tris</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>EDTA</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>EGTA</td>
<td>+</td>
<td></td>
</tr>
</tbody>
</table>

Significant difference considered 10 fold different from the control. All pH were 7.2-7.4.

Abreviations: TE s; Tris 100 mM EDTA 10 mM. TE o; Tris 50 mM EDTA 112 mM.

Tris; Trris-HCl 100 mM. EDTA; 10 mM ethylenediaminetetraacetic acid

EGTA; 10 mM ethylene glycol tetraacetic acid.

+ = Viability not effected
Table A2.3 Affect of EtOH on motility.

<table>
<thead>
<tr>
<th>Minutes</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-2</td>
<td>+++</td>
<td>+++</td>
<td>++</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>++</td>
<td>++</td>
<td>++/+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>15</td>
<td>+</td>
<td>+</td>
<td>+/−</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Bacteria (~1 x 10^8 per ml) were mixed with CCP (this was designated time zero) and immediately spotted (10 µl) onto a glass microscope slide, mounted with a coverslip, and visualized by light microscopy. Motility was assessed by light microscopy and scored as follows: ++++, more than 60% of the bacteria were motile; ++, between 30 and 60% of the bacteria were motile; +, less than 30% of the bacteria were motile; -, none of the bacteria were motile.
Figure A2.1

(A) Percent of total cellular LPS loss from treatment of membrane disrupting chemicals for 15 minutes, as determined by dot blot and densometry. The primary antibody was Sal4, and the secondary a goat anti-mouse IgA-HRP.

(B) Relative amount of LPS loss from treatment of membrane disrupting chemicals for 15 minutes, as determined by ELISA. The coating antibody was Sal4, and the secondary antibody was a biotinylated Sal4 and strepavidin-HRP. The PBS control could not be detected above the background.
TE s; Tris 100 mM EDTA 10 mM. TE o; Tris 50 mM EDTA 112 mM. Tris; Tris-HCl 100 mM. EDTA; 10 mM ethylenediaminetetraacetic acid. EGTA; 10 mM ethylene glycol tetraacetic acid.
Figure A2.2

The affect of membrane binding compounds on the OM by SEM.

*S. Typhimurium* were visualized by SEM following 15 minutes of treatment with: (A) PBS control; (B) Sal4 (5 µg/ml); (C) EDTA (10 mM); (D) Tris-HCl (100 mM); (E) Poly-L-lysine (100 µg/ml); (F) Polymyxin B (50 µg/ml). Arrowheads indicate blebs in C and D, and Sal4 ‘blebs’ in B. Scale bars 200 nm.
Appendix 3 Strains constructed and the strain database

Appendix 3a Strains generated (SJF)

Strains generated during progress on my dissertation.

Appendix 3b Strain database

The strain database contains strains generated or collected during progress on my dissertation. These include strains mentioned in the discussion or elsewhere in the dissertation and not listed in the materials and methods.

The database was done in conjunction with Christopher R. Forbes, M.S. (Computer Security and Information Assurance). The database is accessible on the internet to lab members by password, and is located at http://cforsys.com/steve/index.php
<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Antibiotic Sensitivity</th>
<th>Antimicrobial Species</th>
<th>Parental Strains</th>
<th>Plasmid(s)</th>
<th>Storage Loc</th>
<th>Freeze Date</th>
</tr>
</thead>
<tbody>
<tr>
<td>SIR01</td>
<td>AcroN::kan</td>
<td>Kan50</td>
<td>S. Typhimurium</td>
<td>LT2</td>
<td></td>
<td>ABG1</td>
<td>2005-06-28</td>
</tr>
<tr>
<td>SIR02</td>
<td>AcroN::kan</td>
<td>Kan50</td>
<td>S. Typhimurium</td>
<td>ATCC14028</td>
<td>Box 2; 24</td>
<td></td>
<td>2005-06-28</td>
</tr>
<tr>
<td>SIR03</td>
<td>Acro::kan</td>
<td>Kan50</td>
<td>S. Typhimurium</td>
<td>ATCC14028</td>
<td>Box 2; 28</td>
<td></td>
<td>2006-10-12</td>
</tr>
<tr>
<td>SIR03 + pCP20</td>
<td>Acro::kan</td>
<td>Kan50 Ampi000 Can35</td>
<td>S. Typhimurium</td>
<td>ATCC14028</td>
<td>pCP20</td>
<td>Box 2; 27</td>
<td>2008-04-24</td>
</tr>
<tr>
<td>SIR03 + pKD20</td>
<td>Acro::kan</td>
<td>Kan50 Ampi000 Can35</td>
<td>S. Typhimurium</td>
<td>ATCC14028</td>
<td>pKD20</td>
<td>Box 2; 52</td>
<td>2007-07-10</td>
</tr>
<tr>
<td>SIR04</td>
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Figure A3.1 Strains generated during progress on my dissertation.
Figure A3.2

A screenshot of the Bacteria DB interface, showing the search bar with options for Strain, Genotype, Parental Strain, Plasmids, Species, Order by, and Columns. The search results are not visible in this view.
Figure A3.2 The strain database. The strain database contains strains generated or collected during progress on my dissertation. Strains can be searched on multiple criteria, and reports generated by PDF. It is accessible from any computer on the internet, provided the password is given. Information is supplied on: strain name, genotype, antibiotic resistance, species, parental strain, plasmids, storage location, freeze date, references, and notes on source on generation. Figure A3.1 above is an example of the use of the database.
REFERENCES


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WHO (World Health Organization).


monoclonal immunoglobulin A from hybridoma tumors protects against Vibrio cholerae infection. Infection & Immunity 59, 977-982.


