A CAPSULAR VACCINE CANDIDATE FOR NON-TYPHOIDAL *SALMONELLA*

A Thesis Submitted to the College of
Graduate Studies and Research
In Partial Fulfilment of the Requirements
For the Degree of Master of Science
In the Department of Microbiology and Immunology
University of Saskatchewan
Saskatoon

By

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ABSTRACT

Salmonella infections remain one of the most common food borne diseases worldwide. Gastroenteritis, which can be caused by many non-typhoidal Salmonella (NTS) serovars, is relatively common in North America. One of the main risk factors of NTS gastroenteritis is travel to endemic areas in the developing world. The current treatment of NTS infections with antibiotics is reserved for severe cases. A growing concern with antibiotic use is that clinical isolates are becoming drug resistant. Although most NTS infections are self-limiting in nature, the burden on the body and recovery can take several months. Thus, it is vital to prevent NTS infections rather than solely rely on treatment.

We have previously discovered two novel surface associated polysaccharides in Salmonella: O-Antigen capsule and X-factor. Not only O-Antigen Capsule is considered a common surface antigen, but its' genes were found to be expressed during *in vivo* infections in mice. Such an antigen would be a suitable candidate in developing a vaccine against *Salmonella* induced gastroenteritis. The goal of this research was to evaluate the use of O-Antigen capsule to develop a traveler's vaccine for NTS associated gastroenteritis.

Results and Conclusions: We have developed a purification protocol and purified the capsule and X-factor from *Salmonella* Typhimurium, Enteritidis, and Heidelberg. Lipopolysaccharide (LPS) was co-isolated with O-Antigen capsule, but removed using Triton extraction. *Salmonella* LPS is strain-specific and an adaptive immune response against LPS will not provide cross-protection. We generated specific immune sera in rabbits to recognize O-Antigen capsule and X-factor produced by *Salmonella* Typhimurium and Enteritidis. We used a mouse model to determine the immunization dose of O-Antigen capsule and showed that conjugation is necessary to enhance the immune response in mice.

To boost capsule production, we analyzed P*yihUTSRQPO* activity using a luciferase-based reporter system. Deletion of a putative transcriptional repressor (YihW) resulted in over 100-fold increase in P*yihUTSRQPO* confirming YihW as a repressor. We have also looked at the effect of growth media, temperature, and sugar precursors on P*yihUTSRQPO* activity, and were able to show that P*yihUTSRQPO* has highest activity in Tryptone broth at 30^{o}C in the absence of any additional sugars.

ACKNOWLEDGMENTS

I would like to thank my supervisor Dr. Aaron White for showing me the world of science and for all the guidance extended to me over the years. Thank you for being so patient and understanding even when I made the most obvious mistakes. Also, I would like to thank my committee members Dr. Sylvia van den Hurk, Dr. Harold Bull, and Dr. George Mutwiri for all the guidance and the interesting science discussions. In addition I would like to thank my external examiner Dr. Miroslaw Cygler. I extend my gratitude to Dr. Deanna L. Gibson (UBC Okanagan) for her preliminary work on this project and for supplying the immune serum.

I would also like to thank all the past and present members of our lab, especially Keith MacKenzie, Dylan Shivak, Landon Waldner, and Dr. Yejun Wang. Keith, this degree wouldn't have been possible without you. Thanks for being there for me on sunny days and on rainy days, and for being so kind and always putting others before you. Dylan, you made my time in the lab unforgettable with your constant singing and interesting stories. I couldn't have asked for a better buddy to do, not just one, but two degrees with. Landon, thanks for all the valuable life lessons and for always being there for me. I want to extend my gratitude to Shirley Lam for her continuous guidance and help throughout my work at VIDO, and Will Deck for all the chromatography work. A special thank you to Dr. Hugh Townsend for helping me with statistics; I couldn't have done it alone. Also I want to thank everyone at VIDO who have helped me in numerous ways, especially Dr. Sam Attah-Poku, Neil Rawlyk, Donna Dent, Stacy Strom, Marlene Snider and Animal Care Staff.

This work would not have been possible without the funding agencies: Saskatchewan Health Research Foundation (SHRF), Natural Sciences and Engineering Research Council of Canada (NSERC), The Jarislowsky Foundation, Government of Saskatchewan, and the University of Saskatchewan – thank you.

Lastly, and most importantly, I want to thank my parents. Mommy, I wouldn't have come this far without you. Thanks for always being an inspiration and for sharing your invaluable knowledge with me. Thanks for believing in me and for your endless love. Daddy, thanks for the patience and all the sacrifices you have done for my learning. Life has changed and you no longer carry me to school, but I am here only because you did it back then. Thanks for always being there for me.

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LIST OF ABBREVIATIONS

Abbreviation	Description
Amp	Ampicillin
Cm	Chloramphenicol
CPS	Capsular Polysaccharides
E. coli	*Escherichia coli*
ECM	Extracellular Matrix
ELISA	Enzyme-Linked Immunosorbent Assay
EPS	Extracellular Polysaccharide
EU	Endotoxin Unit
H. influenzae	*Haemophilus influenzae*
H. Somni	*Histophilus somni* (HS25)
i.m.	Intramuscular
IM	Inner Membrane
Kan	Kanamycin
LAL	Limulus Amebocyte Lysate
LB	Luria Broth
LOS	Lipooligosaccharides
LPS	Lipopolysaccharides
N. meningitides	*Neisseria meningitides*
OM	Outer Membrane
O/N	Over Night
PAMP	Pathogen Associated Molecular Patterns
PBS	Phosphate Buffered Saline

PCR	Polymerase Chain Reaction
NTS	Non-Typhoidal *Salmonella*
s.c.	Subcutaneous
S. Enteritidis	*Salmonella enterica* subspecies *enterica* serovar Enteritidis serotype 27655-3b
SDS-PAGE	Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis
S. Heidelberg	*Salmonella enterica* subspecies *enterica* serovar Heidelberg serotype S4825-1.1 PT19
S. pneumonia	*Streptococcus pneumonia*
S. Typhimurium	*Salmonella enterica* subspecies *enterica* serovar Typhimurium serotype ATCC 14028S
TBST	Tris-Buffered Saline with Tween 20
Tet	Tetracycline
TLR	Toll-like Receptor
WT	Wild-Type

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1.0 LITERATURE REVIEW

1.1 Introduction to *Salmonella*

1.1.1 Nomenclature and Classification

Salmonella are a diverse group of bacteria that belong to the same family as *Escherichia* and *Shigella*. *Salmonella* consist of two species known as *bongori* and *enterica* (Figure 1.1.1). *S. bongori* mainly infect cold blooded animals, but can cause rare infections in humans (Fookes et al., 2011). *S. enterica* are Gram-negative, facultative anaerobic bacteria that can invade a wide range of warm blooded animals causing acute and chronic infections. According to the White-Kauffmann-Le Minor scheme *S. enteria* is further divided in to six subspecies: *enterica*, *salamae*, *arizonae*, *diarizonae*, *houtenae*, and *indica*. *S. enterica* subspecies *enterica* is the group responsible for the majority of the human infections. Subspecies *enterica* isolates can be categorized into two main groups referred to as Typhoidal and Non-Typhoidal *Salmonella* (NTS) (Hurley et al., 2014). Typhoidal *Salmonella*, *Salmonella* ser. Typhi and *Salmonella* ser. Paratyphi, tend to be human restricted, while Non-Typhoidal *Salmonella* (NTS) infect a broad range of hosts including; humans, chickens, reptiles, birds, amphibians, pigs, cows, and horses (Hurley et al., 2014).

Classification of *Salmonella* is based on serology to three main surface antigens: O, H, and K antigens. The O antigen refers to oligosaccharides that are part of lipopolysaccharide (LPS) on the outer membrane, the H antigen refers to flagella proteins, and the K antigen refers to capsular polysaccharides. For example, the Vi antigen, being a subtype of capsule's K antigen, is only expressed by *S.* Typhi (McQuiston et al., 2011; Grimont and Weill, 2007). Each unique combination of O, H and K antigens lead to the designation of a new serovar (Grimont and Weill, 2007).

Classification of serovars has evolved over time, where serovar names can indicate the syndrome (*Salmonella* ser. Typhi), relationship (*Salmonella* ser. Paratyphi A, B, and C), syndrome and host specificity (*Salmonella* ser. Abortusequi), or geographical origin (*Salmonella* ser. London). With time, names were maintained only for serovars from subspecies *enterica*, while serovars of other *S. enterica* subspecies and *S. bongori* are designated only by their antigenic formula. *Salmonella enterica*, subspecies *enterica* serovar Typhimurium in abbreviated

form is referred to as *Salmonella* ser. Typhimurium, or *S. enterica* serovar Typhimurium (Grimont and Weill, 2007).

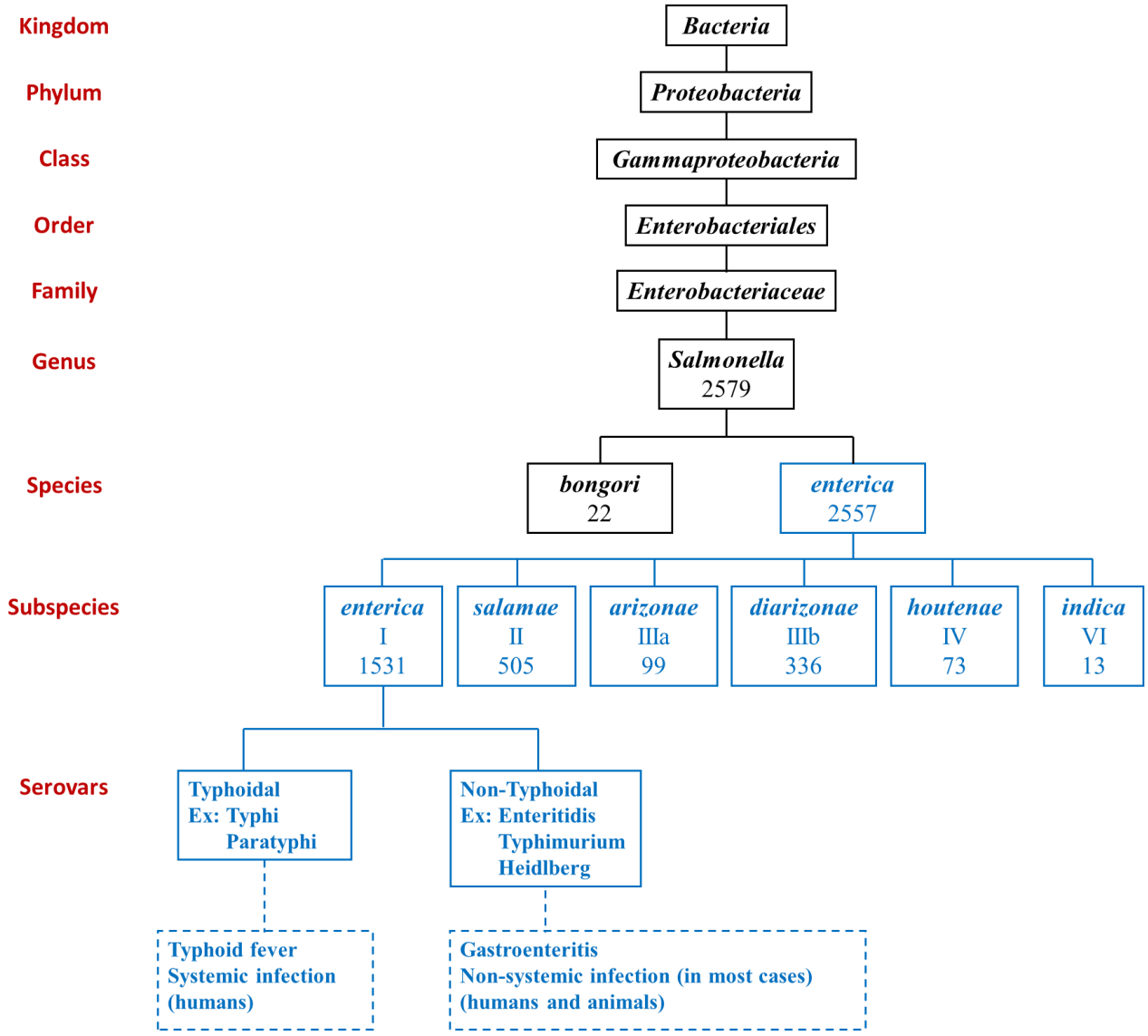


Figure 1.1.1: Phylogenetic tree and White-Kauffmann-Le Minor classification of *Salmonella*. *Salmonella* are divided in to two species, *S. enterica* and *S. bongori*. *S. enterica* is further divided in to six sub-species: *enterica*, *salamae*, *arizonae*, *diarizonae*, *houtenae*, and *indica*. Serovars of *S. enterica* subspecies *enterica* falls in to two main categories called Typhoidal and Non-Typhoidal *Salmonella* (NTS). Below each name is a numerical value corresponding to the current number of serovars in the given category. Figure adopted with modifications from Grimont and Weill, 2007.

S. enterica subspecies *enterica* serovars can also be grouped as host generalist, host adapted, or host restricted. Host generalist serovars like *Salmonella* ser. Typhimurium and *Salmonella* ser. Enteritidis have the ability to infect multiple species. Host adapted serovars like

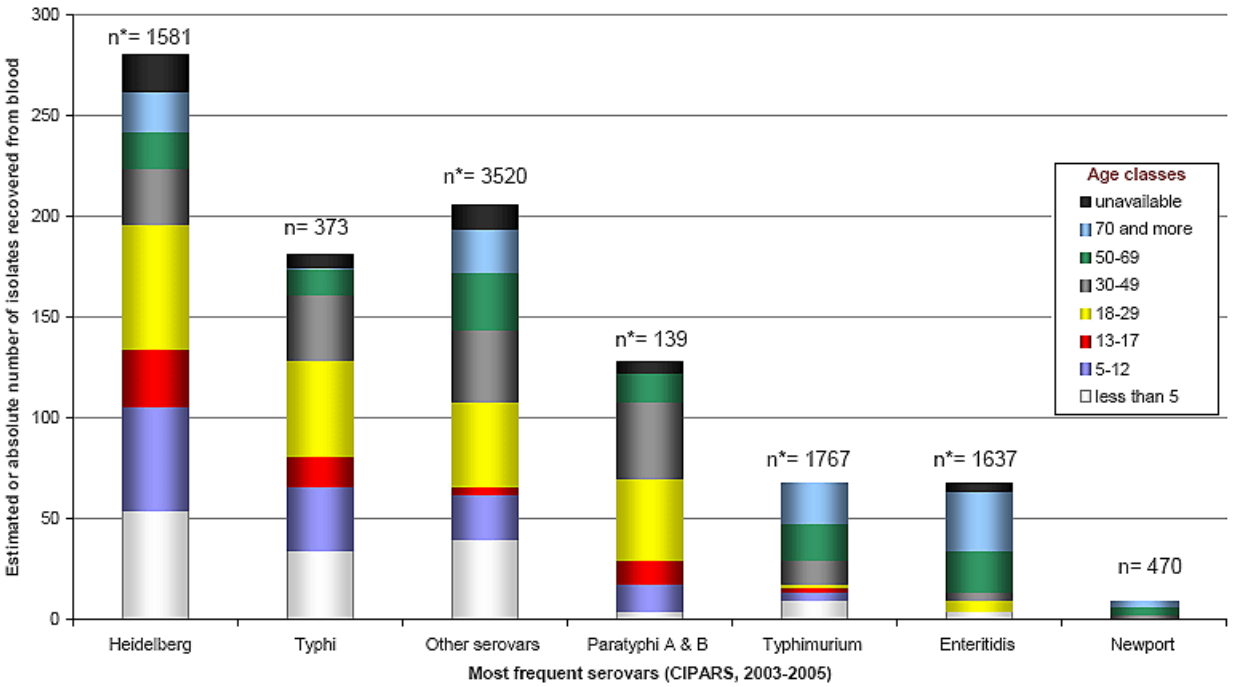
Salmonella ser. Choleraesuis (in swine), and *Salmonella* ser. Dublin (in cattle), usually cause disease in one species, but is capable of causing disease in other species as well. Host restricted serovars like *Salmonella* ser. Typhi, *Salmonella* ser. Paratyphi (human), and *Salmonella* ser. Gallinarum (poultry) cause disease in only one species and are usually characterize by the loss of function of certain virulence genes (Uzzau et al., 2000; Waldner et al., 2012).

1.1.2 Disease and Importance

Salmonellosis is the intestinal illness that arises after ingestion of *Salmonella* contaminated food or water and is one of the most common, yet preventable, food borne diseases present throughout the world (Hurley et al., 2014). The two most common *Salmonella* serovars in North America are *Salmonella* ser. Typhimurium and *Salmonella* ser. Enteritidis (Public Health Agency of Canada 2007). Each year the reported number of *Salmonella* infections range from 6,000-12,000 cases in Canada. However, this number does not reflect the milder, undiagnosed cases that are mostly mistaken for Stomach flu (Canadian Meat Council 2015). Among Canadian travellers, travel to Asia and Caribbean were the most common destinations associated with travel acquired NTS infections (Public Health Agency of Canada 2014). In the four largest provinces of Canada (Ontario, Quebec, British Columbia, and Alberta) *Salmonella* ser. Typhimurium and *Salmonella* ser. Enteritidis isolates are mainly recovered from individuals over 50 years of age. Although *Salmonella* ser. Heidelberg isolates are recovered from all age groups, in one population study more than 200 isolates of *Salmonella* ser. Heidelberg were recovered from blood of individuals over 50 years of age (Figure 1.1.2) (Public Health Agency of Canada 2007).

After eight years, 2012 marked the change in ranking of the three most common *Salmonella* serovars in Canada from *Salmonella* ser. Typhimurium, Enteritidis, and Heidelberg, to *Salmonella* ser. Typhimurium, Heidelberg, and Enteritidis respectively (Public Health Agency of Canada 2014). This is not surprising considering the large number of ceftiofur (a third generation cephalosporin used in animals) resistant *Salmonella* ser. Heidelberg isolates found in Canadian poultry. Of more significance is that ceftiofur resistance is correlated with ceftriaxone, cefoxitin, amoxicillin-clavulanic acid, and ampicillin (antibiotics used in humans) resistance. As extra-intestinal Salmonellosis in young children and pregnant women are treated with

Ceftriaxone, it is recognized as a drug of very high importance to human medicine. Thus, patients infected with even moderately Ceftriaxone resistant *Salmonella* ser. Heidelberg are at elevated risk of ceftriaxone therapy failure (Public Health Agency of Canada 2007).



** n=estimated total number of isolates received (except for S.* Typhi *and S.* Newport*). Values were corrected for differences in submission by the four largest provinces.*

Figure 1.1.2: *Salmonella* isolates recovered from patients' blood samples in Canada; according to the age category. Samples were collected during the first half of each month from 2003-2005 in the largest four provinces: British Columbia, Alberta, Ontario, and Quebec. Figure extracted from Public Health Agency of Canada 2007, reproduced with kind permission from the Public Health Agency of Canada.

In the United States *Salmonella* causes 1.4 million infections and 600 deaths annually (Public Health Agency of Canada 2011). According to the foodborne Diseases Active Surveillance Network, *Salmonella* infections are the leading cause of hospitalizations and death among foodborne pathogens (Centers for Diseases Control and Prevention 2011). In the United States, the economic cost of *Salmonella* infections in terms of lost productivity and medical expenses have been estimated to be in the billions of dollars (Voetsch et al., 2004). Annually NTS cause over 94 million infections and 155 000 deaths worldwide (Majowicz et al., 2010). In developing countries the mortality rate can reach up to 24%, with certain serotypes having higher mortality rates than others. *Salmonella* are also responsible for 20% of childhood diarrhoea and mortality in developing countries (Public Health Agency of Canada 2011). However, 60-80% of

Salmonellosis cases are thought to be either not diagnosed at all or are classified as sporadic cases (World Health Organization 2014).

The number of NTS infections has risen throughout the world in correlation with increased incidences of HIV and malaria, which predispose patients for *Salmonella* infection. In Sub-Saharan Africa, a place notorious for enteric fever, NTS infections are becoming the cause of as many as 50% of diagnosed bacteremia cases (Andrews-Polymenis, et al., 2010). Genomic sequencing has identified that NTS is the primary cause of bacteremia in Malawi children, with up to 75% of these infections being caused by *Salmonella* ser. Typhimurium (Andrews-Polymenis, et al., 2010).

Although most serotypes of *Salmonella* can cause disease in humans, as discussed above, some are host-adopted. When animal host adopted species cause disease in humans, it can be quite severe and even deadly. The host range of individual isolates is affected by genome degradation, differential transcriptional regulation, and prophage repertoire (Kingsley et al., 2009). *Salmonella* ser. Enteritidis and *Salmonella* ser. Typhimurium are the most commonly transmitted serovars from animals to humans (Wold Health Organization 2013). There are a wide range of species acting as reservoirs of *Salmonella*, including livestock and poultry (Alberta Health and Wellness 2011).

NTS can survive for many weeks, even up to years in non-host environments. For example, *Salmonella* can survive up to 63 days in frozen yogurt (el-Gazzar and Marth 1992). *Salmonella* ser. Choleraesuis can survive in dry swine feces for up to 13 months (Gray and Fedorka-Cray 2001), *Salmonella* ser. Dublin can survive in feces spread on rubber, polyester, and concrete for up to 6 years, *Salmonella* ser. Typhimurium can survive in water for up to 152 days, and soil for 231 days (Public Health Agency of Canada 2011). Surprisingly, *Salmonella* can also survive on low moisture products, such as spray dried milk, nuts, cereal, and dry seasonings, for extended periods of time (Podolak et al., 2010; Waldner et al., 2012).

NTS are capable of causing gastroenteritis and bacteremia. Symptoms of gastroenteritis, such as abdominal cramps, diarrhea, nausea, vomiting, chills, fever, and headache, typically develop 6-72 hours after ingestion of *Salmonella* species. These symptoms can last up to 2-7 days depending on the initial concentration of bacteria ingested, serotype, age and the immune status of the host among many other factors (World Health Organization 2014; Public Health Agency of Canada 2011). Although most infected individuals recover without treatment within

few days, clinical manifestation depends on the susceptibility (i.e., immune status) of the host (De Jong et al., 2012). NTS infections are most commonly associated with gastroenteritis. Even though most infections are self-limiting, in rare cases (3-10%) they can lead to bacteremia. The risk of bacteremia is higher in immunosuppressed individuals, infants, and the elderly. Bacteremia can lead to further complications, such as endocarditis, septic shock, infection of liver, spleen, biliary tract, aorta, and urinary tract, mesenteric lymphadenitis, pneumonia, osteomyelitis, pulmonary and brain abscess, empyema, meningitis, CNS infections and death (Public Health Agency of Canada 2011). In addition, perforation of the gut and necrosis of Peyer's patches can result in toxic encephalopathy (De Jong et al., 2012).

Human transmission occurs through ingestion of *Salmonella* contaminated food or water and contact with infected animals, especially food animals and pets, and animal feed. In addition, *Salmonella* can be acquired through ingestion of raw or undercooked poultry and meat among many other food sources. Person to person transmission through fecal oral route is also possible and is further facilitated by inadequate hand washing. Water and food sources soiled with urinary or fecal matter from humans and animals shedding *Salmonella* act as reservoirs and allow easy transmission (Public Health Agency of Canada 2011).

Preliminary diagnosis is mainly based on symptoms and travel history, and can be confirmed by serotyping specimen from stool, rectal swabs, vomit, urine, deep tissue wound, or sterile site at the public health lab (Public Health Agency of Canada 2011; Alberta Health and Wellness 2011). As this process can take over two weeks, treatment options are made available to the patient prior to disease confirmation (Centers for Diseases Control and Prevention 2010).

Treatment is usually based on the symptoms and will include electrolyte replacement (lost through diarrhea and vomiting) and rehydration (World Health Organization 2014). Antimicrobial therapy is usually reserved for immunocompromised patients, infants, children, the elderly and for severe cases of gastroenteritis; as unnecessary administration of antibiotics can select for resistant strains (World Health Organization 2014). Commonly used antibiotics include ciproflaxin, cephalosporins, ampicillin, and Trimethoprim/Sulfamethoxazole (Public Health Agency of Canada 2011).

Multidrug resistant *Salmonella* strains have emerged as far back as 1989 (Public Health Agency of Canada 2011). As of 1997, more than 70% of clinical isolates showed resistance to ampicillin, tetracycline, sulphonamides, streptomycin, and chloramphenicol. A few strains were

even resistant to nalidixic acid, trimethoprim, sulfamethoxazole, and kanamycin (Brisabois et al., 1997). Although vaccines are available for typhoid fever, currently there are no vaccines available for NTS infections.

Preventive measures should be exercised throughout the food chain from production, processing and manufacturing to food preparation at home and in commercial settings. Animal contact should happen with care and infants should be monitored around pet animals. Consuming properly cooked food, especially meat, poultry, and egg; drinking boiled water and pasteurized milk and juices; washing fruits and vegetables eaten raw; and proper hand washing can decrease the incidents of Salmonellosis (World Health Organization 2014). Proper sanitation, access to clean water supplies and treatment of infected individuals are among the best prophylactic measures to prevent the spread of Salmonellosis in endemic regions (Public Health Agency of Canada 2011).

Global surveillance of Salmonellosis is mainly conducted by the Centers for Disease Control and Prevention (CDC) and World Health Organization (WHO). In Canada, there's a hierarchical surveillance system starting from local hospitals to provincial and national health authorities. More specifically, the National Notifiable Disease Summary Program (NDRS), National Enteric Surveillance Program (NESP) and the federal government of Canada. These organizations maintain demographic and epidemiological data related to *Salmonella* outbreaks in Canada (Public Health Agency of Canada 2009). Salmonellosis was the most common pathogen reported to the Canadian National Enteric Surveillance Program (NESP) in 2012 with 220 different serovars and 6979 isolates. From these, *Salmonella* ser. Enteritidis consisted of 30% of recovered isolates (Public Health Agency of Canada 2009).

1.1.3 Clinical Manifestation

Within the host's stomach, *Salmonella* are exposed to a range of stress conditions including low pH, heat shock, and oxidative stress. *Salmonella* that survive these conditions travel to the small intestine and compete with host normal flora for space and nutrients (Chaudhuri et al., 2013; Jones 1997). In addition, *Salmonella* also have to evade killing by bile salts, digestive enzymes, antimicrobial peptides, and secretory IgA prior to attaching intestinal epithelial cells through fimbriae (Haraga et al., 2008).

Salmonella pathogenesis begins when cells cross the intestinal epithelial barrier and enters mesenteric lymph nodes either through active invasion, especially of microfold (M) cells, bacterial mediated endocytosis by non-phagocytic enterocytes, or passive transport through dendritic cells that extend pseudopods through tight junctions into the basolateral side of the intestinal barrier (Jones et al., 1994; Haraga et al., 2008). Bacteria mediated endocytosis lead to intestinal inflammation and diarrhoea (Haraga et al., 2008). A hallmark of Salmonellosis is the massive inflammatory response elicited by the host innate immune system in response to the pathogen associated and secreted effector molecules (Hurley et al., 2014). *Salmonella* use multiple effectors to downregulate the host immune response, thereby facilitating intestinal colonization over long periods of time (Haraga et al., 2008).

Two main categories of proinflammatory stimuli are observed during *Salmonella* infections. These are (a) virulence-associated factors that abuse host processes and cell machinery, ultimately resulting in disease pathology, and (b) pathogen-associated factors stimulating the host innate immune system (Hurley et al., 2014). In *Salmonella* colonization and survival within host cells are mainly regulated by *Salmonella* pathogenicity islands (SPI): clusters of genes acquired through horizontal gene transfer (Ibarra and Steele-Mortimer, 2009). SPI-1 and SPI-2 are very important in *in vivo* infections as Type III secretion systems (T3SSs) encoded by these SPIs are used to secrete effector proteins into the host cell cytoplasm (Galan, 1999).

Mechanisms employed by Salmonella: NTS can invade both phagocytic (dendritic cells and macrophages) and non-phagocytic cells (intestinal epithelial cells) (Portillo and Finlay 1994). During entry *Salmonella* induce the formation of membrane ruffles (protrusions) at the site of attachment (Jones 1997). Actin filament rearrangements enclose and internalize *Salmonella* containing membrane ruffles (Coombes et al., 2005). Internalization of *Salmonella* is mediated by SPI-1 T3SS effector proteins SopE, SopE2, and SopB. These effector proteins can activate host Rho GTPases Cdc42, Rac1 and RhoG leading to cytoskeletal reorganization, formation of membrane ruffles, and internalization of *Salmonella* through micropinocytosis (Haraga et al., 2008). In addition, these effector molecules stimulate transcriptional re-programming in host cells through STAT3 activation. *Salmonella*-induced gene expression changes include signal transduction, membrane trafficking, cytoskeletal architecture, and production of pro-inflammatory cytokines to facilitate survival of *Salmonella* within host cells

(Galan, 1999; Haraga et al., 2008; Hannemann et al., 2013). For example it has been demonstrated that mature dendritic cells produce CCL19, an important chemoatract regulating dendritic cell migration, which binds to CC chemokine receptor 7 on naïve T cells and mature dendritic cells. Mature murine dendritic cells containing *Salmonella* were chemoattracted toward CCL19 produced and secreted in secondary lymphoid organs. Therefore it was proposed that CCL19 secretion by dendritic cells may have a role in dissemination of *Salmonella* throughout the body (Pietila et al., 2005, and Cheminay et al., 2002). Recent evidence suggest that the metabolic pathway utilized by *Salmonella* are specifically adapted to take advantage of the inflamed intestinal environment and outcompete the normal flora that are present (Nuccio and Baumler 2014).

In addition, *Salmonella* remodel surface molecules to evade immune recognition. These include repression of flagella and SPI1 T3SS expression, decrease length of O-Antigen of LPS, alterations to the acyl chain in the lipid-A component, protein content changes in the outer and inner membranes and peptidoglycan layer (Haraga et al., 2008). Effector proteins secreted by SPI-1 T3SS, especially SipA, facilitate bacterial uptake and induce inflammation and recruit polymorphonuclear (PMN) cells across the intestinal epithelium (Haraga et al., 2008). SipA, IL-8 and pathogen-elicited epithelial chemoattractants (PEEC) in turn recruit neutrophils to the site of infection (McCormick et al., 1993). As discussed above, migration of neutrophils to the intestine leads to a massive inflammation, clinically described as acute infection (Bellet et al., 2013). Signaling by SPI-1 released effector proteins also lead to NF-κB signaling and activation of caspase-1, which activate IL-1β, IL-18 and SipB. SipB is a powerful mediator of inflammation and pyroptotic cell death (Hurley et al., 2014). *Salmonella* ser. Typhimurium is also able to induce cell death through caspase-1 and NLRC4 activation, where NLRC4 can recognize accidental injection of flagella by T3SS in to the host cell cytosol (De Jong et al., 2012).

Within macrophages *Salmonella* reside in membrane-bound compartments known as *Salmonella* containing vacuoles (SCV). Within SCV, SPI-2 T3SS-secreted effector proteins delay the endosomal fusion with lysosome and thereby evade the immune recognition (Coombes et al., 2005). In addition, SPI-2 effector proteins are involved in enterocolitis and have a role in pro-inflammatory activity and intracellular persistence.

Mechanisms employed by the host: Host cells recognize *Salmonella* through pathogen associated molecular patterns (PAMP); such as LPS, flagella, peptidoglycan, doublestranded

RNA, and unmethylated CpG motifs, and host derived damage associated molecular patterns (DAMP) such as extracellular matrix (ECM) compounds, secreted immunomodulatory proteins, and stress induced molecules (Tolle and Standiford, 2013; Kawai and Akira, 2010). When PAMPs and DAMPs interact with pattern recognition receptors (PRR) such as Toll-like receptors (TLRs), and NOD-like receptors (NLRs) on host cell surfaces, a signaling cascade is activated to recruit phagocytic cells such as neutrophils and dendritic cells to the site of infection (Hurley et al., 2014). In turn these phagocytic cells secrete pro-inflammatory cytokines IL-6, IL-1β, TNF-α and INF-γ and establish a network between innate and adaptive immune systems. INF-γ is involved in extending macrophage activation and increasing phagosome-lysosome fusion while IL-18 is important for early innate resistant to *Salmonella* (De Jong et al., 2012; Jones 1997). This was shown where mice deficient in caspase-1, IL-1β, or IL-18 had higher titers of bacteria and were readily susceptible for *Salmonella* ser. Typhimurium infection (Raupach et al., 2006). Although both neutrophils and macrophages phagocytose and destroy *Salmonella*, macrophages are capable of killing both self-targeted *Salmonella* and other infected host cells (Delves et al., 2011).

Macrophages target *Salmonella* both through oxygen-dependent and independent mechanisms. Primary oxygen-dependent killing involve secretion of hydrogen peroxide, superoxide, and hydroxyl radicals in to SCV following phagosome-lysosome fusion. Oxygen-independent killing mechanisms include secretion of small bactericidal peptides and acidification of the phagolyssome (Jones 1997). In turn, macrophages present peptides derived from *Salmonella* on major histocompatibility complex class II (MHC II) for CD4 T cell activation (Bellet et al., 2013).

Multiple studies demonstrate that infection of mice with *Salmonella* induces a Th1 response, characterized by the production of large amounts of IFNγ. However, there are also reports indicating that under certain conditions infection with attenuated *Salmonella* can induce Th2 responses, characterized by the production of IL-4 and increased serum levels of IgE (Ramarathinam 1991). The importance of IFNγ in controlling *Salmonella* was demonstrated in mouse mutants deficient in IFNγ receptors, where *Salmonella* had uncontrolled access to host cell colonization (Mittrucker and Kaufmann 2000).

1.2 Capsular Polysaccharides

1.2.1. Importance and Function

The extracellular matrix of bacteria is composed of many components including proteins, lipids, and carbohydrates. Carbohydrates, namely extracellular polysaccharides: lipooligosaccharides (LOS), lipopolysaccharides (LPS), capsular polysaccharides (CPS), teichoic acids, lipoteichoic acids, glycoproteins, and peptidoglycans are found in various Gram-negative and Gram-positive bacteria (Upreti et al., 2003). CPS are unique and distinct from LPS in several ways. First, CPS does not contain Lipid A associated with the LPS core region. In some bacteria CPS have a lower net charge than LPS allowing separation on ion exchange chromatography. CPS usually has several hundred more repeat units, thus higher molecular weight, than LPS molecules. In *Salmonella*, CPS are partially substituted with a glucose side chain, for an example on tyvelose in *S.* Enteritidis and on abequose in *S.* Typhimurium, while this modification is only found on galactose on LPS molecules (Snyder et al., 2006).

CPS are highly hydrated molecules containing 95%-99% water. They are made up of homo or heteropolymers of both organic and inorganic molecules linked together by glycosidic bonds (De Rezende et al., 2005; Costerton et al., 1981; Roberts 1996). The structural diversity and the presence or absence of immunological epitopes on CPS arises from the type of monosaccharides involved, variations in the glycosidic linkages between sugar monomers, number of carbon atoms forming the ring structure (furanose or pyranose form) of sugars, configuration of the anomeric center of the sugar molecule, presence of organic and inorganic molecules, introduction of side chains, and enantiomeric form (D or L form) of sugars (Mazmanian and Kasper 2006; Roberts 1996).

CPS are often associated with virulence, provide barrier protection, desiccation resistance, innate and adaptive immune evasion, and act as lubricating agents and reduce friction over solid surfaces. For example, CPS facilitate swarming by *Proteus mirabilis* on solid surfaces (De Rezende et al., 2005). In addition, CPS can allow bacterial adherence to host cells, surfaces, and to each other; thereby facilitating biofilm formation and colonization (Costerton et al., 1987). Biofilm formation is highly ubiquitous and provides bacteria with nutritional advantages, protection from bacteriophage infection, and phagocytic protozoa (Ledeboer and Jones 2005; Roberts1996). It had been shown that capsulated *E. coli*, *Acinetobacter calcoaceticus*, and

Erwinia stewartii strains are more resistant to desiccation than non-capsulated strains. (Ophir and Gutnick 1994)

Polysaccharide capsules, being associated with the cell surface, are able to regulate interactions between the bacterium and the environment. During capsule biosynthesis, activated precursor molecules (nucleotide monophosphate and diphosphate sugars) are assembled together by inner membrane enzymes to form the growing polysaccharide. Following this, capsule specific translocation proteins move the newly synthesized polysaccharide through the periplasm and across the outer membrane to the surface of the cell. Recent studies have indicated the presence of trans-envelope assembly complexes that coordinate simultaneous biosynthesis, export and translocation of the polysaccharide (Whitfield 2006). In some instances identical translocation pathways are used for certain capsular polysaccharides and LPS O-antigens (Whitfield et al., 1997).

CPS are mainly attached to the cell through covalent linkages to lipid A molecules or phospholipids, while EPS are released to the cell surface and are often involved in slime (Roberts 1996). However, though rarely, CPS can be released from the cell due to poor stability of the phosphodiester interactions between the CPS and the cell surface phospholipid molecules. Similarly, at times, EPS can remain tightly associated with the cell surface (Troy et al., 1971).

Several studies have shown that carbohydrate antigens (i.e. CPS) are T cell-independent activators of B cells. Consequently, CPS antigens often induce IgM responses without a detectable level of IgG production (Figure 1.2.1A). The absence of antibody class switching from IgM to IgG and the inability to produce higher titers of antibody upon subsequent exposure to the antigen are hallmarks of T cell-independent antigens (Mazmanian and Kasper 2006). However, conjugation of polysaccharides to proteins can lead to T cell activation, memory B cell production, and development of polysaccharide specific immune responses (Figure 1.2.1B) (Sood and Fattom 1998).

Capsules can serve different purposes and have differing immunogenicity in different bacteria. CPS producing bacteria are highly diverse and include both animal and plant pathogens. Some examples of CPS producing bacteria include: *Escherichia coli, Salmonella* Typhi, non-typhoidal *Salmonella* serovars such as Typhimurium and Enteritidis, *Shigella* spp., *Neisseria* spp., *Vibrio* spp., *Streptococcus* spp., *Cryptococcus neoformans*, *Staphylococcus aureus*, *Campylobacter jejuni*, *Bacteroides fragilis*, *Haemophilus influenza*, *Aeromonas Salmonicida*,

Acetobacter methanolicus, *Burkholderia mallei*, and *Francisella tularensis* (Sukupolvi-Petty et al., 2006; Weiss et al., 2007; Gibson et al., 2006; DeShazer et al., 2001; Laxalt and Kozel 1979; Karlyshev and Wren 2000; Nelson et al., 2007; Fournier et al., 1984; Mazmanian and Kasper 2006)

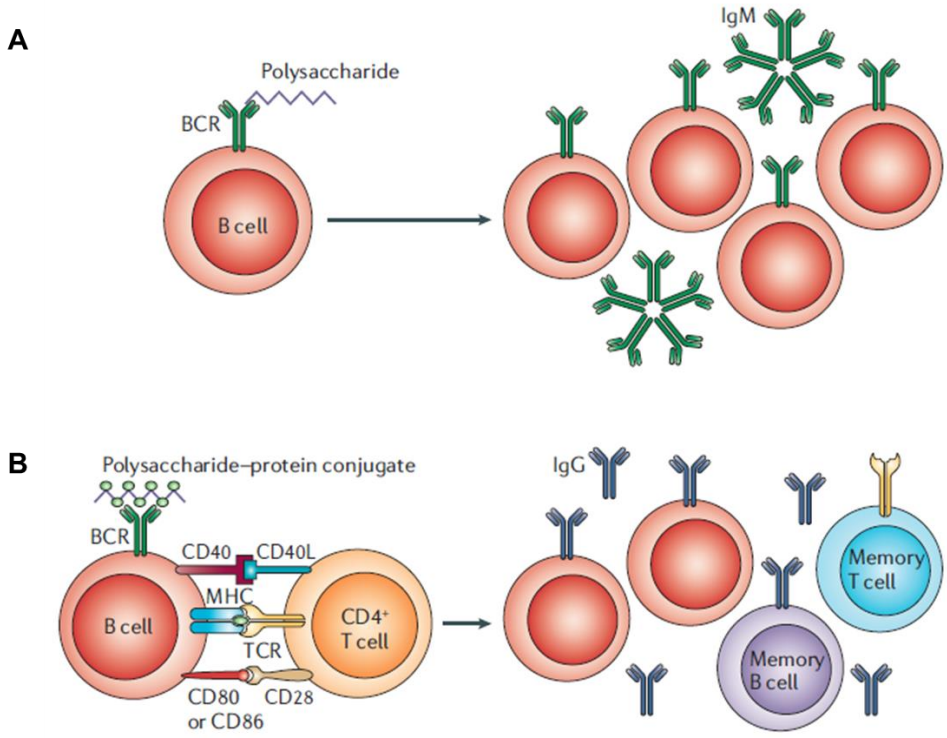


Figure 1.2.1: Antibody production in response to polysaccharide antigens. (A) Polysaccharide antigens alone lead to IgM production, while (B) polysaccharide-protein conjugated antigens lead to IgG production and immune memory. Figure extracted from Mazmanian and Kasper 2006, reproduced with kind permission from Nature Publishing Group.

The following is a look at the capsules and their characteristics in few selected bacteria. Historically, classification of capsules had started with *E. coli*, which has over 80 different CPS, known as K antigens (Orskov and Orskov 1992). Certain K antigens have identical polysaccharide chains, and only differ in the modifications of the polysaccharides (Vann et al., 1983). In general, expression of a given type of CPS is associated with a unique infection. For a example, K1 antigen expressing *E. coli* are the primary cause of neonatal meningitis (Roberts 1996). Interestingly, chemically identical capsular polysaccharides can be produced by different genera of bacteria. For an example, *E. coli* K1 CPS is identical to *Neisseria meningitides* group B CPS, and *E. coli* K18, 22, and 100 CPS are identical to *Haemophilus influenza* serotype b CPS (Grados and Ewing 1970; Roberts 1996).

Classification systems of *E. coli* were first developed by Kauffman in 1940 and were based on O, K and H antigens (discussed 1.1 "Introduction to *Salmonella*"). Since then, the classification scheme had seen many changes. Whitfield and Roberts have introduced an updated version of this system based on genetic and biosynthetic criteria (1999). The primary advantage of this new system is that it is not based on serological assignment and polysaccharide structure. According to this classification system capsules are categorized into four groups (Table 1.2.1). As observed by Whitfield and Roberts even though all polysaccharide K antigens form a capsule structure, all capsules are not composed of K antigens.

Group 1 capsules are acidic polysaccharides containing uronic acid and tend to be similar in structure, and are often inaccurately classified as colanic acid (Whitfield 2006). The most prominent feature distinguishing group 1 capsules and colanic acid is that wild-type bacterial isolates are unable to produce colanic acid when grown at 37^{o}C on common lab media (Whitfield 2006). Group 4 capsules, despite many similarities, are far more diverse than group 1 capsules. Interestingly, group 4 capsules were previously classified as group 1 capsules and as a result became known as the 'O-Antigen capsules' (Goldman et al., 1982). One example is the group 4 representative K40 antigen that was assigned to group 1 due to the presence of acetamido sugars in its repeat unit. K40 antigen was then shown to be surface expressed as smooth LPS and as unlinked O-antigen capsule and was reclassified in to group 4 (Roberts 1996; Amor and Whitfield 1997). This confusion in classification has arisen since K40 antigen is co-expressed with a neutral LPS linked polymer such as O8 or O9 antigen (Whitfield and Roberts 1999). Group 1 and 4 antigens are subgrouped into K_{LPS} and capsular K antigens, where K_{LPS} is made up of low molecular weight K antigenic oligosaccharides containing few repeat units and are linked to the cell surface through lipid A. K_{LPS}, however, are different from LPS with the serological O-antigen found on the same cell (Whitfield 2006). Capsular K antigens are made up of high molecular weight antigens that form the capsule structure responsible for masking O antigen in serotyping. These capsular K antigens are not linked to lipid A core as in LPS molecules (Whitfield and Roberts 1999). When bacteria produce a group 4 capsule as the only serotype specific polysaccharide, it is classified as the O-antigen of that isolate (Whitfield 2006).

Group 2 capsules are linked to the cell surface through α-glycerophosphatidic acid. In some bacteria Kdo (CMP-Kdo synthetase is involved in the biosynthesis of LPS) provides a link between the phospholipid and the polysaccharide. Expression of group 2 capsules is significantly

influenced by the temperature (Whitfield and Roberts 1999). Group 3 capsules are largely similar to group 2 capsules (Whitfield and Roberts 1999). During translocation, group 1 and 4 capsule assembly occur at the periplasmic face of the plasma membrane, while group 2 and 3 capsule assembly occur at the cytoplasmic face of the plasma membrane. In the assembly of capsules from all four groups, the sequential action of glycosyltransferases joins individual repeat units together elongating the polysaccharide (Whitfield and Roberts, 1999).

Characteristic	**Group**			
	1	**2**	**3**	**4**
Former K antigen group	1A	II	I/II or III	IB (O-Antigen capsules)
Co-expressed with O serogroups	Limited range	Many	Many	Often O8, O9, but sometimes none
Co-expressed with colanic acid	No	Yes	Yes	Yes
Thermostability	Yes	No	No	Yes
Thermoregulated (not expressed below 20°C)	No	Yes	No	no
Terminal lipid moiety	Lipid A core in K_{LPS}; unknown for capsular K antigen	α-Glycerophosphate	α-Glycerophosphate (hypothetical)	Lipid A core in K_{LPS}; unknown for capsular K antigen
Genetic locus	*cps* near *his* and *rfb*	*kps* near *serA*	*kps* near *serA*	*rfb* near *his*
Polymerization system	Wzy-dependent	Processive	Processive	Wzy-dependent
Direction of polymer chain growth	Reducing terminus	Non-reducing terminus	Non-reducing terminus (hypothetical)	Reducing terminus
Trans-plasma membrane export system	Wzx (PST)	ABC-2 exporter	ABC-2 exporter (hypothetical)	WZx (PST)

Translocation proteins	Wza, Wzc	KpsD, KpsE (KpsF may be involved)	KpsD (KpsE may be involved)	Unknown
Elevated levels of CMP-Kdo synthetase	No	Yes	No	No
Positively regulated by the Rcs system	Yes	No	No	No
Model system(s)	Serotype K30	Serotypes K1, K5	Serotypes K10, K54	Serotypes K40, O111
Similar to capsules in	*Klebsiella*, *Erwinia*	*Neisseria*, *Haemophilus*	*Neisseria*, *Haemophilus*	Many genera

Table 1.2.1: Classification of *E. coli* capsules. Classification is based on biosynthesis and assembly systems. Rcs system plays an important role in late stages of biofilm development. CMP-Kdo synthetase is involved in the biosynthesis of LPS. Table adopted from Whitfield and Roberts 1999, and Whitfield 2006.

In *E. coli*, expression of genes for the biosynthesis of EPS colanic acid, known as cell surface slime, is increased in the face of harsh environments and desiccation (Ophir and Gutnick 1994). Despite the lack of concrete evidence in the mechanisms behind regulation of capsule expression, it is hypothesized that reduced external osmolarity act as a signal to increase capsule production (Roberts 1996). In support of this argument, it had been shown that reduced external osmolarity leads to increased expression of Vi antigen in *S*. Typhi and anionic polysaccharide alginate expression in *Pseudomonas aeruginosa* (Pickard et al., 1994; Berry et al., 1989).

There are two types of CPS found in *Salmonella*; the Vi capsule (also known as Vi antigen) associated with *S*. Typhi, and the O-Antigen capsule associated with NTS. The Vi capsule increases virulence and disease severity of *S*. Typhi infection by interfering with neutrophil chemotaxis and bacterial clearance (Wangdi et al.2014; De Jong et al., 2012). Deletion of genes for Vi capsule biosynthesis can remarkably enhance the neutrophil chemotaxis *in vitro*. In addition, it had been shown that Vi capsule prevent complement activation through alternative pathway, as C3b is unable to deposit on the capsule surface and promote opsonophagocytosis (Wangdi et al., 2014; Wilson et al., 2011). It had also been demonstrated that Vi capsule promotes immune evasion by masking LPS molecules and preventing their recognition through pathogen recognition receptors (PRR). As a consequence *S*. Typhi does not

induce neutrophil influx and is able to disseminate systemically and cause a persistent infection (De Jong et al., 2012). Furthermore, the capsule-mediated suppression of TNF-α production by human monocytes lead to low serum concentrations of pyrogenic cytokines, and thereby prevent the development of septic shock (Raffatellu et al., 2006; Hirose et al., 1997).

The O-Antigen capsule produced by NTS is co-regulated with cellulose and fimbriae to be part of the ECM. The group 4 O-Antigen capsules were shown to be important for attachment to surfaces, colonization, and desiccation resistance (Gibson et al., 2006). *S.* Typhimurium O-Antigen capsule was shown to be expressed both at lower and higher temperatures indicating that the capsule may play a role in bacterial survival inside and outside the host, as had been described for other bacterial species, such as *Hyphomonas* strain MHS-3 (Quintero and Weiner 1995). Mutations in O-Antigen capsule assembly and translocation gene, *yihO*, or cellulose synthesis gene, *bcsA*, significantly reduced the colonization of alfalfa sprouts by NTS (Barak et al., 2007). In addition, the promoter for O-Antigen capsule biosynthesis was expressed during *in vivo* infection of mice (White et al., 2008).

*Campylobacter jejun*i (*C. jejuni*) produces LOS and CPS that are genetically and biochemically similar to polysaccharide capsules in other Gram-negative bacteria (Karlyshev and Wren 2001). Due to the presence of hypervariable polysaccharide biosynthesis genes, the structure of these polysaccharide monomers can vary between *C. jejuni* strains. These heat stable groups 2 or 3 CPS are part of the Penner antigen used for *C. jejuni* serotyping. It had been shown that *C. jejuni* CPS can be released to the surrounding environment in either naïve or lipid free form depending on the presence or absence of deoxycholate; a component of bile salt. Even though the specific purpose of released EPS is unknown, it is regarded as important for survival and pathogenesis of *C. jejuni* in foreign environments (Karlyshev and Wren 2001). Treatment of *C. jejuni* CPS with phospholipase release a lipid free product to the medium indicating that these CPS are substituted with a phospholipid inplace of a lipid A molecule (Karlyshev and Wren 2001).

Another frequently researched CPS is of *Staphylococcus aureus* (*S. aureus*). Clinical isolates of *S. aureas* are classified in to eight groups based on their CPS. The type 8 CPS is the most prevalent among bacteremic isolates and confers resistance to complement mediated opsonisation and phagocytosis (Fournier et al., 1984).

Bacteroides fragilis (*B. fragilis*) produced zwitterionic CPS was able to activate $CD4^+$ T cells and correct immune defects such as dysregulated systemic cytokine production found in the absence of bacterial colonization and $CD4^+$ T cell reduction in splenic lymphocyte populations (Mazmanian and Kasper 2006). It had also been shown in immunogenicity studies that administration of *B. fragilis* CPS alone protects Wistar rats against subsequent infection with *B. fragilis* (Kasper et al., 1979)

Although CPS are often associated with Gram negative bacteria, many Gram-positive bacteria synthesize lipoteichoic acids or teichoic acids containing sugar alcohols with phosphodiester linkages. These sugar alcohols play a key role in attachment of bacteria to surfaces and in their interactions with host innate and adaptive immune systems (Kenzel and Henneke 2006).

1.3 Vaccines

1.3.1 Polysaccharide Vaccines

Polysaccharide vaccines are composed of capsular polysaccharides from Gram-negative bacteria and are classified as inactivated subunit vaccines. To date pure polysaccharide vaccines have been produced for four encapsulated bacteria: *Streptococcus pneumonia* (pneumococcus), *Neisseria meningitides* (meningococcus), *Haemophilus influenzae* type b (Hib) and *Salmonella* Typhi (Pollard et al., 2009). The adaptive immune response to polysaccharide antigens is usually T-cell independent, implying that polysaccharides are capable of activating B cells in the absence of T helper cells (MacLennan et al., 2014). However, the lack of T cell induction and in turn failure of B cell stimulation by T cells result in the absence of immunoglobulin class switching, induction of memory B cells, affinity maturation, prolonged antibody response, and lead to hyporesponsiveness to subsequent vaccination and/or to natural infection. In addition T cell independent antigens, such as polysaccharides, are poorly immunogenic in infants and young children under the age of two (MacLennan et al., 2014).

Polysaccharide capsules are composed of numerous identical epitopes in close proximity within each other and can crosslink immunoglobulin receptors on the surface of B cells for B cell activation. However, these polysaccharides are not processed and presented on MHC class II molecules for T helper cell activation (Lesinski and Westerink 2001). Although polysaccharide antigens are unable to induce the production of memory B cells they can activate previously formed memory cells, leading to terminal differentiation of memory cells into plasma cells. However, this depletes the reservoir of memory B cells specific for a given polysaccharide antigen and could result in a condition called hyporesponsivness, which is the inability to respond to subsequent exposure to the same antigen due to lack of memory B cells (Blanchard-Rohner and Pollard 2011). Repeated administration of the same polysaccharide antigen(s) can exacerbate this situation. In contrast, repeated administration of most protein vaccines leads to a booster effect resulting in progressively higher antibody titers. In addition, antibody produced in response to polysaccharide antigens are predominantly IgM and have less functional activity than IgG antibody (Blanchard-Rohner and Pollard 2011).

Marginal zone (MZ) B cells and B1 cells (and naïve B cells to a lesser extent) are the most important at recognizing polysaccharide antigens. These cells are able to produce low affinity

IgM (natural) antibodies in response to polysaccharides. B1 and MZ B cells have higher expression of CD21 receptor and are able to bind to complement coated polysaccharides (Blanchard-Rohner and Pollard 2011). The spleen, being a vital organ of the immune system, is crucial for MZ B cell production and their survival (Kruetzmann et al., 2003). For example, individulas with congenital asplenia, splenic hypofunction or who have undergone splenectomy are highly susceptible to encapsulated bacterial diseases, as they have depleted number of functional MZ B cells and are defective at removing opsonized bacteria (Blanchard-Rohner and Pollard 2011). Furthermore, the immune response to polysaccharide antigens is age-dependent as children under the age of two are also susceptible to encapsulated bacteria, since prior to this age the MZ is not fully developed and is unable to facilitate the development of B cells (Kruschinski et al., 2004). In addition, the presence of maternal antibody and the effect of suppresser T cells also contribute to the lack of response to polysaccharide antigens in infants (Sood and Fattom 1998). However, some zwitterionic polysaccharides (have both positive and negative charge) such as capsules from *Bacteroides fragilis, S. pneumonia* serotype 1 and 3, and *N. meningitides* serogroup A possesses characteristics of T cell dependent antigens and are presented in an MHC class-II dependent manner. These antigens are immunogenic in even infants and young children (Jokhdar et al., 2004; Kalka-Moll et al., 2002).

Since the late 20th century, polysaccharide vaccines have helped save lives of millions of people worldwide. However, there are several limitations associated with pure polysaccharide vaccines. These include: hyporesponsiveness, short term impact on bacterial carriage where individuals have been shown to carry bacteria for several months and even years without symptoms and remained communicable, and poor immunogenicity in children under the age of two (Wilder-Smith 2009). In addition, the response for polysaccharide antigens among two to five year old children, the elderly, and the immunocompromised individuals are not optimal, while these populations remain at an elevated risk of contracting bacterial diseases (Sood and Fattom 1998). Furthermore, pure polysaccharide vaccines are unable to provide herd immunity, which is a key element in prevention of invasive diseases. Herd immunity is achieved when the majority of a community is immunized against a given infectious disease, such that even those who have not received immunization are protected as the spread of the disease is contained (Vaccines.gov 2015).

Antibody titers induced in infants after immunization with *N. meningitides* serogroup C (MenC) vaccine fell below the threshold of protection in 50% of vaccinees by one year of age and only 12% of vaccinees maintained protection at four years of age (Snape and Pollard 2005). There is very little evidence that immunization of infants with the MenC vaccine confers protection beyond one year, as antibody titers decrease rapidly following infant immunization (Trotter et al., 2004). When administered after one year of age, polysaccharide vaccines induce a more persistent antibody response. However, sustained levels of protection were not observed until much later in childhood in many children (Snape et al., 2008). In addition, the decline in protective immunity following infant immunization was also noted for *H. influenzae* type b vaccine (Pollard et al., 2009). Therefore, the duration of protective immunity following immunization with polysaccharide antigens depends on the persistence of functional antibodies, maintenance of immunological memory, and establishment of herd immunity (Pollard et al., 2009).

To address the concerns of T-cell independent pure polysaccharide vaccines, in late 1980s it was discovered that chemical conjugation of the polysaccharide antigen to a carrier protein resulted in a polysaccharide-protein conjugate that was T cell dependent and highly immunogenic (Makela and Kayhty 2002). The first polysaccharide-protein conjugate vaccine was produced for *H. influenzae* type b. A conjugate vaccine for *S. pneumonia* was produced in 2000 and for *N. meningitides* in 2005. Conjugate vaccines have resulted in a significant reduction in the disease burden associated with encapsulated bacteria when these vaccines have been introduced in the immunization schedules of multiple countries (Ramsay et al., 2003(a); Heath and McVernon 2002). These vaccines are effective due to their ability to induce memory B cell production, induction of immunity in children under the age of 2, ability to provide herd immunity, and the capacity to overcome hyporesponsiveness (Heath and McVernon 2002; Ramsay et al., 2003(b)).

Although immunogenic, the magnitude of the antibody response to conjugate vaccines and their persistence is age-dependent. It had been observed that in infants and toddlers, the serum antibody concentration following immunization with conjugate vaccines declined rapidly even though the immunological memory lasted much longer (Borrow et al., 2010; Snape et al., 2006). Despite multiple doses being administered to infants, the effectiveness of MenC and Hib vaccines have declined after one year from initial immunization in the absence of a booster dose

after the age of one (Ramsay et al., 2003(a); Trotter et al., 2004). The importance of persistent serum antibodies was demonstrated in vaccine failures where despite being immunized and having memory B cells, some children succumbed to meningococcal and Hib infections (Auckland et al., 2006; McVernon et al., 2003). This is explained by the fact that encapsulated bacteria are able to invade and cause disease within a few hours, while memory B cells require several days to mount a protective antibody response (Snape et al., 2006; Blanchard-Rohner et al., 2008). Thus, induction of immune memory alone is not sufficient to protect against encapsulated bacterial infections and require high titers of persistent serum antibodies. Nonetheless, upon the introduction of the Hib conjugate vaccine in 1988, the disease burden among children in the United States declined by 50% per year from 1988 to 1991 (Schoendorf et al., 1994). In clinical trials, a reduction in the nasal carriage with vaccine-specific *S. pneumonia* serotypes in children was also observed following immunization with two multivalent pneumococcal conjugate vaccines (Dagan et al., 1996; Sood and Fattom 1998). Furthermore, in older children and adults, even a single dose of a conjugate vaccine lead to highly persistent anti-capsular antibodies (Snape et al., 2006; Snape et al., 2008). In Vaccine development bi-, tri-, tetra-, and/or multi- 'valent' means that the given number of separate protein or polysaccharide antigens is combined together in a single vaccine.

Although pure polysaccharide vaccines have been effective at saving lives for years, conjugation of polysaccharide antigens from *H. influenzae* type b, *S. pneumonia*, *N. meningitides*, and *S.* Typhi have generated T cell-dependent antigens that are effective in even the very young children (Plotkin 2003). The first polysaccharide vaccine against *N. meningitides* was used in the United States in 1984, and was given to all the new military personnel upon entering basic training. Upon the administration of this tetravalent polysaccharide vaccine, no cases of meningococcal infections related to serogroup Y or W135 had been reported in the United States military (Sood and Fattom 1998). However, this vaccine was poorly immunogenic among infants and young children (Sood and Fattom 1998). A quadrivalent vaccine containing polysaccharide antigens from *N. meningitides* serogroup A, C, Y, and W135 was initially licensed in the United States in 1981. A bivalent vaccine containing polysaccharide antigens from serogroup A and C is also available in some countries (Pollard et al., 2009).

Serogroup C (MenC) conjugate vaccine containing either tetanus toxoid or cross reacting material 197 (CRM_{197}; which contains a glycerine to glutamic acid point mutation at position 52

in the A subunit of diphtheria toxoid) was first licensed in the United Kingdom in 1999, while a quadrivalent A, C, Y, and W135 diphtheria toxoid conjugate vaccine became first available in North America in 2005 (Nair 2012). In addition, several other conjugate vaccines are currently being produced and in clinical trials including A, C, Y, and W135 conjugated to tetanus toxoid and A, C, Y, and W135 conjugated to CRM_{197} (Pollard et al., 2009). As serogroup B capsular polysaccharide is highly similar to the cell surface glycoprotein on fetal brain tissue and result in tolerance and reduced immunogenicity, development of a polysaccharide based vaccine, either pure or conjugate, remains a significant hurdle in vaccine development against *N. meningitides* serogroup B (Nair 2012). One study compared the effectiveness between the meningococcal serogroups A and C pure polysaccharide vaccine (MACP) vs the conjugate vaccine against serogroup C (MCC), since the use of MACP vaccine had raised concerns that this vaccine is leading to hyporesponsiveness to serogroup C polysaccharide antigen (Richmon et al., 2000). In this study individuals who received a primary immunization of MACP were given a second immunization of either MACP or MCC. Those who received MCC produced significantly higher titers of IgG antibody than the individuals who received MACP as the second dose. In addition, the response to the second MACP vaccine was considerably lower than that following the first immunization. This supported the observation that repeated vaccination with MACP vaccine is ineffective and leads to hyporesponsiveness towards the meningococcal serogroup C polysaccharide antigens in adults. However, it was possible to overcome the MACP induced hyporesponsiveness with subsequent vaccination with MCC (Richmon et al., 2000).

A pure polysaccharide hexavalent vaccine against *S. pneumonia* was first licensed in 1947, but was eventually withdrawn due to low sales and lack of acceptance (Sood and Fattom 1998). A polyvalent vaccine containing 14 polysaccharide antigens became available in the United States in 1977, and was recommended for at risk populations including the elderly, immunocompromised patients, and individuals with chronic cardiac, pulmonary, or renal diseases (Shapiro et al, 1991). The vaccine had 93% efficacy following three years from initial immunization among immunocompetent individuals under 55 years of age. However in vaccinees over 85 years of age, the efficacy was only 46% after three years (Shapiro et al., 1991). Furthermore, the vaccine's efficacy was shown to decline over time. However, this can be explained by the waning immunity and the exposure to additional pneumococcal serotypes over the years (Halloran et al., 1991; Shapiro et al., 1991).

The widely used current polyvalent pneumococcal vaccine, licensed in 1983, is made of 23 immunologically distinct polysaccharide antigens and is effective in preventing disease in immunocompetent individuals (Shapiro et al., 1991). In addition, there are several other pneumococcal vaccines that are also in use. These include the seven-valent conjugate vaccine (PCV7) that contain the serotypes 4, 6B, 9V, 14, 18C, 19F, and 23F, and the ten-valent conjugate vaccine additionally containing serotypes 1, 5, and 7F. The eleven-valent and thirteen-valent conjugate vaccines are currently undergoing clinical trials (Pollard et al., 2009; Nair 2012). As there are multiple pathogenic *S. pneumonia* serotypes, development and conjugation of each polysaccharide to carrier proteins remains a complex process and is quite challenging (Nair 2012).

A pure polysaccharide vaccine for *H. influenzae* type b (Hib) was first used in the United States in 1985, but due to poor efficacy in children younger than 18 months of age and immunological variability in older children, 88% to -69%, where a negative efficacy means higher disease risk for the immunized population compared to the non-vaccinated group, the vaccine was withdrawn from use in 1988 (Robbins et al., 1992). Pure polysaccharide Hib vaccine elicited an age-dependent antibody response, where older children and adults developed protective levels of antibodies with long lasting efficacy (Rodrigues et al., 1971; Smith et al., 1973), while young children generate protective, but lower levels of antibody titers that decrease after one year from initial immunization. In addition, infants, being the most susceptible to Hib infection, were non-responsive to the pure polysaccharide Hib vaccine (Smith et al., 1973; Robbins et al., 1992).

In 1989 the pure polysaccharide vaccine was replaced by a polysaccharide-protein conjugate vaccine linking the Hib polysaccharide polyribosylribitol phosphate to diphtheria toxoid, tetanus toxoid, or meningococcal outer membrane protein (Pollard et al., 2009). The United Kingdom had introduced the Hib conjugate vaccine to the infant immunization schedule in 1992 (Pollard et al., 2009). These vaccines are administered to date either alone or in combination with other vaccines for the protection of preschool children (Pollard et al., 2009). Meningitis and other infections caused by Hib have been completely eliminated in both vaccinated and unvaccinated children due to herd immunity in countries that have incorporated Hib conjugate vaccine in the infant immunization schedule (Adams et al., 1993; Robbins et al., 1992). The antibody subclass highly induced and mainly sustained by pure polysaccharide Hib or

Hib conjugate vaccines in adults is IgG2. Subsequent immunizations with pure polysaccharide Hib or Hib conjugate vaccines do not generate booster responses in adults (Schneerson et al., 1986; Robbins et al., 1992). However, multiple immunizations with Hib conjugate vaccine elicit booster responses in infants with elevated IgG1 titers (Parke et al., 1991; Claesson et al., 1988; Robbins et al., 1992).

The pure polysaccharide vaccine against *S.* Typhi is made up of the Vi capsular polysaccharide antigens. The serum antibody towards Vi antigen is mostly IgM driven. In one study only 2 out of 32 subjects generated sufficient amount of IgG towards the Vi antigen (Tacket et al., 2004). However, in typhoid fever endemic regions, immunization with the Vi vaccine resulted in 55% and 75% efficacy rates among adults and children over the age of 5 years (Sood and Fattom 1998). Vaccines for Typhoid fever are discussed in more detail in Section 1.3.2 "*Salmonella* Vaccines: Past, Present, and Future".

Another polysaccharide-protein conjugate vaccine candidate is *Shigella* O-specific polysaccharide (O-SP) domain of LPS. It had been shown that serum IgG to O-SP induced protective immunity to Shigellosis (Passwell et al., 2001). In order to increase immunogenicity in young children, O-SP was covalently linked to carrier proteins. In one clinical study it was shown that conjugation of *Shigella sonnei* and *Shigella flexneri* 2a O-SP to succinylated *Pseudomona aeruginosa* exotoxin A mutants or *Corynebacterium diphtheria* toxin mutants was highly immunogenic, and resulted in high titers of serum IgG against O-SP within one week of immunization (Passwell et al., 2001). Out of the different vaccine candidates *P. aeruginosa* conjugate vaccine resulted in the highest IgG titers and the highest efficacy was observed for *S. sonnei* conjugate vaccine at 74% (Passwell et al., 2001; Ferreccio et al., 1991). Following immunization with *S. sonnei* conjugate vaccine, IgG and secretary IgA antibody had been detected in urine, implying that this vaccine was able to induce mucosal immunity (Cohen et al., 1996). In addition, a single immunization with *S. sonnei* rEPA conjugate vaccine conferred specific protection against shigellosis in young adults (Cohen et al., 1997).

Staphylococcus aureus type 5 and type 8 capsular polysaccharides are of high clinical importance. Immunization with *S. aureus* capsular polysaccharide conjugate vaccines generated high titers of specific antibodies. These antibodies were able to recognize variations in capsular polysaccharide that occur among clinically significant *S. aureus* isolates (Fattom et al., 1998). In addition, the *Vibrio cholera* O1 serotype Inaba polysaccharide and cholera toxin conjugate

vaccine was shown to induce serum anti-LPS IgG with bactericidal activity. *V. cholera* specific antibodies, whether acquired through natural immunity, following colonization by the bacteria, or after immunization, are all directed against the LPS molecules (Gupta et al., 1998). Furthermore, antibody generated against the O-specific polysaccharides of *V. cholera* serotype Inaba were able to cross recognize polysaccharides from *V. cholera* serotype Ogawa (Gupta et al., 1998). In another study, *S.* Typhimurium O-antigens 4 and 12 covalently linked to bovine serum albumin (BSA) when administered with Freund adjuvant led to the production of significantly high O4 specific antibody titers in rabbits; comparable to those elicited by the immunization with heat killed bacteria (Svenson and Lindberg 1981).

1.3.2 *Salmonella* Vaccines: Past, Present and Future

Development of an effective *Salmonella* vaccine for humans remains a pressing issue today. *Salmonella* are able to survive both extracellularly and intracellularly within monocytes and macrophages. This is a significant challenge for vaccine development, as both humoral and cell mediated immunity are needed to efficiently target and clear both extracellular and intracellular bacteria (Mastroeni et al., 1993; Salerno-Goncalves et al., 2002). Antibodies mediate pathogen killing through opsonisation, thereby facilitating phagocytosis and preventing the spread of disease through blood (Mastroeni et al., 2009). T cells, on the other hand, are involved in destroying *Salmonella* residing within phagocytic cells (Blanden et al., 1966; Mackaness et al., 1966) (discussed in detail in 1.1.3 "Clinical Manifestation'). The requirement of both arms of the immune system had been demonstrated through animal studies, where mice lacking T cells were unable to control *Salmonella* infections (Sinha et al., 1997). In other studies, passive transfer of specific antibodies provided significant clearance of *Salmonella* in vivo (Mastroeni et al., 1993; McSorley and Jenkins 2000).

The first vaccine against *Salmonella* was developed in 1896 against *S.* Typhi, and was used by British and United States military personnel. Over the next 100 years the vaccine had been in use, this inactivated whole cell vaccine had dramatically reduced the number of deaths associated with typhoid fever (MacLennan et al., 2014; Hawley and Simmons 1934). Although this vaccine had an efficacy of 73% over three years post immunization, it also had high reactogenicity and therefore its use was discontinued (Engels et al., 1998; Ivanoff et al., 1994; Wahdan et al., 1975).

Currently, there are two vaccines against *S*. Typhi: Ty21, a live attenuated vaccine, and Vi capsule-based vaccine made up of purified Vi capsular polysaccharides (Table 1.3.2(a)). Even though Ty21 strain was derived from non-specific chemical mutagenesis of the *S*. Typhi Ty2 strain, Ty21 does not express Vi capsule, and as a result, the immune response to live attenuated Ty21 strain is not directed against the Vi capsule (Germanier and Furer 1975). The Ty21vaccine requires three doses to induce best protective immunity and in combination the three doses have an efficacy of 51% (Engels et al., 1998; Fraser et al., 2007). This vaccine is not thermostable over long periods of time and requires a cold chain. This is a setback as the vaccine is most needed in developing countries where access to refrigeration is difficult (MacLennan et al., 2014). It had been shown that freeze drying can increase the thermal stability of the vaccine, but this measure is currently not in place (Ohtake et al., 2011). Despite the Ty21vaccine being licenced to use in adults and children over five years, seroconversion is greatly reduced in young children compared to adults (Cryz et al., 1993). Nonetheless, the Ty21 vaccine can induce T cell immunity and had shown cross protection against *S*. Paratyphi B in clinical studies. In addition, *in vitro* cross protection had been shown for *S*. Paratyphi A and B (Pakkanen et al., 2012; Wahid et al., 2012). As the antibody response is mostly directed against the O:9 O-antigen epitope of *S*. Typhi, it had been shown that the Ty21 vaccine is able to elicit cross protective immunity against iNTS and NTS serovars also expressing the O:9 or O:9,12 epitopes (Pakkanen et al., 2014; MacLennan et al., 2014; Kantele et al., 2012).

Although the immune response to the Vi capsule based vaccine is primarily directed towards the Vi capsule, recent evidence had indicated that the immune response could also be due to other *Salmonella* components in the vaccine (Kantele et al., 2012). However, the vaccine is considered to be non-immunogenic in infants, due to Vi capsule being a T-independent antigen. Therefore it is only licenced for children over 2 years of age (MacLennan et al., 2014). In contrast to Ty21 vaccine, the Vi capsule-based vaccine requires a single dose and has a efficacy of 55% over three years. This vaccine also requires a cold chain to prolong the life and effectiveness of the vaccine (MacLennan et al., 2014). It had been shown that following vaccination with Vi capsule based vaccine, circulating plasmablasts (immature plasma cells) express systemic homing receptors (L selectin), while vaccination with Ty21 vaccine lead to the expression of mucosal homing receptors ($\alpha_4\beta_7$), as with natural infection (Kantele et al., 2012). No clinical trial had been performed to date to examine the efficacy and protection of these two

vaccines combined (MacLennan et al., 2014). However, several clinical trials have tested the efficacy and type of immune response mounted following immunization with inactivated whole cell *Salmonella* vaccine candidates (Nath et al., 1977; Rajagopalan et al., 1982).

Name	Description	Developer	Stage of Development
Vaccines currently available and in development against *S*. Typhi			
Ty21a	Live attenuated	Vivotif (Crucell)	Licensed for adults and children over 5 years
Vi CPS	Vi polysaccharide	Typherix (GSK), Typhim Vi (Sanofi), Tybar Vi (Bharat Biotech), Typho Vi (BioMed) and Vax-tyVi (Finlay Institute), also manufactured by 6 other endemic countries	Licensed for adults and children over 2 years
Vi-TT	Vi conjugate	Peda-Typh (BioMed)	Licensed in India
		Tybar-TCV (Bharat Biotech)	Licensed in India
Vi-rEPA	Vi conjugate	National Institutes for Health (NIH)	Phase 3
		Lanzhou Institue (China)	Licensed in China
Vi-CRM	Vi conjugate	Novartis Vaccine Institute for Global Health (NVGH) (technology transfer to Biological E. Limited underway)	Phase 2
Vi-DT	Vi conjugate	International Vaccine Institute (IVI)/Shanta Biotech	Phase 1
Vi conjugated to fusion protein PsaA-PdT	Vi conjugate	Harvard Medical School	Preclinical
O:9-DT	O:9 conjugate	International Vaccine Institute (IVI)	Preclinical
M01ZH09	Live attenuated	Emergent Biosolutions	Phase 2 in adults and children; evaluation in *S.* Typhi human challenge
CVD 909	Live attenuated	University of Maryland	Phase 2
Ty800	Live attenuated	Avant immunotherapeutics	Phase 2

OmpC and OmpF	Outer membrane protein	Institute Mexicano del Seguro Social	Phase 1 in Mexico

Vaccines in development against *S*. Paratyphi A			
O:2-TT	O:2 conjugate	National Institutes for Health NIH)	Phase 2
		Technology transfer from NIH to Lanzhou Institute (China)	Phase 2
		Technology transfer from NIH to Chengdu Institute (China)	Preclinical
		Changchun Institute of Biological Products	Preclinical
O:2-DT (development in combination with Vi-DT conjugate against *S*. Typhi)	O:2 conjugate	International Vaccine Institute (IVI) (Seoul, Korea)	Preclinical
O:2-CRM (development in combination with Vi-CRM conjugate against S. Typhi)	O:2 conjugate	Novartis Vaccine Institute for Global Health (NVGH) (technology transfer to Biological E. Limited underway)	Preclinical
VCD 1902 (development in combination with CVD 909)	Live attenuated	University of Maryland	Phase 1

Vaccines in development against invasive non-Typhoidal *Salmonella* (iNTS)			
O:4,5/O:9-flagellin	O:4,5/O:9 conjugate	University of Maryland	Preclinical
O:4, 12-TT	O:4-TT conjugate	National Institutes for Health NIH)	Preclinical
Os-po	O:4-porin conjugate	National Bacteriology Laboratory, Stockholm	Preclinical
O:4,5/O:9-CRM	O:4,5/O;9 conjugate	Novartis Vaccine Institute for Global Health (NVGH)	Preclinical
WT05	Live attenuated	Microscience, Wokingham Berkshire	Phase 1
CVD 1921 and	Live attenuated	University of Maryland	Preclinical

CVD 1941			
S. Typhimurium ruvB mutant	Live attenuated	Seoul National University	Preclinical
Salmonella hfq deletion mutant	Live attenuated	Indian Institute of Science Bangalore	Preclinical
SA186	Live attenuated	Istituto Superiore di Sanita Roma	Preclinical
MT13	Live attenuated	KIIT University Odisha	Preclinical
Various	Live attenuated, DNA adenine methylase mutants	University of California, Santa Barbara	Preclinical
Various	Live attenuated, regulated delayed attenuation	Arizona State University	Preclinical
Porins	*S*. Typhimurium porins	National Bacteriology Laboratory, Stockholm	Preclinical
OmpD	Outer membrane protein	University of Birmingham, UK	Preclinical
S. Typhimurium and *S*. Enteritidis GMMA	Generalized Modules for Membrane Antigens	Novartis Vaccine Institute for Global Health (NVGH)	Preclinical

Table 1.3.2(a): Vaccines currently in use and vaccine candidates being developed for humans against *S*. Typhi, *S*. Paratyphi A, and iNTS. Note that most vaccine candidates for *S*. Typhi are currently in clinical trials, while those for *S*. Paratyphi A and iNTS are still in preclinical trials. This is not a comprehensive list of all the vaccine candidates that are being tested. Table extracted from MacLennan et al., 2014, reproduced with kind permission from Human Vaccine & Immunotherapeutics.

The Vi capsule-based vaccine primarily induces humoral immunity, since almost always polysaccharides are T-independent antigens and require conjugation to carrier proteins to induce T cell-dependent immune responses (Suz et al., 2014; Pollard et al., 2009). The importance of antibody in clearing *S*. Typhi infections was demonstrated by efficacy data for Vi capsule based vaccine and Vi conjugate vaccine candidate phase 3 studies (Engels et al., 1998; Fraser et al., 2007; Lin et al., 2001; Thiem et al., 2011). A phase 3 study of a glycoconjugate vaccine combining *S*. Typhi Vi capsule with *P. aeruginosa* exoprotein A (Vi-rEPA), conducted over a decade ago in Vietnamese children between two and five years of age, found a 91% efficacy after 27 months and 89% efficacy after 46 months from primary immunization (MacLennan et

al., 2014). However, the delay in seeing new vaccines in the market is mainly due to a lack of commercial interest in developing vaccines against typhoid fever and iNTS disease, since these are largely diseases of low-income countries, which have little or no money to invest in vaccines (MacLennan 2013). New vaccines are expected to provide improved protection and efficacy, especially in young children, and to reduce the cost of delivery these vaccines are to be administered as part of the national Expanded Programmes on Immunization (MacLennan et al., 2014). Vaccines for *S*. Typhi and/or *S*. Paratyphi are to be given at nine months, as disease peaks at two years of age, and vaccines for iNTS are to be given between 2 and 4 months of age, prior to the disease peak at one year of age (Podda et al., 2010; MacLennan et al., 2008; Feasey et al., 2010).

Although a humoral response is sufficient to clear *S*. Typhi infections, both antibody and cell-mediated immunity are required for proper clearance of iNTS. This had been shown in field studies in sub-Saharan Africa, where age-related prevalence of iNTS disease in children declined with acquisition of specific antibody (MacLennan et al., 2008). In addition, serum antibodies had shown *in vitro* bactericidal activity and oxidative killing of iNTS (MacLennan et al., 2008; Gondwe et al., 2010). It had also been demonstrated that O-antigen-based conjugate vaccine candidates can induce sufficient protection against otherwise lethal doses of *Salmonella* in mice (Simon et al., 2011; Watson et al., 1992). Passive transfer of monoclonal antibodies specific for O-antigen was also able to confer protection against lethal doses of *Salmonella* challenge (Carlin et al., 1987; Singh et al., 1996). Immunization of animals with heat-killed invasive African *S*. Typhimurium 313 strain leads to majority of antibody to be mounted against O-antigen (Rondini et al., 2013). On the other hand, the importance of cell-mediated immunity in clearing iNTS is apparent through the association between HIV infection and predisposition to iNTS, especially when the $CD4^+$ count is below 200 cell/uL (Gordon et al., 2002). HIV is also known to increase susceptibility to NTS through dysregulation of cytokine response, humoral immunity, and disruption of the integrity of the gastrointestinal mucosa (Mackaness et al., 1966; Lazarus and Neu 1975). Therefore, vaccine candidates that induce protective mucosal immunity, preventing gastroenteritis and NTS invasion from the gastrointestinal tract, would be highly beneficial for HIV-infected individuals (MacLennan et al., 2014).

Currently there are several different types of novel *Salmonella* vaccine candidates being tested (Table 1.3.2(a)) which fall in to three main categories: glycoconjugate, live attenuated, and

subunit vaccines. Glycoconjugate vaccines covalently link a poorly immunogenic antigen, such as a bacterial surface polysaccharide, to a protein carrier molecule to activate $CD4^+$ T cells. Thus, glycoconjugation is able to convert the Vi capsule or O-antigen of *Salmonella* from T independent to T-dependent antigens and facilitate the production of specific antibodies and memory B cells (MacLennan et al., 2014). For glycoconjugation most *Salmonella* vaccine candidates use the common carrier proteins tetanus toxoid, diphtheria toxoid, a nontoxic recombinant of diphtheria toxoid, or *P. aeruginosa* exoprotein A (rEPA) (MacLennan et al., 2014). It had been proposed that glycoconjugate vaccines using *Salmonella* carbohydrates linked to *Salmonella* proteins could be more effective at inducing *Salmonella*-specific antibody than using exogenious carrier proteins, since antibody can be produced against both *Salmonella* protein and carbohydrate (Simon and Levine 2012; Svenson et al., 1979). In addition, conjugation to *Salmonella* proteins can also lead to the generation of *Salmonella*-specific T cells. It had been shown that conjugation of *S.* Typhimurium O:4 to its porins lead to better protection of mice upon challenge, instead of using porins alone or O:4 conjugated diphtheria toxoid (Svenson et al., 1979). As flagellin is the only *Salmonella* surface typing protein antigen it had been investigated for the ability to generate protective immunity. It had been shown in mice that immunisation with flagellin alone, or in conjugation with O-antigen lead to the induction of protection (Simon et al., 2011; Eom et al., 2013; Simon et al., 2013). For a example, immunization with O:4,5 and O:9 conjugated to *Salmonella* flagellin in iNTS vaccine candidates lead to enhanced protective immunity (Simon et al., 2011; Bobat et al., 2011). Flagellin, being the primary ligand for TLR5 activation, can signal to the innate immune system and lead to immunomodulatory effects in mice (Simon et al., 2013 Cunningham et al., 2004). One concern is that in some *Salmonella* serovars, such as in *S.* Typhimurium, flagellin can have phase variable expression and is not constitutively expressed during infection (MacLennan et al., 2014).

Live, attenuated vaccines are able to induce both humoral and cellular immunity, and thereby facilitate *Salmonella* specific T cell responses. These vaccines are easy to deliver (orally) and can induce strong mucosal immune responses (Kantele et al., 2012). In addition, the availability of numerous *Salmonella* antigens on live bacteria (Table 1.3.2(b)) can allow the development of broad protective immune responses and even allow cross recognition between *Salmonella* serovars. The primary challenge with developing live attenuated vaccines is in balancing the level of immunogenicity with proper and sufficient level of attenuation

(MacLennan et al., 2014). Currently several live attenuated *S.* Typhimurium strains are being tested for their efficacy in preclinical and clinical studies (Table 1.3.2(a)). Also several recombinant *Salmonella* strains expressing heterologous antigens are being assessed for their ability to protect against various pathogens (Mittrucker and Kaufmann 2000). Furthermore, it will be useful to develop vaccines that require one or two doses to mount an optimal immune response, compared to the required three doses of the current Ty21 vaccine (MacLennan et al., 2014).

Mutated gene	Gene function(s)
galE	Conversion of UDP-galactose to UDP-glucose
aro	Biosynthesis of PABA, DHB and aromatic aminoacids
pur	Biosynthesis of adenine
htrA	Protection against heat stress and oxidative stress
cya/crp	Biosynthesis of cAMP and expression of the AMP receptor
ompR	Regulation of the expression of outer membrane protein and Vi antigen
ompC, ompF	Biosynthesis of outher membrane proteins C or F
phoP/phoQ	Two-component system that is a key regulator of many virulence genes
waaN	Secondary acylation of lipid A
recA, recBC	DNA recombination and repair
guaBA	Biosynthesis of guanine nucleotides
nuoG	Expression of NADH dehydrogenase-I
surA	Biosynthesis of a peptidylprolyl-*cis*, *trans*-isomerase
dam	Expression of DNA adenine methylase

Table 1.3.2(b): *Salmonella* genes mutated in live-attenuated vaccines. Immunization with live *Salmonella* mutated in these genes decrease the virulence and allow the induction of strong protective cell mediated and humoral immune responses. Table 1 of Mastroeni et al., 2001 with modifications, reproduced with kind permission from Elsevier.

Protein-based subunit vaccines are composed of multiple antigenic epitopes, and can have broad coverage. A subunit approach is currently in use for multivalent pneumococcal and meningococcal conjugate vaccines. However, subunit vaccines, especially those using glycoconjugate technologies, are costly to produce and since most vaccines are to be used in the poorest countries, affordability remains a key factor in vaccine development. In order to balance the cost effectiveness, it had been proposed that multivalent vaccines composed of 5 to 6

antigens could provide cross protection against the majority of the iNTS serovars (Simon and Levine 2012). Another strategy to develop broad coverage, cross protective vaccines are to use highly conserved protein antigens produced through recombinant technology (Gil-Cruz et al., 2009; Secundino et al., 2005; Salazar-Gonzalez et al., 2004). Proper antigens can be selected using bioinformatics analysis of whole genome sequences and reverse vaccinology (Sette and Rappuoli 2010). Examples of highly conserved protein antigens include flagellin and porins (i.e. OmpC, F, and D). Currently, recombinant and purified protein vaccine candidates are being tested against conserved protein epitopes (World Health Organization 2014). Subunit vaccines can induce both T cell responses and antibody production; a balanced Th1-Th2 approach that is key to proper clearance of *Salmonella*. One of the downsides of subunit vaccines developed through recombinant technology is that it is difficult to maintain and preserve the proper conformation of proteins, especially when there are several membrane spanning domains. This can result in induction of a poor antibody response. One approach is to purify proteins from whole *Salmonella* rather than relying on recombinant proteins (Salazar-Gonzalez et al., 2004).

As glycoconjugate vaccines primarily rely on the production of antibodies specific for the surface carbohydrate moieties, it may not be sufficient to efficiently deal with growing iNTS disease. The development of new live-attenuated, protein based, or GMMA (Generalized Modules for Membrane Antigens) based vaccines will surve to provide broader protection by activating *Salmonella* specific T cell responses (MacLennan et al., 2014). GMMA technology is used to deliver outer membrane proteins and surface polysaccharides in the correct orientation and confirmation to induce protective immunity. The use of GMMA has adjuvant activity by co-delivering multiple PAMPs along with the target antigens (World Health Organization 2014). However, to date, vaccine development has been hindered due to a lack of understanding regarding specific antigenic epitopes of *Salmonella*, complications in protein purification, and manufacturing issues with respect to preservation of proper protein confirmation (MacLennan et al., 2014).

This is a very brief discussion of *Salmonella* vaccines for animals. Out of all food animals, *Salmonella* vaccine industry is most developed for poultry (Table 1.3.2(c)), with the use of live attenuated, inactivated, and subunit vaccines (Desin et al., 2013). In poultry *S*. Gallinarum and *S*. Pullorum are the main causative agents of fowl typhoid fever and pullorum disease respectively. In addition, *S*. Enteritidis, *S*. Typhimurium, *S*. Heidelberg, and *S*. Kentucky cause asymptomatic

disease, and in rare cases systemic infection in mature chickens (Desin et al., 2013). Unlike in humans, vaccination of chickens does not lead to clearance of *Salmonella*, but a reduction of the level of *Salmonella* associated with the chicken upon infection (Desin et al., 2013). In addition to the previously discussed issues associated with live attenuated vaccines, one additional concern is that the live strain could survive for extended periods in chickens posing a health risk for humans (Tan et al., 1997). However, live attenuated strains are capable of horizontal transfer among birds, and therefore promising to be highly efficient vaccine antigens (Tan et al., 1997). Inactivated vaccines are made of killed whole *Salmonella*, and birds are immunized intramuscularly or subcutaneously and require a minimum of two doses (Barrow 2007; Gast 2007). Although inactivated vaccines are capable of inducing strong antibody responses, they can be eliminated within a short time span by the host. In addition, these vaccines require adjuvants to confer optimal immune responses (Barrow 2007; Gast 2007). *Salmonella* subunit vaccines are mainly administered intramuscularly or subcutaneiously; oral administration requires the use of proper formulations to minimize antigen degradation in the intestine. Although, these vaccines do not pose many of the health risks associated with live attenuated vaccines, they are usually poorly immunogenic and require adjuvants. However, subunit vaccines for *Salmonella* are still at an early stage of development (Mutwiri et al., 2011). Currently there are many vaccine candidates being tested for use in poultry including nucleic acid, bacterial and viral vector based vaccines (Kaiser 2010). In addition, there are vaccines and vaccine candidates being tested for swine, cattle, and equines (Farzan and Friendship 2010; Singh 2009; Smith et al., 2015).

Name	**Description**	**Frequency of immunization**	**Effect**
Nobilis® SG 9R	Live-attenuated	Twice (6 and14-16 weeks)	Protection (2.5% flocks were positive relative to 11.5% of control
TAD *Salmonella* vac® E	Live-attenuated	Three doses (1 day, 6 weeks, 16 weeks)	Protection (12/28 liver, 6/28 oviduct, 9/35 egg samples positive relative to control 22/30, 15/29, 15/35 respectively)
Megan® Vac 2	Live-attenuated	Three doses (1 day, 2 weeks, 5 weeks)	Protection (38% cecal and 14% reproductive tract samples positive relative to control 68% and 52% respectively)
Salenvac®	Inactivated	Twice (1 day and 4 weeks)	Protection from shedding and reduction of colonization

AviPro® *Salmonella* vacT	Live-attenuated	Broiler: 1 dose, Layers/Breeders: 1 day, 7 weeks, 16 weeks	Limited persistence in internal organs and long lasting protecton from S. Typhimurium
AviPro® *Salmonella* vacE	Live-attenuated	Three doses	Short duration of shedding and protection from *S.* Enteritidis for laying hens
Layermune SE®	Inactivated	Twice (5 and 9 weeks)	Reduction of colonization in layers only
Poulvac SE®	Inactivated	Twice (12 and 20 weeks)	Protection in day old chicks (7/25 positive chicks relative to control 25/25)
Corymune® 4K and 7K	Inactivated	Twice (5 and 9 weeks)	Minor effect on colonization

Table 1.3.2(c): Commercial *Salmonella* vaccines for poultry. A brief summary of some widely used commercial live attenuated and inactivated vaccines in poultry against *Salmonella* colonization. Table adopted from Opinion of the scientific panel 2004 and Desin et al., 2013.

Our group has previously discovered a novel surface associated polysaccharides termed O-Antigen capsule in Non-Typhoidal *Salmonella* (NTS). The O-Antigen capsule is considered to be a common surface antigen, since the biosynthesis genes and cross-reactive material have been detected in all subgroups of *Salmonella*. In addition, the O-Antigen capsule genes were found to be expressed during *in vivo* infections in mice. Such an antigen would be a suitable candidate in developing a vaccine against *Salmonella* induced gastroenteritis. The goal of this research was to evaluate the potential of using O-Antigen capsule as a vaccine antigen to develop a traveler's vaccine for gastroenteritis caused by NTS serovars.

2.0 HYPOTHESIS AND OBJECTIVES

HYPOTHESES:

- Deletion of the putative repressor of the O-Antigen capsule operon will boost the activity of the promoter and lead to more capsule production.
- Immunization with purified *S.* Typhimurium O-Antigen capsule will generate an antibody response in rabbits and mice against the O-Antigen capsule.

OBJECTIVES:

1. To purify the O-Antigen capsule and to determine the best possible method to increase the yield of the capsule, while decreasing LPS contamination.
2. To determine the appropriate dosage of O-Antigen capsule required to obtain the best possible immune response in mice immunized with purified *S.* Typhimurium O-Antigen capsule.
3. To characterize the gene expression of the O-Antigen capsule operon.

3.0 MATERIALS AND METHODS

3.1 Bacterial Strains and Growth Conditions

Unless otherwise stated, the following were the standard conditions used in bacterial culture preparations. O/N cultures were grown in LB +/- antibiotic and incubated at 37^{o}C. Antibiotic concentrations were as follows: Amp 100ug/mL (Amp_{100}), Cm 10ug/mL (Cm_{10}) or 30ug/mL (Cm_{30}), Tet 7ug/mL (Tet_{7}), and Kan 50ug/mL (Kan_{50}). For O-Antigen capsule purification, cells were grown on EPS agar and/or 1% Tryptone agar at 28^{o}C-30^{o}C for 5 days (detailed description in 3.4.1 "O-Antigen Capsule and X-factor Purification").

Luciferase assays were carried out in 1% Tryptone media with 50ug/mL Kan at 30^{o}C. Bacterial strains used in this study are presented in Table 3.1. Some strains will be discussed in more detail in the 4.0 "Results" section.

Strain	Source
E. coli DH10B	Quandt J., and Hynes M.F. (1993). *Gene*. 127(1):15-21.
H. Somni (HS25)	Dr. Andrew Potter's Lab, VIDO-InterVac, University of Saskatchewan: Alberta field lot isolate from 1983 (lung isolate from a female cow with pneumonia)
S. Enteritidis 27655-3b Δ*bcsA*	White et al., (2003). *Journal of Bacteriology*. 185(18):5398-5407.
S. Heidelberg S4825-1.1 phagetype19	Agri-Food Laboratories Branch, Food Safety and Animal Health Division, Alberta Agriculture and Rural Development (liver/spleen isolate from a chicken)
S. Typhimurium ATCC 14028S WT	American Type Culture Collection (ATCC), Manassas, VA, USA.

S. Typhimurium ATCC 14028S Δ*yihQ*	Gibson et al., (2006). *Journal of Bacteriology*. 188(22):7722-7730.
S. Typhimurium ATCC 14028S Δ*bcsA*	This Study
S. Typhimurium ATCC 14028S Δ*bcsA* Δ*yihVW*	This Study
S. Typhimurium ATCC 14028S Δ*bcsA* Δ*yihW*	This Study
S. Typhimurium ATCC 14028S WT pBR322-*yihVW*	This Study
S. Typhimurium ATCC 14028S WT pCS26-Pac-P*yihU*::lux	This Study
S. Typhimurium ATCC 14028S WT pCS26-Pac-P*yihV*::lux	This Study
S. Typhimurium ATCC 14028S WT pBR322-*yihVW* pCS26-Pac-P*yihU*::lux	This Study
S. Typhimurium ATCC 14028S WT pBR322-*yihVW* pCS26-Pac-P*yihV*::lux	This Study
S. Typhimurium ATCC 14028S Δ*bcsA* pBR322-*yihVW*	This Study
S. Typhimurium ATCC 14028S Δ*bcsA* pCS26-Pac-P*yihU*::lux	This Study
S. Typhimurium ATCC 14028S Δ*bcsA* pCS26-Pac-P*yihV*::lux	This Study
S. Typhimurium ATCC 14028S Δ*bcsA* pBR322-*yihVW* pCS26-Pac-P*yihU*::lux	This Study
S. Typhimurium ATCC 14028S Δ*bcsA* pBR322-*yihVW* pCS26-Pac-P*yihV*::lux	This Study
S. Typhimurium ATCC 14028S Δ*bcsA* Δ*yihVW* pBR322-*yihVW*	This Study
S. Typhimurium ATCC 14028S Δ*bcsA* Δ*yihVW* pCS26-Pac-P*yihU*::lux	This Study
S. Typhimurium ATCC 14028S Δ*bcsA* Δ*yihVW* pCS26-Pac-P*yihV*::lux	This Study
S. Typhimurium ATCC 14028S Δ*bcsA* Δ*yihVW* pBR322-*yihVW* pCS26-Pac-P*yihU*::lux	This Study
S. Typhimurium ATCC 14028S Δ*bcsA* Δ*yihVW* pBR322-*yihVW* pCS26-Pac-P*yihV*::lux	This Study
S. Typhimurium ATCC 14028S Δ*bcsA* Δ*yihW* pCS26-Pac-P*yihU*::lux	This Study
S. Typhimurium ATCC 14028S Δ*bcsA* Δ*yihW* pCS26-Pac-P*yihV*::lux	This Study
S. Typhimurium ATCC 14028S Δ*bcsA* Δ*yihW* pBR322-*yihVW* pCS26-Pac-P*yihU*::lux	This Study
S. Typhimurium ATCC 14028S Δ*bcsA* Δ*yihW* pBR322-*yihVW* pCS26-Pac-P*yihV*::lux	This Study

Table 3.1: Bacterial Strains used in this study.

3.2 Generating *S.* Typhimurium Mutants

3.2.1 Cellulose Mutant Δ*bcsA*

An in-frame deletion removing 1,998 bp in *bcsA* (encoding amino acids 165 to 828 in BcsA) was generated as previously described (Zogaj et al., 2001) with some modifications. Primer YHJ05 was modified to contain an *Eco*RI site instead of a *Bam*HI site, and a new YHJ07 primer (CCACTGCAGATTCGCGCCGCCTTCAGTAA [a *Pst*I site is underlined]) was generated since *S.* Typhimurium *bcsA* contains a unique *Eco*RI site corresponding to amino acids 828 to 829; primers YHJ06 and YHJ08 were used as described previously. Regions of DNA surrounding the *bcsA* gene were PCR-amplified from the *S.* Typhimmurium 14028S WT. The YHJ05+YHJ06 PCR product was digested with *Eco*RI and *Pst*I to generate fragment #1 and the YHJ07+YHJ08 product was digested with *Pst*I and *Hin*dIII to generate fragment #2. These fragments were sequentially cloned into pTZ18R, and then removed together by digesting with EcoRI and *Hin*dIII. The resulting product was ligated into pHSG415 and electroporated into *S.* Typhimurium 14028S (Figure 3.1.1). The allelic exchange procedure used is described in White et al., 2007. In potential *bcsA* mutant strains, the *bcsA* deletion was confirmed by a reduced product size upon PCR-amplification with Taq polymerase (New England Biolabs - NEB) and primers *bcsA*ko1 (CGGCCCGTTACCTCATTCAG) and *bcsA*ko2 (TTCAGCACCGCTTTCGACGC); reaction conditions were as recommended by the manufacturer.

3.2.2 Mutants *S.* Typhimurium Δ*yihVW* and *S.* Typhimurium Δ*yihW*

PCR products containing the *cat* gene, which codes for chloramphenicol resistance, and 50 bp flanking regions corresponding to the beginning of *yihV* and the end of *yihW* were generated from pKD3 (Datsenko and Wanner, 2000) using Phusion polymerase (Fisher Scientific) and primers *yihVW*koFOR (TTCGTGAAATTAAAATGAGCACATCGAAAATGCTTGAGGAA TGACCATGGGTGTAGGCTGGAGCTGCTTC) and *yihVW*koREV (TTGGCCGGATAAAG CGCTGACGCGACCCTCCGGCGCAAGGGCGCTTGTCACCTCCTTAGTTCCTATTCCG): reaction conditions as recommended by the manufacturer. This PCR product was electroporated

into *S.* Typhimurium cells containing pKD46, which encodes the lambda-red recombinase gene products (*γ,β,exo* encoding Gam, Bet and Exo) required for chromosomal recombination. Gam inhibits RecBCD exonuclease V activity allowing Bet and Exo to gain access to DNA to promote recombination with *cat* (Datsenko and Wanner 2000). Recipient cells were plated on LB + Cm_{10} agar and incubated O/N at 37°C. Positive clones were re-streaked on to LB + Cm_{30} agar and incubated O/N at 37°C. Positive clones were checked by PCR for the replacement of *yihVW* with the Cm^R marker using Taq polymerase (NEB) and primers *yihVW*detect1 (GCACATCGAA AATGCTTGAGGA) and *yihVW*detect 2 (ATATCGCCTGCATCACAGCG); reaction conditions were as recommended by the manufacturer.

Deletion of *yihW* was performed in the same manner as deletion of *yihVW* described above. The only difference was that pKD3 amplification was performed using Phusion polymerase and primers *yihW*koF (TAATATGAGCAGTAGGAAGC TTTTAGAGGAATGCTCATGAGTGTA GGCTGGAGCTGCTTC) and *yihVW*koREV; reaction conditions were as recommended by the manufacturer. Positive clones were confirmed as described above.

P22 phage was used to move the mutation into a clean *S.* Typhimurium background. This avoids the possibility of any secondary mutations generated as part of the lambda-red recombination process. In this experiment P22 was used to move the *yihVW::cat* and *yihW::cat* mutations into S. Typhimurium Δ*bcsA* strain, following standard procedures (Maloy et al., 1990). To generate final, unmarked mutant strains, the *cat* gene was removed from the chromosome using a helper plasmid (pCP20). The pCP20 encodes FLP recombinase which acts on FRT (FLP recognition target) sites flanking the *cat* gene. For this pCP20 was electroporated into *S.* Typhimurium Δ*bcsA* Δ*yihVW::cat* strain and grown O/N at 30°C on LB+ amp_{100} agar. One isolated colony was removed from the agar using a sterile loop and re-suspended in Phosphate Buffered Saline (PBS). Serial dilutions were performed, and 100 μL of the 10^{-5} and 10^{-6} dilutions were inoculated onto LB agar and incubated at 42°C O/N. This step cures the cells of pCP20. Loss of the *cat* gene from the chromosome and the pCP20 plasmid was confirmed by patch plating of ~30 colonies onto LB agar or LB agar supplemented with Cm_{30} or Amp_{100}. Colonies that grew on LB agar only were selected for further screening: 1) drop in PCR product size using Taq polymerase (NEB) and primers *yihVW*detect1 and *yihVW*detect2 and 2) DNA sequencing of the amplified PCR products.

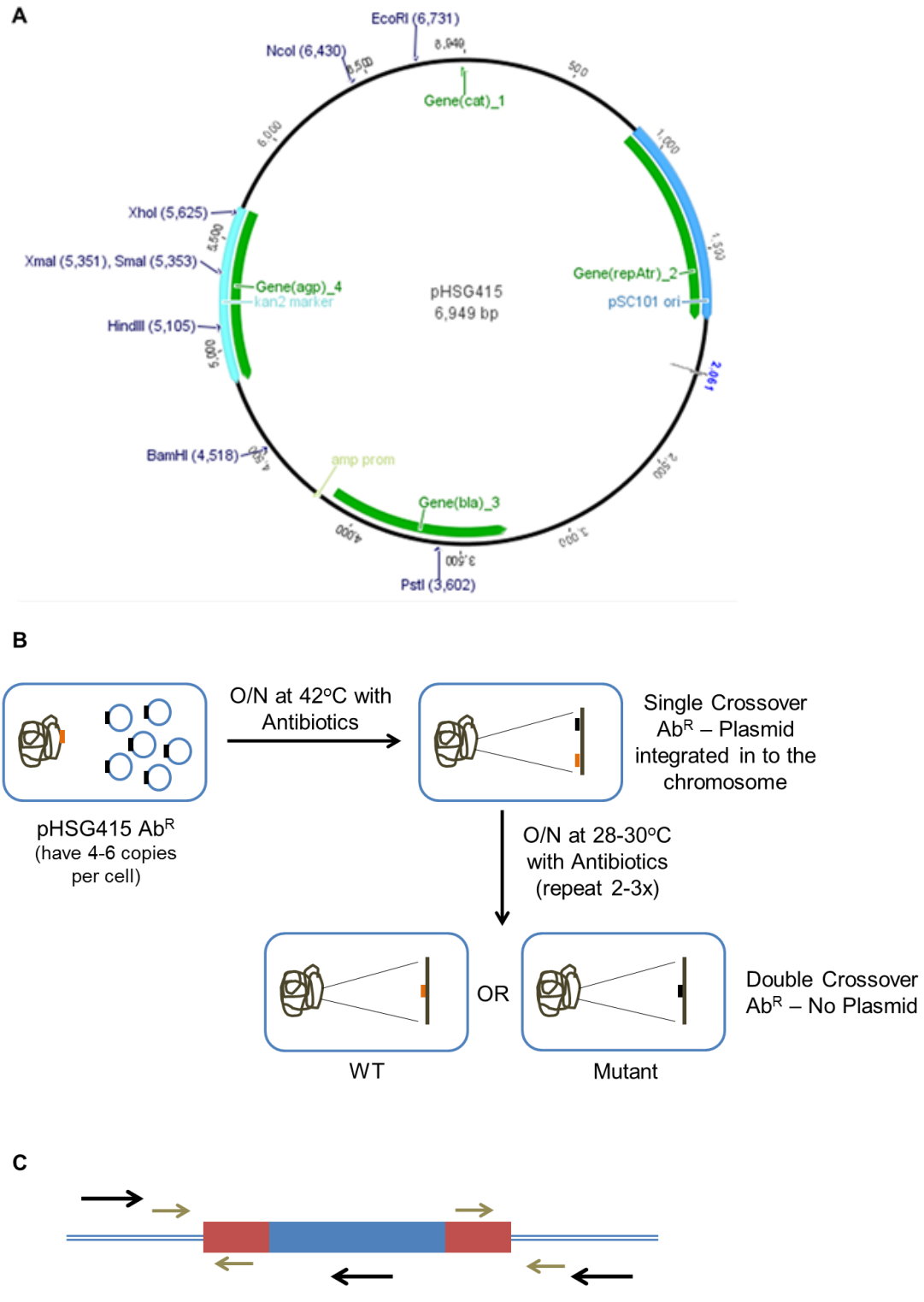


Figure 3.1.1: Creation of cellulose mutant (Δ*bcsA*) *S.* Typhimurium strain. (A) Map of pHSG415 containing the antibiotic resistance markers. (B) Schematic representation of allelic replacement procedure. (C) Different PCR reactions used to generate and detect the mutation; the deleted region (blue), the gene of interest (red), PCR primers for detecting the deletion (black arrow), and PCR primers used for making the deletion (grey arrows). Figure adopted with modifications from White et al., 2007, reproduced with kind permission from Canadian journal of Microbiology.

3.3 Cloning

3.3.1 *yihVW* Cloning

The DNA region containing *yihVW* (1918bp) was PCR amplified from *S*. Typhimurium 14028S genome using Phusion polymerase (Fisher Scientific) and primers *yihVW*FOR (CGCGCTGCAGCTGTTTGTGATCGTATTTGTAATTTAT) and *yihVW*REV (GATCGACGT CGCATCACAGCGCCGTTTTATTG); reaction conditions as recommended by the manufacturer. The resulting PCR product was digested with *Aat*II and *Pst*I and ligated into *Aat*II/*Pst*I cut pBR322 prior to electroporation into *S*. Typhimurium WT, *S*. Typhimurium Δ*bcsA* and *S*. Typhimurium Δ*bcsA* Δ*yihVW* strains. Positive clones were selected by growing on LB + Tet_7 agar. The pBR322-*yihVW* ligation was confirmed by sequencing using primers *yihVW*seqF (GATCTTGCCGGGAAGCTAGAGTAAG) and *yihVW*seqR (GATCTTCTTGAAGACGAAAG GGCCT).

3.3.2 O-Antigen Capsule Operon Cloning

The 9650bp *yihUTSRQPO-yshA* operon was PCR-amplified from the *S*. Typhimurium 14028S genome using Phusion polymerase (Fisher Scientific) and primers CloneFOR (GACTA GTACTTCCTCAAGCATTTTCGATGTGC) and CloneREV (GACTAGTACTACCCGCTGTG ATGCTGATTC); reaction conditions as recommended by the manufacturer. Since the region of DNA was so large, there were no unique restriction sites available for cloning purposes, thus blunt-ended ligations were performed. In one cloning strategy, *yihUTSRQPO-yshA* PCR product and pACYC184 were both digested with ScaI and ligated together. In another strategy, *yihUTSRQPO-yshA* PCR product was digested with ScaI while pBR322 was digested with ScaI and ZraI. The *yihUTSRQPO-yshA* cloning was also tried by only digesting pACYC184 with ScaI.

Once it was determined that *yshA* was not part of the O-Antigen capsule operon (RNA-seq; KD MacKenzie, Y Wang and AP White, unpublished data), a second blunt end cloning was performed. PCR amplified *yihUTSRQPO* (8890bp) from S. Typhimurium 14028S genome using Phusion polymerase and primers CloneFOR and CloneREV2 (GACTAGTACTTTCAAATATA

GGGAAGCCGC); reaction conditions were as recommended by the manufacturer. This PCR product was naturally blunt ended. Then the *yihUTSRQPO* PCR product and pACYC184, which was digested with EcoRV, were ligated together.

3.4 Purification

3.4.1 O-Antigen Capsule and X-factor Purification

An O-Antigen capsule purification method was adopted from Gibson et al., 2006 with modifications. 300uL of *S.* Typhimurium O/N cultures were spread on agar plates (d=140mm) containing either 1% Tryptone media or EPS (extracellular polysaccharide) media (pH 8.23) that contain 0.05% yeast extract, 1% D-glucose, 1.5% agar, 10mM sodium phosphate dibasic anhydrous, 0.1% ammonium chloride, and 0.3% potassium phosphate monobasic. Cells were incubated at 28^{o}C-30^{o}C for five days, following which cells were scraped off agar surfaces, re-suspended in 1%, 5% or 10% phenol, and gently mixed at room temperature for 1h to lyse the cells. This was followed by centrifugation (16,000xg, 4h, 4^{o}C) to separate polysaccharides from other cell material. Polysaccharides were precipitated by mixing the supernatant with four volumes of ice cold acetone while continuously stirring. The pellet containing cellular debris was discarded. Precipitated material was stored overnight at -20^{o}C to allow for any further precipitation. The precipitate in the acetone/supernatant solution was collected by centrifugation (6000xg, 20min, 4^{o}C), air dried, dissolved in ddH_2O and dialysed (10K MWCO – SnakeSkin Dialysis Tubing, Thermo Scientific) against dH_2O for 48h at 4^{o}C. This solution was lyophilized (Freeze-dried) and the dry weight of the polysaccharide material was measured prior to further purification using anion and size exclusion chromatography.

The fractions eluted at the end of anion exchange chromatography of O-Antigen capsule contained the X-factor. Therefore, at this stage, both the X-factor and the O-Antigen capsule were extracted together from the cells.

<u>Anion Exchange Chromatography</u>

The lyophilized polysaccharides were dissolved in 95mL of buffer A (15mM NaOAc, 0.05% Triton X-100 pH 5.5) and 0.01% sodium azide, and heated at 37^{o}C for 10-15min. The sample (100mL of diluted curde sample, pH 5.53, electrical conductivity 540uS/cm) was filtered through a 0.22µm filter prior to loading onto the Q Sepharose FF xk50/11.5 column. The flow

rate was 8.5mL/min, and the fraction size was 20mL. Then the column was washed with 2 column volumes of 100% buffer A followed by 1.6 column volumes of 7% buffer B (1.5M NaOAc, 0.05% Triton X-100, pH 5.5). Next, buffer B concentration was increased sequentially to 17% (1.25 column volumes), 50% (1.25 column volumes) and 100% (1.5 column volumes) prior to flushing the resin with 2 column volumes of buffer D (2M NaCl). The cleanest fraction of the O-Antigen capsule eluted with 17% buffer B, whereas less pure fractions eluted with 7% and 17% buffer B. Western blotting confirmed which peaks or fractions corresponded to the O-Antigen capsule after anion exchange chromatography. The capsule fractions that eluted with 7% buffer B were run separately on size exclusion resin. Final fractions, eluted in 2M NaCl, contained the X-factor, which was collected and run separately on size exclusion chromatography.

Size Exclusion Chromatography

Fractions from anion exchange were pooled together according to purity and concentrated (50K MWCO - centrifugal filters, Millipore). These fractions (2.0-7.5mL) were filtered through a 0.22µm syringe tip filter prior to loading on to the Superdex S300 prep grade xk26/95 column. The flow rate was 0.45mL/min and the fraction size was 7.5mL. Although the O-Antigen capsule was expected to elute in 1 column volume (185-190mL of retention volume), the sample was eluted with 3 column volumes.

Fractions containing the O-Antigen capsule were concentrated either with a Millipore-Amicon Ultra-15 Centrifugal Filter Device or using a vacuum concentrator. Then these fractions were mixed with SDS sample buffer, boiled for 5min at 95^{o}C, centrifuged at 3800xg for 5min and run on either a 7.5% resolving SDS-PAGE gel alone or on a 12% resolving and 5% stacking SDS-PAGE gel (Molecular weight standard - Precision Plus protein dual colour standard, Bio-Rad.) Following this, samples were transferred to nitrocellulose membrane using a Trans-Blot SD semi-dry transfer cell (Bio-Rad Laboratories) in buffer recommended by the manufacturer. After transfer, the membrane was blocked in 5% skim milk either for 1h at 37^{o}C or overnight at 4^{o}C. Following this the membrane was incubated for 1h in primary antibody: rabbit polyclonal immune serum specific for *S.* Typhimurium (or *S.* Enteritidis) O-antigen capsule (1:1000), or rabbit polyclonal immune serum specific for *S.* Typhimurium (or *S.* Enteritidis) X-factor (1:1000). Initial screening was carried out using rabbit serum specific for *S.* Enteritidis O-antigen capsule (obtained from Deanna Gibson, UBC-Okanagan). Each step was followed by washing

with1% TBST (Tris-Buffered Saline with 1% Tween 20). The secondary antibody (alkaline phosphatase conjugated goat anti-rabbit serum) was used at 1:2000 dilution. Immunoreactive material was visualized by incubating in BCIP (5-bromo-4-chloro-3-indolylphosphate) and NBT (4-nitroblue tetrazolium chloride) solution. Dot blots were performed similar to Western-blots. However, instead of running the sample on a SDS-PAGE gel, it was directly pipetted on to the Nitrocellulose membrane and allowed to air dry. Then the membrane was developed in the same manner as a Western blot.

3.4.2 Endotoxin Removal

Twenty grams of Triton X-114 (Sigma-Aldrich) and 16mg of 2,6-Di-tert-butyl-4-methylphenol (Aldrich) were mixed and dissolved at 4^{o}C, and this mixture was buffer exchanged with 10mM Tris (pH7.4) and 150mM NaCl (Bordier 1981). This solution was poured into a separator funnel and incubated at 30^{o}C until the mixture separated into a large aqueous phase and a small Triton-enriched phase. The smaller phase was buffer-exchanged two more times with an equal volume of Tris-NaCl solution and incubated at 30^{o}C. The resulting 11% Triton X-114 solution was stored at room temperature. Following size exclusion chromatography, O-Antigen capsule-containing fractions were combined together and dialyzed in dH_2O for 48h at 4^{o}C (10K MWCO – SnakeSkin Dialysis Tubing, Thermo Scientific). The dialyzed material was lyophilized and the resulting powder was dissolved in dH_2O at a concentration of 0.5mg/mL. An aliquot of this solution was saved as a "pre-LPS removal" control for use in subsequent experiments. Using the 11% Triton X-114 stock solution (described above), a 1% Triton X-114 solution was prepared and this was mixed with 0.5mg/mL O-Antigen capsule solution. This mixture was initially cloudy, but turned clear (after stirring for 30min on ice or O/N at 4^{o}C). After this step, the solution was dispensed in to 25mL centrifuge tubes and incubated for 30min at 37^{o}C (after which the solution turned cloudy). The tubes were centrifuged for 30min at 1200xg at 25^{o}C, and the solution separated in to two phases. The upper phase containing the O-Antigen capsule was mixed with 2% Triton X-114 and extracted as per 1% Triton. The lower LPS containing phase was stored separately at 4^{o}C. The 2% Triton step was repeated for a total of three times. Centrifugation speed and time for each step was adjusted to obtain the best

separation of the two phases. This procedure was adopted with modifications from Adam et al., 1995.

To remove Triton X-114 from the O-Antigen capsule-containing solution, Triton-capsule solution was mixed with three volumes of a 2-methanol:1-chloroform solution and poured in to a separator funnel. Once the solution separated into two phases (at room temperature), the Triton X-containing lower phase was eluted. The O-Antigen capsule-containing upper phase was mixed again with the methanol-chloroform solution; this procedure was repeated two additional times. Any remaining methanol-chloroform was removed from the sample using a water aspirator. Following this, the sample was dialysed for 48h in dH_2O at 4^oC, lyophilized, and the resulting powder (shiny, brown powder) was weighed and recorded as the yield of the final-purified capsule. Purified capsule was resolved by SDS-PAGE and tested on Western blots for the presence of O-Antigen capsule and LPS. Capsule was detected using rabbit polyclonal immune serum specific for *S.* Typhimurium (or *S.* Enteritidis) O-Antigen capsule (1:1000) and LPS was detected using mouse monoclonal antibody specific for *S.* Typhimurium LPS [1E6] (Abcam: ab65922). Alternatively, the presence/absence of LPS was verified using silver staining as described by Kropinski et al., 1986.

The amount of LPS associated with the O-Antigen capsule was quantified using the Limulus Amebocyte Lysate (LAL) assay (QCL-1000, Lonza, Walkersville, MD, USA), according to the manufacturer's guidelines. In this assay, the LPS quantity of crude capsule (prior to LPS extraction) was compared to that of the purified capsule (after LPS extraction).

3.5 Immune Serum Generation

Four New Zealand white rabbits were immunized subcutaneously (s.c.) with a specific concentration of purified (LPS removed) O-Antigen capsule or X-factor. Group A received *S.* Typhimurium X-factor, Group B received *S.* Enteritidis X-factor, Group C received *S.* Typhimurium O-Antigen capsule, and Group D received *S.* Enteritidis O-Antigen capsule. Each antigen was dissolved in PBS (phosphate buffered saline) and mixed with 30% Emulsigen, which was used as an adjuvant.

Prior to primary immunization, 1-2mL of blood was collected from each rabbit as a pre-immunization control. The immunization doses and schedule were as follows: Day0, primary

immunization with 100ug O-Antigen capsule or X-factor; Day21, immunization with 50ug O-Antigen capsule or 100ug X-factor; Day42, immunization with 50ug O-Antigen capsule or X-factor. After each immunization 1-2mL of blood was collected from each rabbit and used to determine the antibody titer (ELISA). Animals were euthanized on Day 52 and blood samples were collected into Serum Separator Tubes.

The blood samples were centrifuged at 3700xg for 10min at 4^{o}C, which separated the blood in to three phases: the lowest phase containing cell material, the middle phase containing protein and the upper phase containing serum. Serum was collected and stored at -20^{o}C. For ELISAs, Immulon II Immunoassay 96 well plates (Thermo Scientific) were coated with 0.5ug/mL O-Antigen capsule in coating buffer (1.32g Na_2CO_3 and 3.16g $NaHCO_3$ in 1L dH_2O, pH 9.6) and incubated O/N at 37^{o}C. The plates were washed with 0.5% TBST (Tris-Buffered Saline with 0.5% Tween 20) prior to pipetting 5% skim milk into each well and incubating for 3h at 37^{o}C. Serially diluted primary antibody (rabbit serum) was added to each well and incubated for 1h at room temperature. Each step was followed by washing with1% TBST (Tris-Buffered Saline with 1% Tween 20). The secondary antibody (alkaline phosphatase conjugated goat anti-rabbit serum) was used at 1:2000 dilution. The reaction was developed with PNPP (p-nitrophenyl phosphate di(tris) salt crystalline – Sigma N3254) substrate (10uL PNPP stock [1g PNPP in 10mL substrate buffer] and 1mL substrate buffer [10mL Diethanolamin – Sigma D8885 and 1mL 500mM $MgCl_2$ in 1L dH_2O, pH 9,8]). The reaction was stopped with 0.3M EDTA (pH 8.0) and absorbance measured at 490nm reference wavelength using Spectramax Plus 384 Microplate Reader (Molecular Devices, Sunnyvale CA).

3.6 O-Antigen Capsule Dosage Trial

Seven groups of ~8 week old female BALB/c mice (n=6) were immunized twice intramuscularly as follows: Group A: 5ug O-Antigen capsule + adjuvant, Group B: 5ug O-Antigen capsule, Group C: 25ug O-Antigen capsule + adjuvant, Group D: 25ug O-Antigen capsule, Group E: 50ug O-Antigen capsule + adjuvant, Group F: 50ug O-Antigen capsule, and Group G: 50ug O-Antigen capsule + 30% Emulsigen (positive control group) The "triple-combo" adjuvant was developed at VIDO-InterVac and contained CPG ODN, Poly I:C (a host

defense peptide), and polyphosphazeen at a 1:1:2 ratio. Mice from different groups were co-housed in a formally randomized manner and we were blinded as to the group designations until the final data had been collected. All the mice in the positive control group were placed in one cage since Animal Care can only put six mice in a cage and there were seven groups.

Mice were immunized on days 0, and 28 with the antigens as described above, while blood samples (50uL) were collected on days 0, 21, and 42. Mice were euthanized on Day 52 and all the blood was collected. Blood samples were separated by centrifugation, as described in 3.5 "Immune Serum Generation" and the serum was stored at -20^{o}C until ELISA was performed. ELISAs were performed as described in 3.5 "Immune Serum Generation". For ELISAs, Immulon II Immunoassay 96 well plates (Thermo Scientific) were coated with 1.25uL/well O-Antigen capsule in coating buffer. The yield of O-Antigen capsule was quite low after purification, and it was not possible to determine the weight. Thus with an antigen titration ELISA it was determined that the optimal amount of O-Antigen capsule for screening was at 1.25uL/well. The mouse serum (pre-bleed, post 1st immunization serum, and post 2nd immunization serum) was used at 1:50 and 1:200 dilutions. The secondary antibody (alkaline phosphatase conjugated goat anti-mouse serum) was used at a 1:2000 dilution.

3.7 Luciferase Assays

Expression of the divergent operons (*yihUTSRQPO* and *yihVW*) coding for O-Antigen capsule biosynthesis was measured using promoter-luciferase fusions. DNA regions containing Promoter *yihUTSRQPO* (P*yihUTSRQPO*) and Promoter *yihVW* (P*yihVW*) were cloned into pCS26-Pac plasmid containing *luxCDABE* of *Photorhabdus luminescens* (Bjarnason et al., 2003 and Gibson et al., 2006). The resulting plasmids were transformed into each *Salmonella* strain; promoter activation resulted in expression of *luxCDABE* which was measured through light production. Different intensities of promoter activity were reflected in the light output. To perform luciferase assays, O/N cultures of each reporter strain were diluted 1:600 into a final volume of 150uL/well containing the desired growth medium (EPS media, EPS media with 2,2'-dipyridyl, 1% Tryptone, or LB +/- sugars) in 96-well clear-bottom, black plates (9520 Costar; Corning Inc.). The optical density (at 590nm) and light production (counts per second) was measured every 30min for 48h using a Wallac-Victor X^3 multi-label plate reader (Perkin-Elmer

Life Sciences, Boston MA). The strain containing pCS26-Pac with either P*yihUTSRQPO* or P*yihVW* was also used in assessing the light production by individual colonies. For this, each strain was streaked for single colonies on LB agar + Kan_{50} and incubated O/N at 37^{o}C. Light production by individual colonies was measured using an IVIS Lumina II (Perkin-Elmer Life Sciences).

3.8 Capsule Staining and Microscopic Imaging

Capsule staining was performed using Maneval's stain, where 10uL of Congo red dye was placed on a clean slide and mixed with a small amount of a colony from the desired strain (A single colony was touched with a pipette tip and the tip was swirled in the Congo red dye on the slide). The slide was allowed to air dry for ~1h. Following this, the slide was placed on a staining tray and flooded with Maneval's solution (Merlan Scientific Ltd. Mississauga, ON, Canada) and incubated for 2min. Then the excess stain was washed off with dH_2O and the slide was allowed to air dry for ~1h prior to visualization with a light microscope (63X objective lens).

4.0 RESULTS

4.1 Purifications

4.1.1 Crude Capsule Purification

The purification of O-Antigen capsule was difficult in the presence of cellulose (Gibson et al., 2006), resulting in lower yields than what had been previously reported by others (Snyder et al., 2006). This was because cellulose non-specifically traps all polysaccharides, including O-Antigen Capsule, in the extracellular matrix. In *Salmonellae*, *bcsA* codes for the catalytic subunit of cellulose synthase (Solano et al., 2002; Barak et al., 2007). To allow for more efficient capsule purification in the absence of cellulose, a deficient strain (*S.* Typhimurium Δ*bcsA*) was generated with allelic replacement. The final confirmation of the *bcsA* deletion was done by analyzing the colony morphology of potential mutant strains. While WT colonies were characterized by a dry, rough texture, undefined edges, and a pattern on top, the *bcsA* mutant strain colonies were smooth, shiny, and had round edges (Figure 4.1.1(a)).

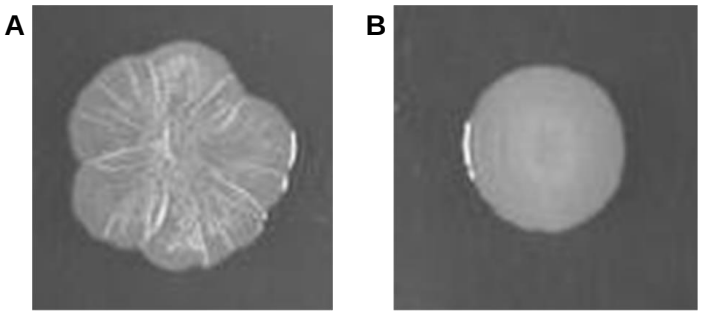


Figure 4.1.1(a): Phenotypes of WT and *bcsA* mutant *S*. Typhimurium strains. (A) *S.* Typhimurium WT. (B) *S.* Typhimurium Δ*bcsA.* Both strains were grown on 100ug/mL Congo red + 1% Trypton agar at 28ºC for 5 days.

Initially, the O-Antigen capsule was purified from both *S.* Typhimurium Δ*bcsA* and *S.* Enteritidis Δ*bcsA* strains, but for subsequent experiments only the *S.* Typhimurium Δ*bcsA* strain was used. The crude polysaccharides were white, powdery material that had the texture of cotton (Figure 4.1.1(b)). Following crude purification of the polysaccharides, samples were further purified using anion exchange followed by size exclusion chromatography. Fractions from size exclusion chromatography were tested by Western blotting to identify O-Antigen capsule-

containing fractions (Figure 4.1.1(c)). The size exclusion chromatograph contained two peaks, where the first peak was always associated with O-Antigen capsule and the composition of the second peak was unknown. In the absence of a stacking gel, crude polysaccharides were smeared on Western blots from top to bottom of the lane. Laddering seen on the bottom of lanes were associated with low-molecular weight LPS. Upon confirmation on Western blots, O-Antigen capsule-positive fractions were pooled together and used in subsequent purifications.

Figure 4.1.1(b): Physical appearance of crudely purified polysaccharides. Crude polysaccharides were white, cotton like material soluble in water upon vigorous stirring or with gentle heating.

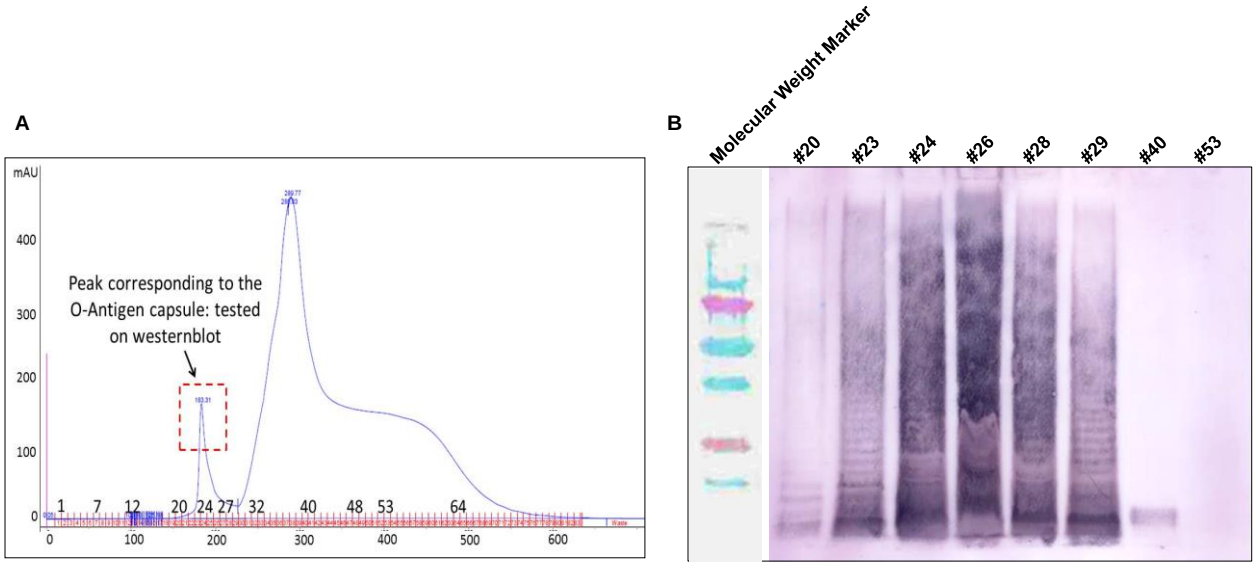


Figure 4.1.1(c): Crude O-Antigen capsule detection following chromatography. (A) Size exclusion chromatogram. (B) Western blot showing crudely purified O-Antigen capsule fractions from *S.* Typhimurium Δ*bcsA* following size exclusion chromatography detected using rabbit polyclonal immune serum specific for *S.* Typhimurium O-Antigen capsule (1:1000).

For developing an effective vaccine against NTS it is important to ensure that there will be immune memory and cross-protection between different serovars. Therefore, it was important to determine early on if cross recognition of O-Antigen capsule from different serovars was possible for serum generated against the capsule of a given serovar. Prior to specific serum generation (discussed in 4.2 "Animal Trials"), *S.* Typhimurium O-Antigen capsule was visualized on Western blots using the serum specific for *S.* Enteritidis O-Antigen capsule (obtained from Deanna Gibson, UBC-Okanagan) (Figure 4.1.1(d)). In addition, crude polysaccharides were extracted from *Salmonella* serovar Heidelberg; a previously uncharacterized serovar with respect to O-Antigen capsule production. *S.* Heidelberg O-Antigen capsule was further purified using anion exchange (Figure 4.1.1(e)-A) and size exclusion chromatograms and the resulting fractions were tested on dot blots. Rabbit polyclonal serum specific for *S.* Enteritidis O-Antigen capsule was used to detect the presence of capsule in *S.* Heidelberg samples (Figure 4.1.1(e)-B). O-Antigen capsule from *S.* Typhimurium and *S.* Enteritidis were used as positive controls.

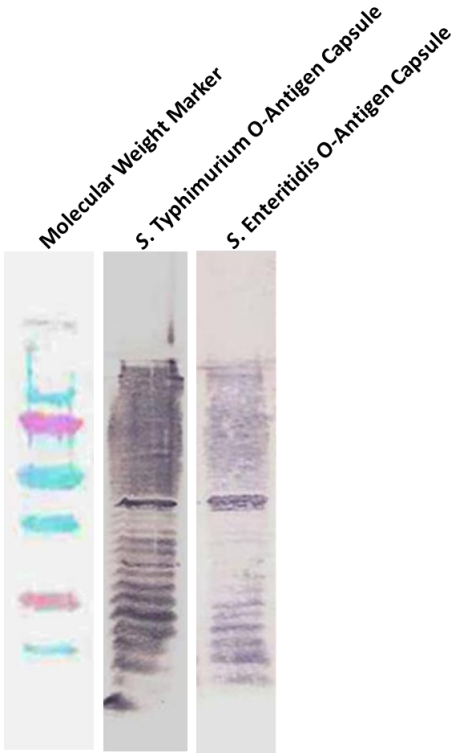


Figure 4.1.1(d): Cross recognition of *S.* Typhimurium O-Antigen capsule by serum specific for *S.* Enteritidis capsule: Western blot showing the crudely purified O-Antigen capsule following size exclusion chromatography detected using rabbit polyclonal immune serum specific for *S.* Enteritidis O-Antigen capsule (1:1000) – serum obtained from Deanna Gibson, UBC-Okanagan

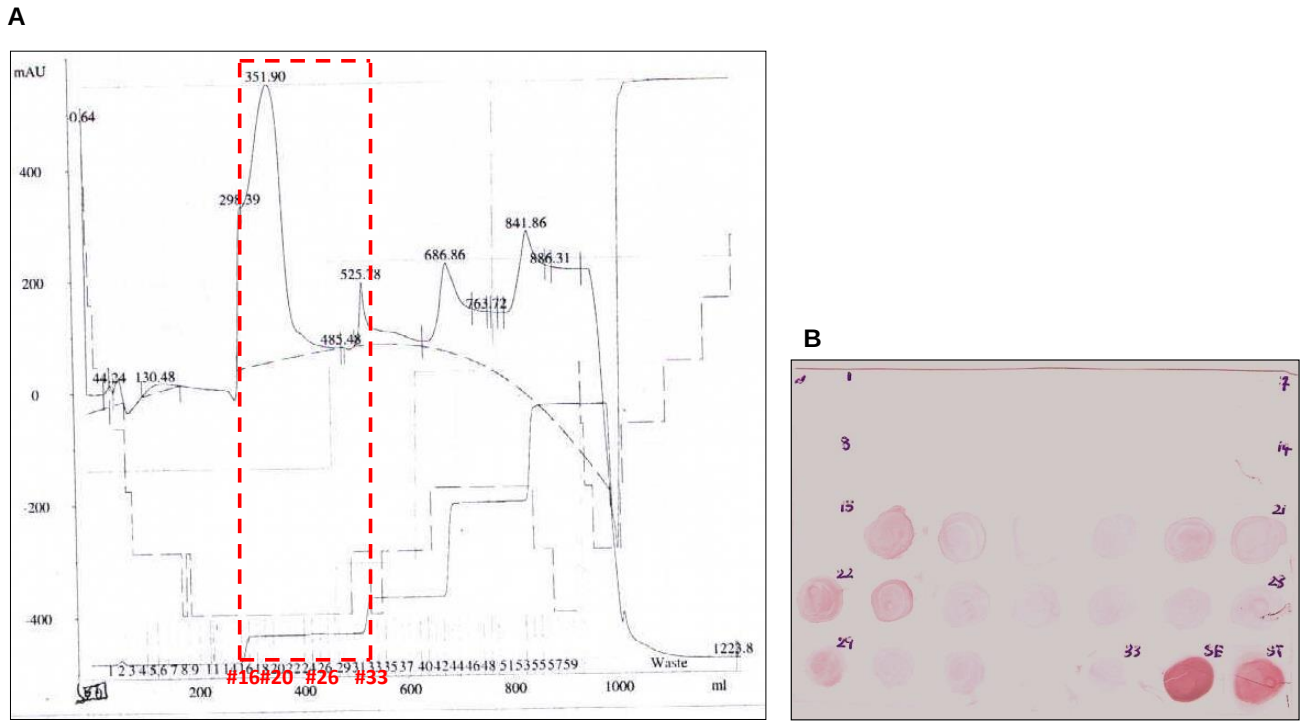


Figure 4.1.1(e): Cross recognition of *S*. Heidelberg O-Antigen capsule by serum specific for *S*. Enteritidis capsule: (A) Anion exchange chromatogram and peaks corresponding to the O-Antigen capsule. (B) Dot blot showing the crudely purified *S.* Heidelberg O-Antigen capsule following size exclusion chromatography detected using rabbit polyclonal immune serum specific for *S.* Enteritidis O-Antigen capsule (1:1000) – serum obtained from Deanna Gibson, UBC-Okanagan. S. Enteritidis (SE) and S. Typhimurium (ST) O-Antigen capsules are used as controls.

4.1.2 O-Antigen Capsule Purification from Additional Mutant Strains of *S*. Typhimurium

Through gene expression studies (discussed in 4.3 "Gene Expression Studies") it was observed that the promoter for the O-Antigen capsule operon (P*yihUTSRQPO*) mediated elevated expression in the absence of the repressor YihW. Thus, the standard purification protocol using *S.* Typhimurium Δ*bcsA* strain grown on EPS agar was changed to include the *S.* Typhimurium Δ*bcsA* Δ*yihVW* strain grown on 1% Tryptone agar. However, during the first attempt, it was difficult to obtain any crude capsule from this strain. As it was not explicitly clear whether this was due to an inability to produce capsule by the *S.* Typhimurium Δ*bcsA* Δ*yihVW* strain or that the cell lysis method was inadequate, given that the absence of YihVW may have altered the cell surface dynamics, it was considered best to increase the phenol concentration during cell lysis. Thus, a range of phenol concentrations were tested from 2%, 5% and 10%, and 10% phenol was considered best in replacing the existing 1% phenol in lysing cells.

As the deletion of *yihVW* resulted in *S.* Typhimurium Δ*bcsA* Δ*yihVW* not producing any crude polysaccharides, it was considered that maybe YihVW were necessary in activating or positively regulating the O-antigen capsule operon. Thus, *yihVW* were cloned in to pBR322 and introduced in to *S.* Typhimurium Δ*bcsA* and *S.* Typhimurium Δ*bcsA* Δ*yihVW* strains. However, during purifications, the yield of crude polysaccharides from pBR322-*yihVW* containing strain was half as much as that from the *S.* Typhimurium Δ*bcsA* strain. As a result it was unclear whether YihVW were acting as activators or repressors of the O-Antigen capsule operon. One explanation was that maybe YihV (a kinase) was involved in capsule production, and deletion of *yihV* interfered with this process. Therefore, a *yihW* deletion strain was constructed to determine whether the absence of YihW and the presence of YihV may lead to an increase in capsule production. During purification *S.* Typhimurium Δ*bcsA* Δ*yihVW* produced 0.6x and *S.* Typhimurium Δ*bcsA* Δ*yihW* produced 2.5x crude polysaccharides compared to *S.* Typhimurium Δ*bcsA* strain. Therefore YihV may have a regulatory role in promoting capsule production.

Crude polysaccharides from all three strains: *S.* Typhimurium Δ*bcsA*, *S.* Typhimurium Δ*bcsA* Δ*yihVW* and *S.* Typhimurium Δ*bcsA* Δ*yihW,* were further purified through anion exchange and size exclusion chromatography and resulting O-Antigen capsule fractions were visualized on a Western blot (Figure 4.1.2). Capsules from all three strains were detected using rabbit polyclonal serum specific for *S.* Typhimurium O-Antigen capsule. Although *S.* Typhimurium Δ*bcsA* Δ*yihW* produced more crude polysaccharides, capsule material from all three strains visually appeared the same and had bands of same thickness on the Western blot. The O-Antigen capsule was associated with the top of the resolving gel while the lower molecular weight LPS bands appeared below the capsule.

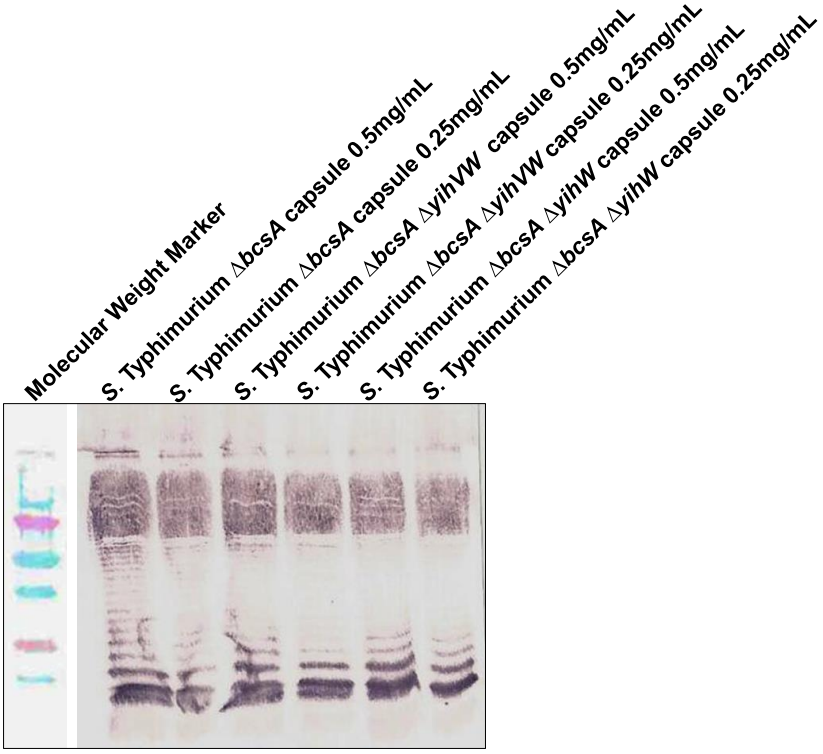


Figure 4.1.2: Comparison of Crude O-Antigen capsule between three different strains. Crudely purified O-Antigen capsule samples from *S.* Typhimurium Δ*bcsA*, *S.* Typhimurium Δ*bcsA* Δ*yihVW*, and *S.* Typhimurium Δ*bcsA* Δ*yihW* following size exclusion chromatography were detected using rabbit polyclonal immune serum specific for *S.* Typhimurium O-Antigen capsule (1:1000).

4.1.3 Endotoxin Removal

Size exclusion chromatography separates molecules based on their size. Following size exclusion chromatography, LPS, being a lower molecular weight molecule, was expected to be separated from higher molecular weight O-Antigen capsule. However, in Western blots conducted following chromatography, there was a distinct LPS banding pattern at the bottom of each lane containing the O-Antigen capsule samples (Figure 4.1.3(a) left lane). To specifically test for the presence of LPS, crude O-Antigen capsule samples were separated on a SDS-PAGE gel and either transferred to a membrane and detected using mouse monoclonal serum specific for *S.* Typhimurium LPS [1E6] (Abcam: ab65922) (Figure 4.1.3(a) right lane) or silver stained; a procedure highly sensitive to bacterial LPS in polyacrylamide gels. Both of these methods were able to confirm that LPS was associated with crudely purified O-Antigen capsule even after chromatography.

The presence of LPS in a vaccine is not desired as LPS is pyrogenic, can lead to inflammation, endotoxic shock and even death. For purposes of this vaccine, the concern is that if LPS is present in the vaccine, the immune response mounted will be against LPS and not the O-Antigen capsule. Immune response to LPS is known to be strain specific and will not provide cross-protection. In this case, the vaccine will not be effective. As such, there was a pressing need to remove LPS from O-Antigen capsule.

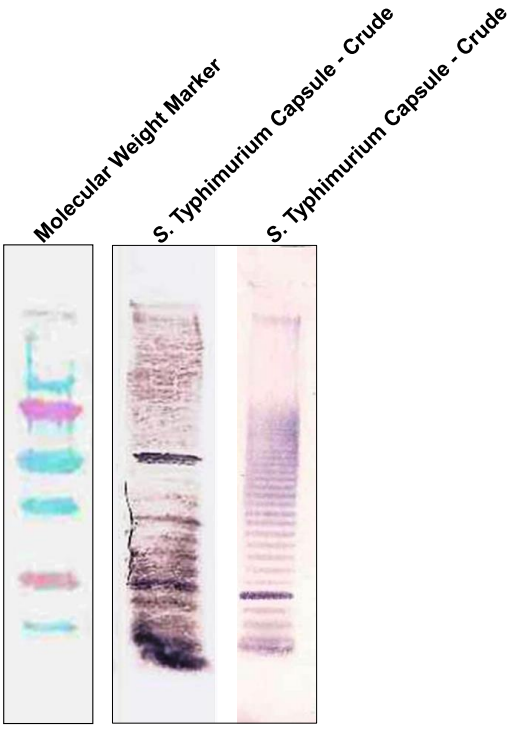


Figure 4.1.3(a): Detection of LPS associated with crude O-Antigen capsule. O-Antigen capsule and LPS were visualized using: left lane - rabbit polyclonal serum specific for *S.* Typhimurium O-Antigen capsule, right lane - mouse monoclonal serum specific for *S.* Typhimurium LPS [1E6] (Abcam: ab65922).

LPS and O-Antigen capsule are both polysaccharides associated with the ECM. However, there are unique distinguishing features between the two polysaccharides. For example, in *S.* Typhimurium O-Antigen capsule, both abequose and galactose residues are partially substituted with a polymeric glucose side chain. However in LPS only galactose residue is substituted with a glucose side chain (Figure 4.1.3(b)). In addition, O-Antigen capsule has over 2300 repeat units while LPS has only 20-30 repeat units. Also, LPS is attached to the cell surface through the lipid-A core region, while the O-Antigen capsule is covalently attached to an uncharacterized glycolipid anchor (Snyder et al., 2006 and Gibson et al., 2006). Furthermore, LPS has an overall

lower net charge than O-Antigen capsule and can be separated by anion exchange chromatography.

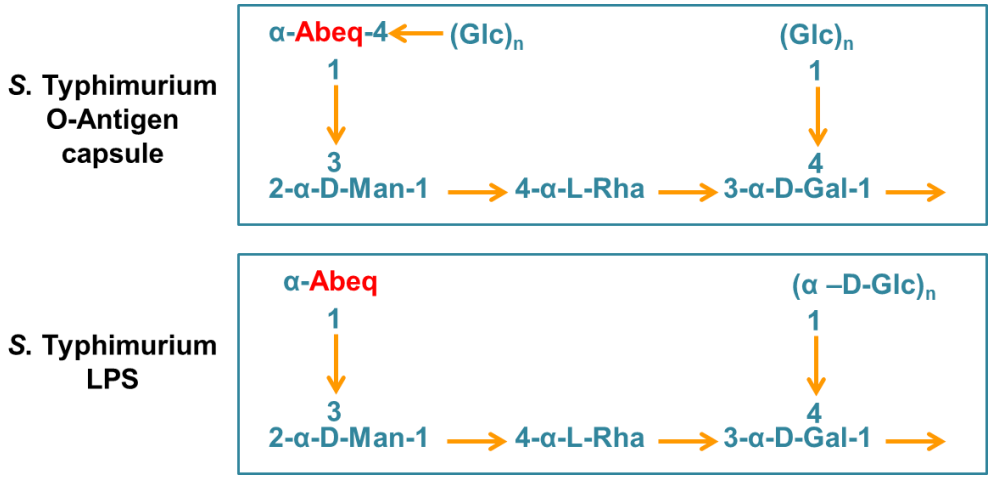


Figure 4.1.3(b): Schematic representing the linear configuration of the *S*. Typhimurium O-Antigen capsule and LPS. Both abequose and galactose residuces on the O-Antigen capsule are partially substituted with a glucose side chain, while only galactose in LPS has this modification.

Following chromatography O-Antigen capsule-containing fractions (confirmed using Western blots) were pooled together and mixed with Triton X-114, which acts as a detergent and dissociated LPS molecules from O-Antigen capsule. This procedure was adapted from Adam et al., 1995. At room temperature Triton X-114 solution separated into two phases where the dissociated LPS molecules were associated with the micellar rich lower phase, while the O-Antigen capsule was associated with the micellar poor upper phase (Figure 4.1.3(c)) (Wang and Coppel 2002). The original protocol was to remove LPS by performing a series of seven Triton X-114 extractions (Adam et al., 1995). However, it was found that four Triton X-114 extractions of the O-Antigen capsule sample removed a significant amount of LPS and left a detectable amount of O-Antigen capsule. In capsule purification, the additional incubation steps with Triton X-114 reduced the amount of LPS, but also decreased the final concentration of O-Antigen capsule. Upon removal of Triton X-114 from the sample with a methanol-chloroform mixture, the presence of the O-Antigen capsule and reduction of LPS was confirmed with Western blots (Figure 4.1.3(d)). O-Antigen capsule was faintly visible when using serum specific for *S.* Typhimurium O-Antigen capsule, as most of the capsule had been lost during the purification. However, LPS was not present at a detectable level in the purified capsule sample.

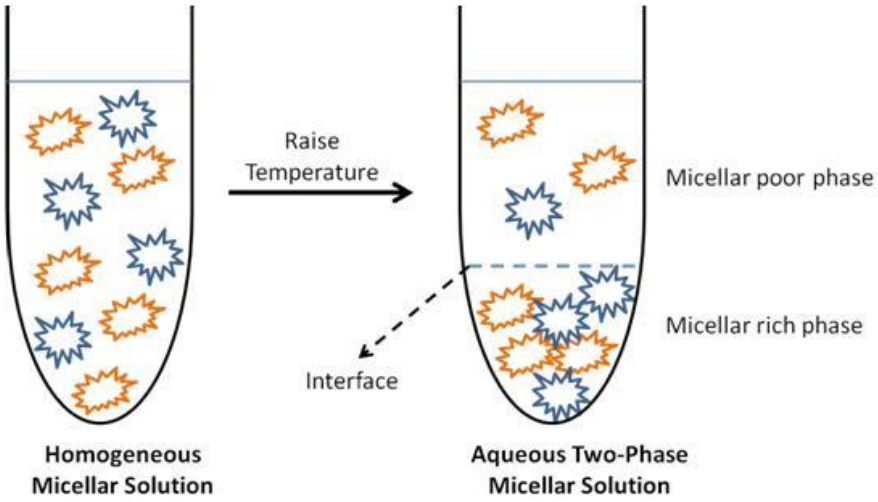


Figure 4.1.3(c): Schematic representation of Triton X-114 phase separation. Triton X-114 separated at room temperature in to a micellar poor upper phase containing O-Antigen capsule and a micellar rich lower phase containing LPS. Figure extracted from Magalhaes et al., 2007, reproduced with kind permission from Journal of Pharmacy and Pharmaceutical sciences.

The amount of remaining LPS associated with O-Antigen capsule was quantified with Limulus Ameobocyte Lysate (LAL) assays (www.lonza.com). LAL is a extremely sensitive assay and thus the results were considered highly accurate. In one purification round, crudely purified *S.* Typhimurium O-Antigen capsule, prior to LPS removal, had an LAL value of $1.84*10^8$ EU/mg. Upon LPS removal with Triton X-114, the LAL value was reduced to $2.5*10^3$ EU/mg. Although the LAL value was not zero, this was a significant reduction in the concentration of LPS compared to that of the crude material, and was within the biologically safe limit to administer to mice. According to Beutler et al., 1985, LD_{50} was achieved when $4.55*10^6$ EU/ml of edotoxin was administered to a 20g mouse. Copeland et al., 2005 demonstrated that administration of $1.0*10^3$EU/mL of endotoxin to a 20g mouse was non-lethal. The final purified O-Antigen capsule had $2.5*10^3$ EU/ml of edotoxin, and the highest dose of antigen administered to mice was 50µg. Therefore in 50µg there was $1.25*10^2$EU of LPS, which was within the biologically acceptable limit to administer to mice.

In previous purifications it was determined that it was not possible to remove all the LPS without greatly reducing the O-Antigen capsule concentration as well. Thus, at this level, there was sufficient LPS removed for the vaccine to be biologically safe and sufficient O-Antigen capsule remaining to mount a protective immune response. Throughout the purification process,

the concentration of O-Antigen capsule had been greatly reduced that at the end only a small fraction of the starting material was still present (Figure 4.1.3(e) and Table 4.1.1).

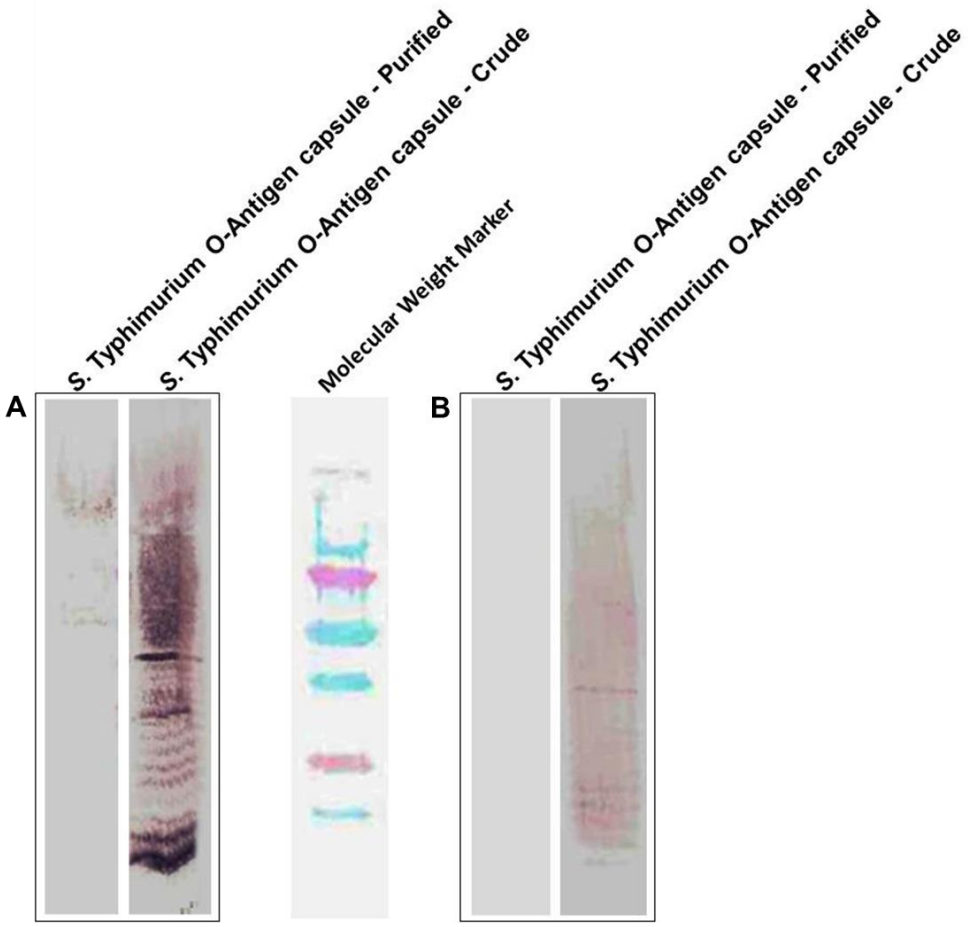


Figure 4.1.3(d): Purified O-Antigen capsule detected on western blots. Triton X-114 treated O-Antigen capsule tested on Western blots for the presence of capsule and reduction of LPS. Purified capsule treated four times with Triton X-114. Crude capsule prior to LPS removal was used as a control. Detected using: (A) rabbit polyclonal serum specific for *S*. Typhimurium O-Antigen capsule, (B) mouse monoclonal serum specific for *S*. Typhimurium LPS [1E6] (Abcam: ab65922).

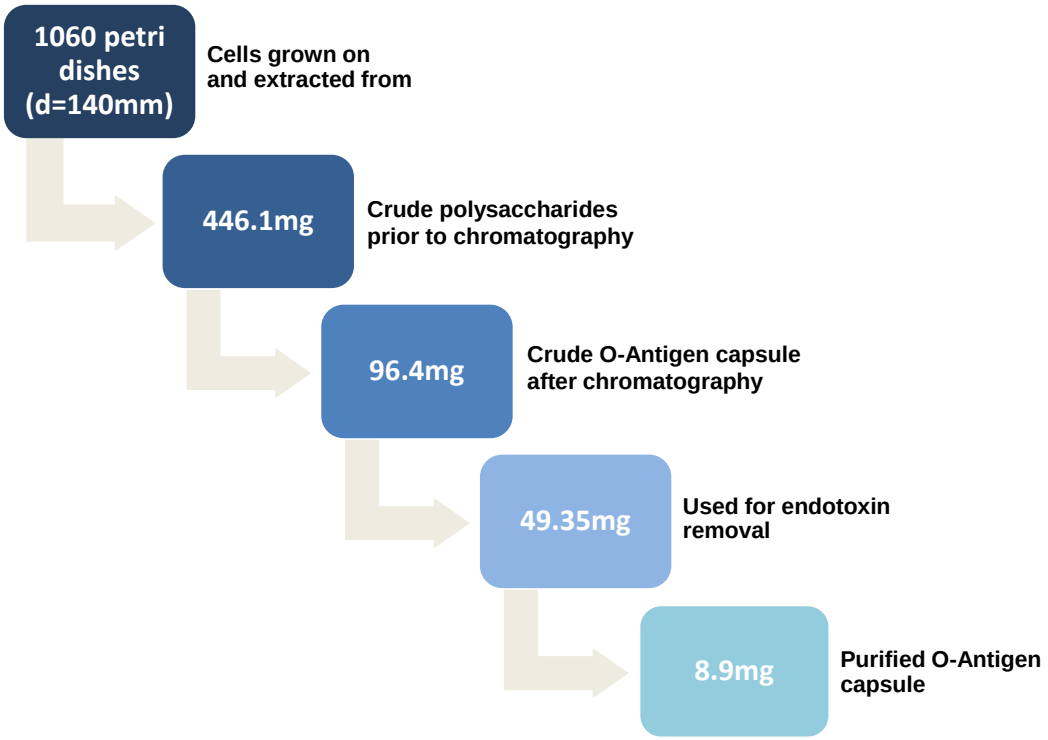


Figure 4.1.3(e): The yield of O-Antigen capsule throughout the purification process.

4.1.4 Influence of Growth Media on Capsule Yield

In order to optimize O-Antigen capsule production, many different growth conditions were assessed both for P*yihUTSRQPO* activity and the yield of crude polysaccharides. It was observed that growth of *S.* Typhimurium *ΔbcsA* Δ*yihVW* on Tryptone agar produces ~2.3 times more crude polysaccharide compared to growth on EPS agar. However, this increase in yield did not reflect the100-fold increase in P*yihUTSRQPO* activity observed in luciferase assays (discussed in 4.3 "Gene Expression Studies"). Thus, initially the low yield was considered to be a result of improper cell lysis. However, further reasoning indicated that even if the cells were not being lysed properly, O-Antigen capsule had to be produced, as P*yihUTSRQPO* was highly expressed under the same growth conditions. Using Western blots, the presence of the capsule was tested in the following samples: cells scraped off agar surfaces (cells), the cell debris pellet following phenol lysis and centrifugation (pellet), the polysaccharide precipitate following acetone treatment (precipitate), and the supernatant of this precipitate (supernatant). Both the cells and the pellet were normalized to 1.0 OD_{600},

(OD_{600} is optical density or absorbance of a samples when the wavelength is at 600nm) and the precipitate and supernatant were lyophilized and used at 0.5mg/mL.

The majority of O-Antigen capsule was present in the precipitate sample with high molecular weight material corresponding to the O-Antigen capsule (Figure 4.1.4(a)). Cells and the pellet had low levels of capsule, while the supernatant did not appear to contain capsule. This assay was repeated with identical results for *S*. Typhimurium Δ*bcsA* Δ*yihVW* pBR322-Δ*yihVW* grown on EPS agar, and *S*. Typhimurium Δ*bcsA* Δ*yihVW* grown on EPS argar and 1% Tryptone agar. This indicated that although not a direct correlation of the P*yihUTSRQPO* activity observed, the cell lysis method was adequate and most of the capsule was collected with the polysaccharide precipitate.

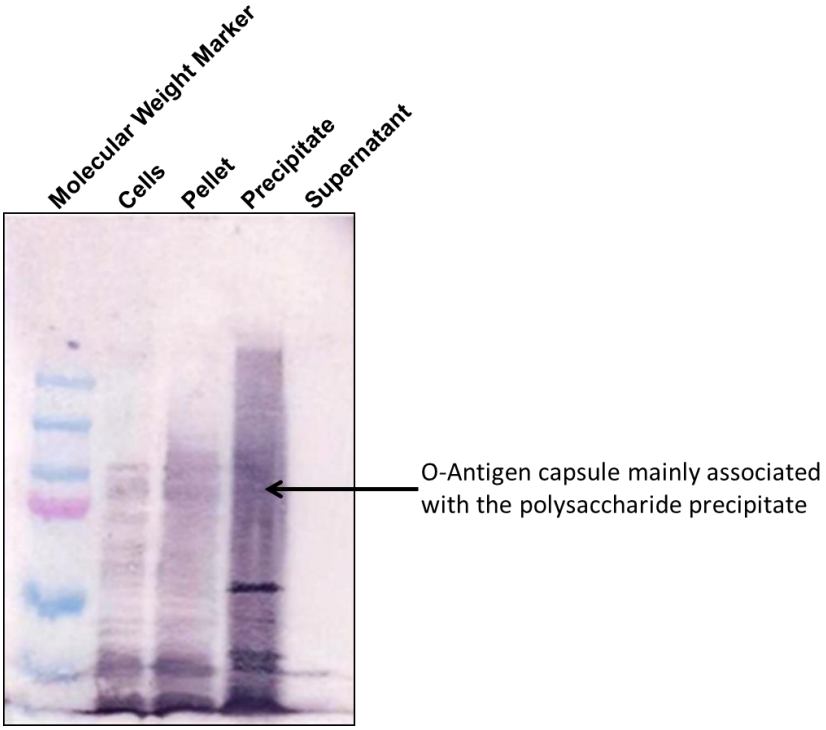


Figure 4.1.4(a): Locating the capsule. Crude O-Antigen capsule of *S*. Typhimurium Δ*bcsA* grown on EPS agar detected on a Western blot. Cells: cells scraped off agar surfaces. Pellet: cell debris pellet following phenol lysis. Precipitate: polysaccharides precipitate following acetone treatment. Supernatant: supernatant of the polysaccharides precipitate containing acetone. Capsule was detected using rabbit polyclonal serum specific for *S*.Typhimurium O-Antigen capsule.

Strain	# of plates	Growth media	% of phenol	PYBC	mg/plate	WLDC	PYAC	PYAC (mg/plate)	FWPC (ALR)
SE bcsA	40	EPS	1%	49.1mg	1.23	-	-		-
ST bcsA	40	EPS	1%	24.8mg	0.62	-	-		-
SE bcsA	46	EPS	1%	50.0mg	1.09	-	-		-
ST bcsA	46	EPS	1%	30.0mg	0.65	-	-		-
SH WT	100	EPS	1%	34.0mg	0.34	-	-		-
SE bcsA	168	EPS	1%	35.1mg	0.21	-	-		-
SE bcsA	224	EPS	1%	108.3mg	0.48	-	-		-
SE bcsA	331	EPS	1%	183.5mg	0.55	73.7% (135.3mg)	48.2mg	0.15 mg/plate	1.2mg/mL DNR, S-20°C
SE bcsA	306	EPS	1%	143.0mg	0.47	82.2% (117.5mg)	25.5mg	0.08 mg/plate	8.9mg
SE bcsA	164	EPS	1%	115.0mg	0.64	-	-		NDY, S4°C
ST bcsA	165	EPS	1%	124.9mg	0.76	-	-		2.8mg
ST bcsA	130	EPS	1%	267.2mg	1.19	79.2% (211.8mg)	55.4mg	0.25 mg/plate	1.1mg/mL DNR, S-20°C
ST bcsA	95	EPS	1%						
ST bcsA yihVW	95	EPS	1%	Few specks		-	-		-
ST bcsA	83	EPS	1%	62.8mg	0.76	-	-		-
ST bcsA pBR-yihVW	83	EPS	1%	31.6mg	0.38	-	-		-
ST bcsA	150	EPS	1%	196.0mg	0.65	-	-		-
ST bcsA	150	EPS	5%			-	-		-
ST bcsA	150	EPS	5%	128.4mg	0.43	-	88.5mg	0.30 mg/plate	-
ST bcsA	150	EPS	5%						
ST bcsA yihVW	25	EPS	5%	48.3mg	1.93	-	-		-
ST bcsA yihVW	25	1% Tryptone	5%	110.7mg	4.43	-	-		-
ST bcsA	25	EPS	5%	61.4mg	2.46	-	-		-
ST bcsA yihVW pBR-yihVW	25	EPS	5%	54.4mg	2.18	-	-		-
ST bcsA yihVW	100	1% Tryptone	10%	455.7mg	4.56	66.5% (303.2mg)	152.5mg	1.53 mg/plate	FSD in 1mL dH_2O
ST bcsA yihVW	100	1% Tryptone 1% Glucose 1% Galactose	10%	270.2mg	2.7	24.9% (67.2mg)	203.0mg	2.03 mg/plate	FSD in 1mL dH_2O
ST bcsA yihVW	60	1% Tryptone	10%	55.6mg	0.93	-	S4°C		-
ST bcsA	60	1% Tryptone	10%	86.6mg	1.44	-	S4°C		-
ST bcsA yihW	60	1% Tryptone	10%	215.2mg	3.59	77% (165.7mg)	49.5mg	0.83 mg/plate	FSD in 1mL dH_2O

PYBC = Polysaccharide yield before chromatography

WLDC = Weight loss during chromatography

PYAC = Polysaccharide yield after Chromatography

FWPC (ALR) = Final weight of purified capsule(After LPS removal)

DNR = does not react

NDY = not done yet

S4°C = stored at 4°C

FSD = few specks dissolved

Table 4.1.1: Amount of O-Antigen capsule isolated after each step in the purification process. Each column represents the yield of O-Antigen capsule following a purification step. Each row represents a single purification carried out for a given strain. Unavailable data is represented by a dash line "-".

The O-Antigen capsule of *Salmonella* is made up of four repeating sugars: galactose, rhamnose, mannose and abequose (in *S.* Typhimurium and *S.* Heidelberg) or tyvelose (in *S.* Enteritidis). In addition, the galactose residue is partially substituted with a glucose side chain (Figure 4.1.4(b)) (Gibson et al., 2006). One possible explanation for the discrepancy between 100-fold increased P*yihUTSRQPO* expression and only 2.3 times more crude capsule production was that there may have been inadequate sugar precursors for the cells to synthesize capsule. To

determine if the addition of sugar would result in an obvious increase in capsule production, which would be signified by a change in colony morphology, *S.* Typhimurium Δ*bcsA* Δ*yihVW* was inoculated on to the following agar containing media: EPS, LB, 1% Tryptone, Terrific broth, Tryptic Soy, SOC, MacConkey, Brain-Heart Infusion, 2X YT, Selenite, and Brilliant Green, supplemented with 1%: glucose, galactose, rhamnose, mannose, fructose, lactose, maltose, cellobiose, sorbose, sucrose, arabinose, or ribose. However, there was no difference in colony morphology between cells grown on these different conditions.

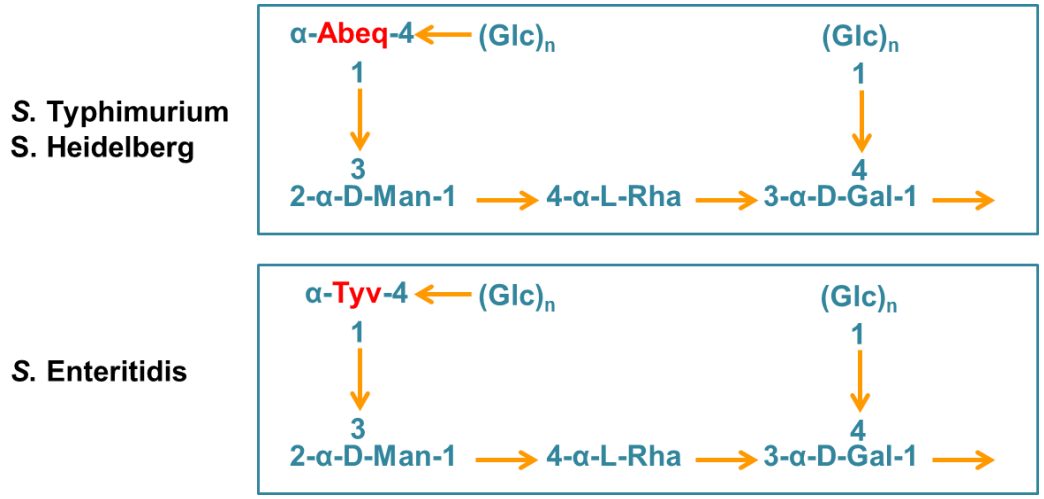


Figure 4.1.4(b): Sugar precursors of O-Antigen capsule. Repeat units of the sugar precursors of *S.* Typhimurium, *S.* Heidelberg and *S.* Enteritidis in making the O-Antigen capsule. In addition to Glucose, Galactose, Mannose and Rhamnose, *S.* Typhimurium and *S.* Heidelberg have abequose, while *S.* Enteritidis has Tyvelose as part of the O-Antigen capsule.

In order to have a closer look at the precursor sugars' influence on colony morphology, single colonies of *S.* Typhimurium Δ*bcsA* were grown on 1% tryptone agar supplemented with 100 ug/mL of the dye congo red and 1%: glucose, galactose, rhamnose, or mannose. In the base media without sugar or media supplemented with 1% rhamnose, or 1% mannose, colonies were shiny, raised, and red/orange in colour (Figure 4.1.4(c)). However, when grown in media supplemented with 1% glucose or 1% galactose, colonies were dry, flat, and maroon/brown in colour. Since the addition of rhamnose or mannose had no noticeable influence on the colony morphology, we did not work with them further. A crude capsule purification was performed on cells grown on 1% Tryptone agar without sugar or supplemented with 1% glucose and 1% galactose: referred to as "no sugar" and "sugar" conditions respectively. Western blotting of crude O-Antigen capsule purified from *S.* Typhimurium Δ*bcsA* Δ*yihVW* grown in no sugar and

sugar conditions revealed no noticeable difference between the capsules (Figure 4.1.4(d)). However, based on weight, the no sugar condition produced twice as much crude polysaccharide precipitate as the sugar condition. Therefore, it is concluded that the lack of O-Antigen capsule production by the Δ*yihVW* mutant cannot be attributed to a deficiency in precursor sugars.

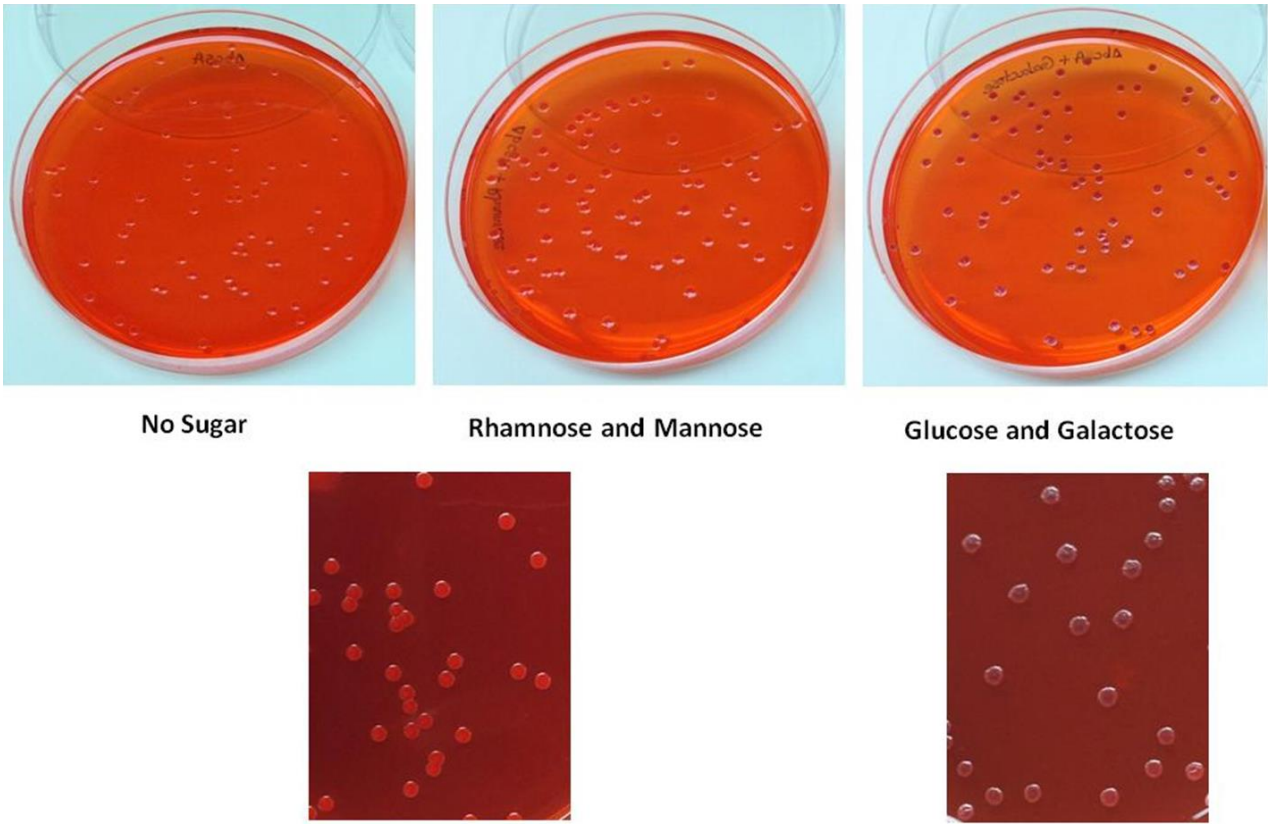


Figure 4.1.4(c): Effect of Sugar Precursors on *S*. Typhimurium Δ*bcsA* Δ*yihVW* colony morphology. Cells were grown at 28ºC for 2 days on 100ug/mL Congo Red + 1% Tryptone agar with no sugar, 1% glucose, 1% galactose, 1% rhamnose or 1% mannose. Addition of Rhamnose, mannose or no sugars produced red/orange colonies, while glucose and galactose produced maroon/brown colonies. Identical results were obtained for *S*. Typhimurium Δ*bcsA* strain.

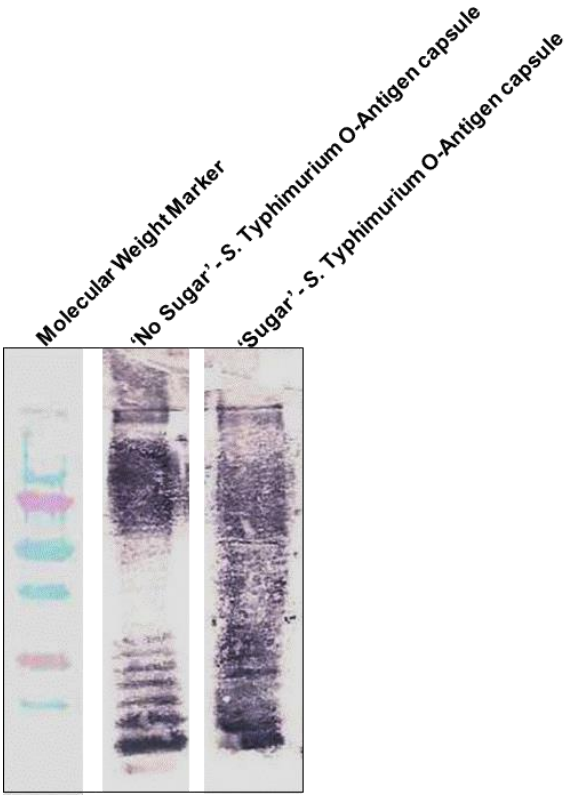


Figure 4.1.4(d): Comparison of crude O-Antigen capsule grown on "sugar" and "no sugar" conditions. Western blots showing the presence of crude O-Antigen capsule following size exclusion chromatography, detected using *S*. Typhimurium O-Antigen capsule specific serum. Cells grown on: (A) 'no sugar condition' - 1% Tryptone agar (B) 'sugar condition' 1% Tryptone agar, 1% glucose, and 1% galactose.

4.1.5 Capsule Staining and Microscopic Imaging

To determine differences in capsule production, individual cells were stained for capsule and visualized under a light microscope. Three *S*. Typhimurium strains were analyzed: WT, Δ*bcsA*, and Δ*bcsA* Δ*yihW*, that were grown on 1% Tryptone agar for 2, 7, or 29 days. *Histophilus somni* was included as a negative control, since it is known to not produce a capsule (Sandal et al., 2011). Cells from each strain were stained with Maneval's Capsule Stain, which is a negative stain, where the cells and the background are stained, but the capsules remained unstained. Upon visualization with a light microscope, the cells stained pink, background stained blue and capsule remained unstained appearing as a white halo (Figure 4.1.5). The cells from each strain changed remarkably over the course of the experiment. On day 2 small, individual pink cells could be seen surrounded by white halos in a blue background. *H. somni*, being a capsule negative strain,

had no white halos, but pink cells in a blue background. On day 7, many cells appeared as long filaments. The cells in these filaments were no longer individually surrounded by a capsule halo, but only had a capsule halo between two cells within the filament. There were also individual cells such as those seen on day 2. *S.* Typhimurium WT and *S.* Typhimurium Δ*bcsA* Δ*yihW* had another set of cells that were in short filaments, but with large white halos surrounding the filaments. By day 29, the colonies of each *Salmonella* strain were dry and rough, while microscopically cells were in very short filaments made up of few cells. Once again small individual cells were seen as in day 2. It was interesting to note that the majority of long filaments from day 7 were no longer present.

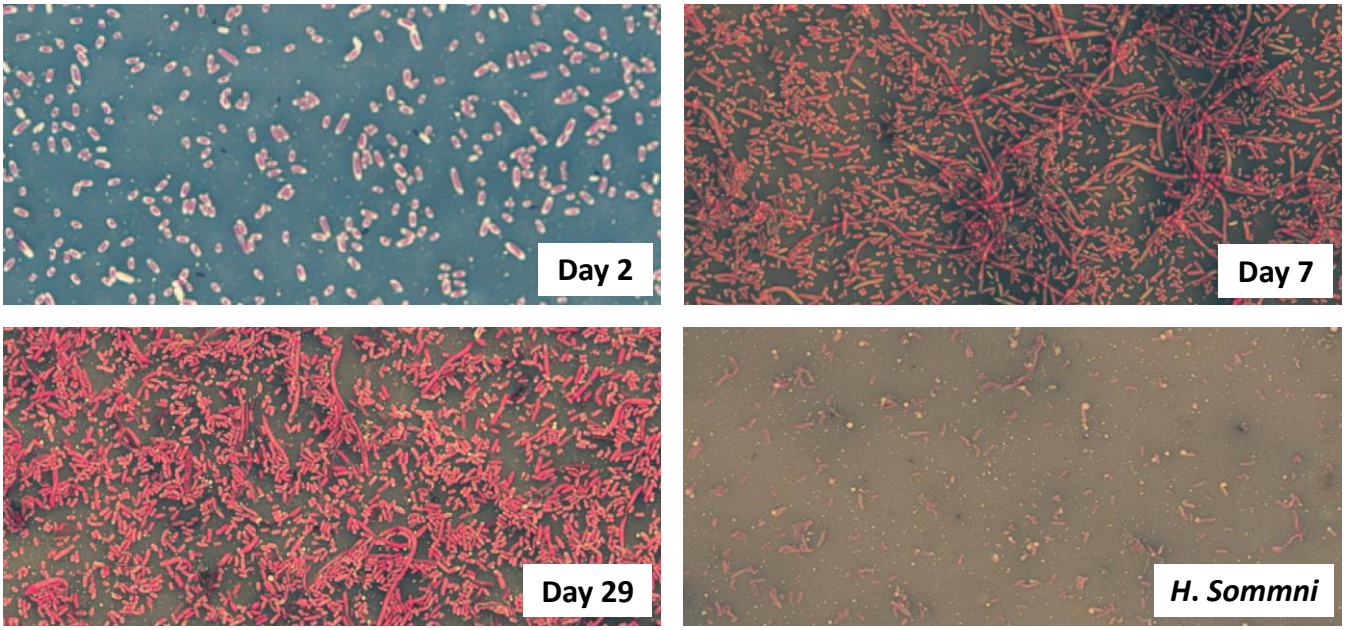


Figure 4.1.5: Visualization of *S*. Typhimurium mutants under a light microscope. *S.* Typhimurium WT, *S.* Typhimurium Δ*bcsA*, *S.* Typhimurium Δ*bcsA* Δ*yihW*, and *H. Somni* cells stained with Maneval's Capsule Stain and visualized on days 2, 7, and 29. Images represent S. Typhimurium Δ*bcsA* Δ*yihW*; identical results were observed for all three strains.

4.1.6 X-Factor Purification

Gibson et al. described a second uncharacterized acidic polysaccharide, known as X-factor that was purified as part of the extracellular matrix (2006). This polysaccharide was negatively charged and was comprised of 4 main sugars: glucose, galactose, xylose, and glucuronic acid, as well as 4 monosaccharides that did not match any known standards (DL Gibson; *Salmonella* Enteritidis This Aggregative Fimbriae and the Extracellular Matrix, PhD Thesis, University of

Victoria, 2000, 208 pages). The X-factor was presumed to elute differently than the O-Antigen capsule during anion exchange chromatography due to the O-Antigen capsule being nearly neutral in overall charge (Gibson et al., 2006). After analyzing all the peaks from the anion exchange column on Western blots using serum specific for *S.* Enteritidis X-Factor (obtained from Deanna Gibson, UBC-Okanagan) it was determined that the X-factor was retained on the column following elution of all other extracellular matrix components (including the O-Antigen capsule). The X-factor was found to elute at the end of the run, when the anion exchange column was 'cleaned' with 2M NaCl (Figure 4.1.6(a)). Furthermore, this X-factor material was found to be cross-reactive with O-Antigen capsule-specific serum (obtained from Deanna Gibson, UBC-Okanagan).

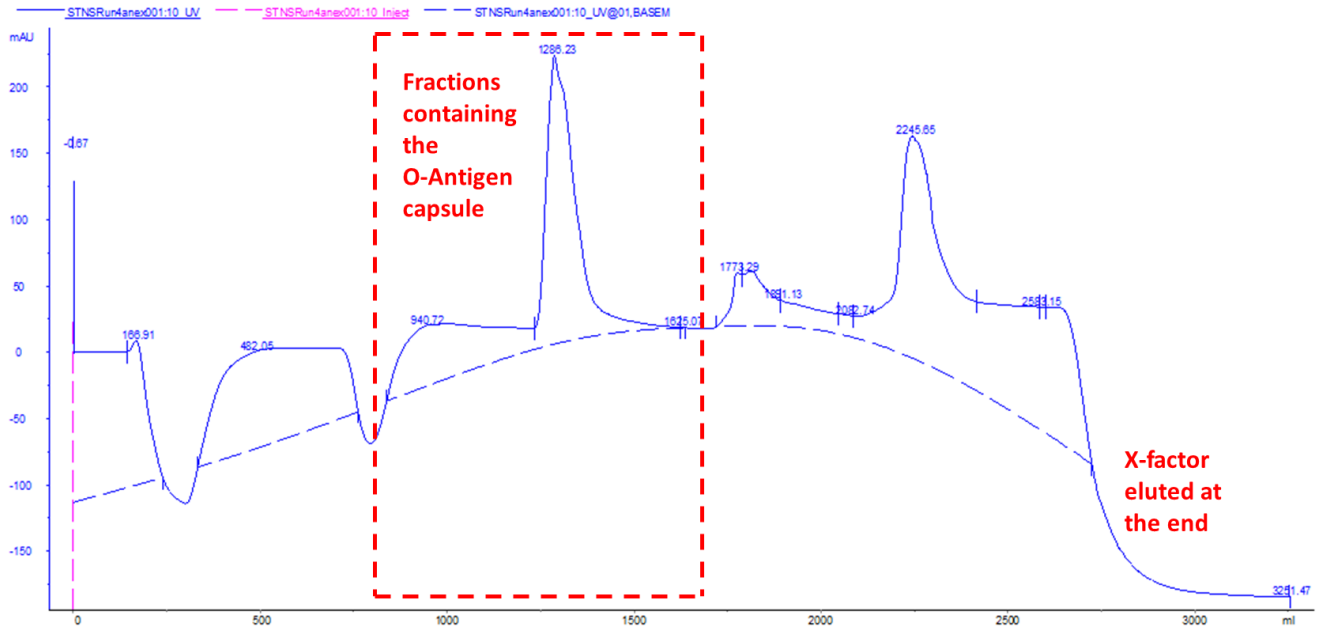


Figure 4.1.6(a): Detection of X-factor during anion exchange chromatography. Following anion exchange chromatography of the O-Antigen capsule, the column was washed with 2M NaCl, which eluted out the X-factor that reacted with X-factor specific serum.

To isolate X-factor, end fractions from the anion exchange chromatography of *S.* Typhimurium O-Antigen capsule were further separated on size exclusion chromatography (Figure 4.1.6(b)-A). The resulting fractions were tested on Western blots using *S.* Typhimurium X-factor-specific serum (obtained from Deanna Gibson, UBC-Okanagan) (Figure 4.1.6(b)-B). As with the O-Antigen capsule, X-factor was also found on the stacking and upper resolving part of SDS gels. The laddering on the bottom of the blot corresponds to low-molecular weight LPS

bands. Serum specific for *S.* Typhimurium O-Antigen capsule recognized crude X-factor from *S.* Typhimurium, and serum specific for *S.* Typhimurium LPS recognized LPS associated with X-factor (Figure 4.1.6(c)). However, since X-factor and O-Antigen capsule elute at different points during the anion exchange chromatography, it is unlikely that the capsule specific serum is recognizing O-Antigen capsule associated with X-factor. The most probable explanation is that capsule-specific serum is either recognizing LPS or being polyclonal, also has specificity to X-factor. During endotoxin removal and subsequent purifications, X-factor fractions were treated in the same manner as O-Antigen capsule (discussed in 4.1 "Purifications").

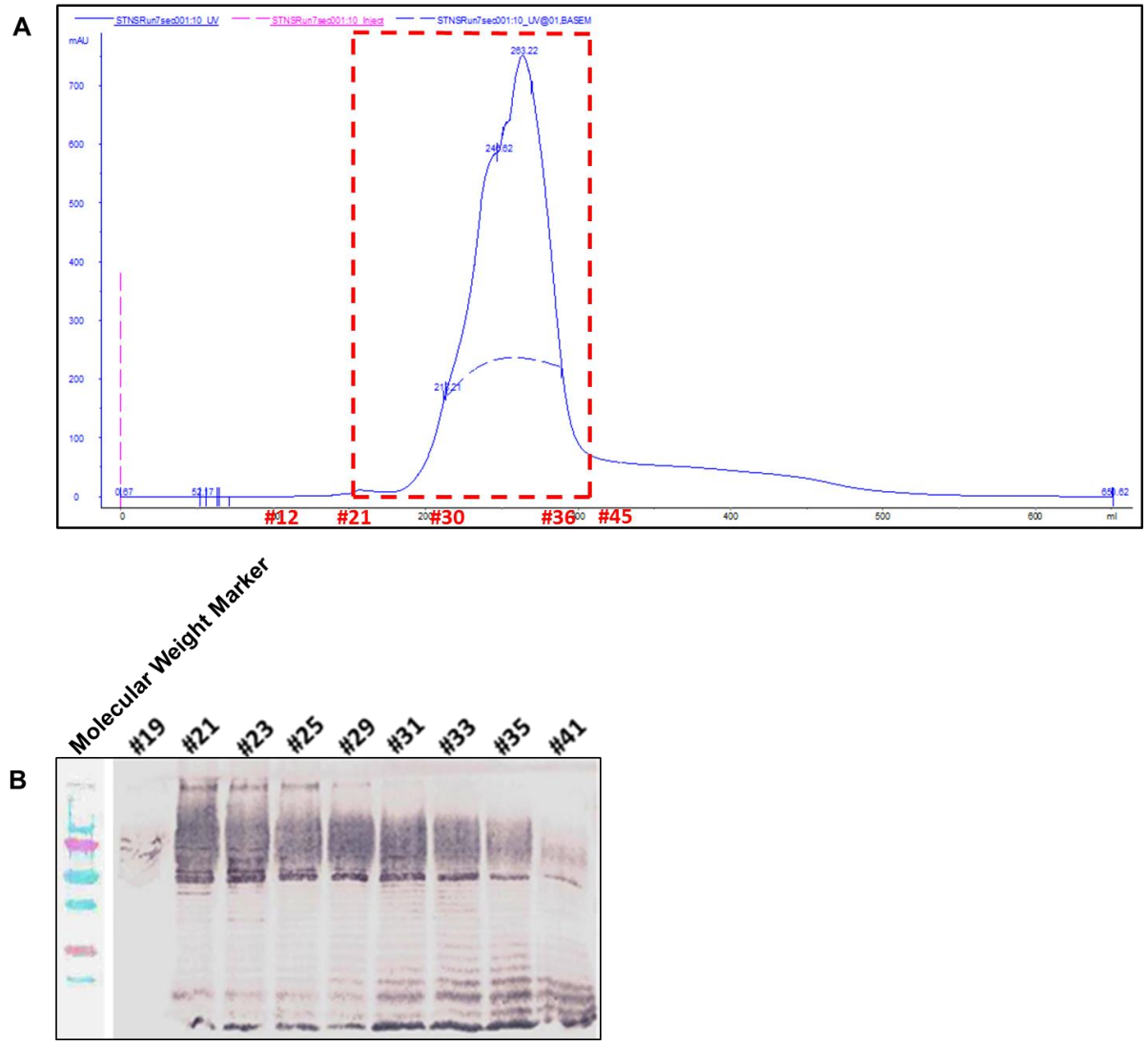


Figure 4.1.6(b): The X-factor. (A) Size exclusion chromatogram for X-factor. Fractions reacted with the X-factor specific serum is indicated by the dashed line. (B) Western blot of crudely purified X-factor samples following size exclusion chromatography detected using rabbit polyclonal immune serum specific for *S.* Typhimurium X-factor (1:1000).

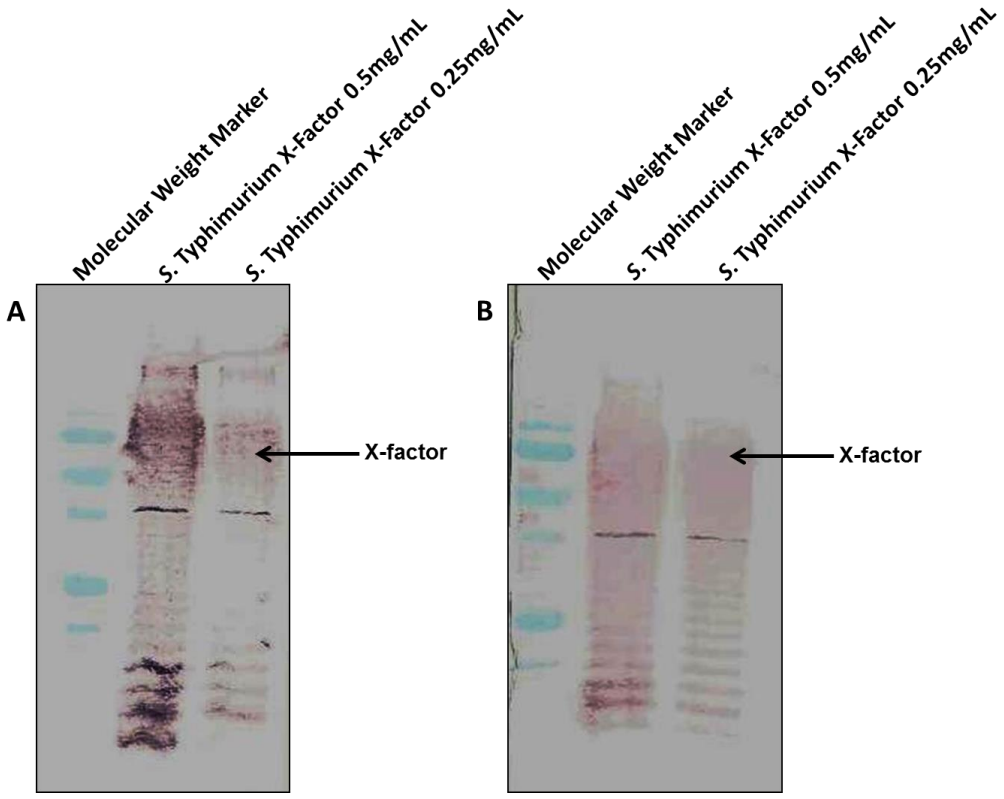


Figure 4.1.6(c): Detection of X-factor by O-Antigen capsule and LPS specific serum. *S*. Typhimurium X-factor recognized by: (A) rabbit polyclonal serum specific for *S*. Typhimurium O-Antigen capsule, (B) mouse monoclonal serum specific for *S*. Typhimurium LPS [1E6] (Abcam: ab65922). The high molecular weight material corresponds to X-factor, while the low molecular weight material corresponds to LPS.

4.2 Animal Trials

4.2.1 Immune Serum Generation

O-Antigen capsule samples were analyzed on Western blots using polyclonal rabbit serum specific for *S.* Typhimurium O-Antigen capsule (Figure 4.2.1(a)). Initially, *S.* Enteritidis O-Antigen capsule-specific serum was used (obtained from Deanna Gibson, UBC-Okanagan), but there was an insufficient amount for screening of O-Antigen capsule production. Thus, rabbits were immunized with purified (endotoxin removed) *S.* Typhimurium and *S.* Enteritids O-Antigen capsule and X-factor to generate immune serum against these antigens.

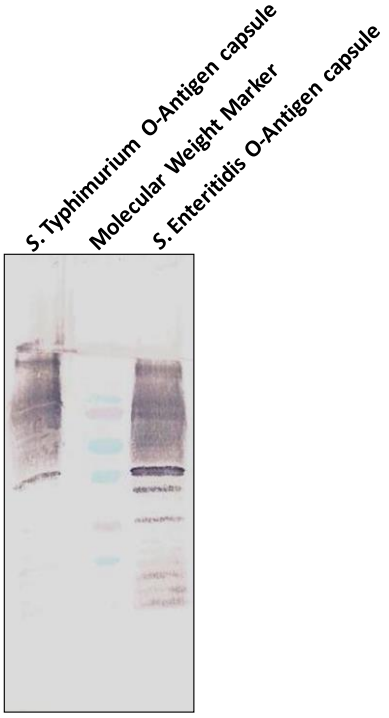


Figure 4.2.1(a): Detection of O-Antigen capsule using specific serum. *S.* Typhimurium and *S.* Enteritidis O-Antigen capsules detected on a Western blot using polyclonal immune serum specific for *S.* Typhimurium O-Antigen capsule.

After three sub-cutaneous (s.c.) immunizations, given at days 0, 21 and 42, the serum antibody titer for each antigen was compared against the corresponding pre-bleed titers (Figure 4.2.1(b)). For *S.* Typhimurium and *S.* Enteritidis O-Antigen capsule and X-factor-specific serum, 1:1000 dilutions of serum were used to achieve a good signal to noise ratio, and optimal use of serum. Each of the pre-bleed sera had poor reactivity against the antigens, as expected. The specificity of these different serums was further tested against the native antigen used for

immunization, along with testing for cross-reactivity between *S.* Typhimurium and *S.* Enteritidis. Both anti *S.* Typhimurium and *S.* Enteritidis O-Antigen capsule serum can cross recognize the capsule from the other serovar (Figure 4.2.1(c)). However, serum against *S.* Typhimurium and *S.* Enteritidis X-factor was not able to cross recognize X-factor between serovars.

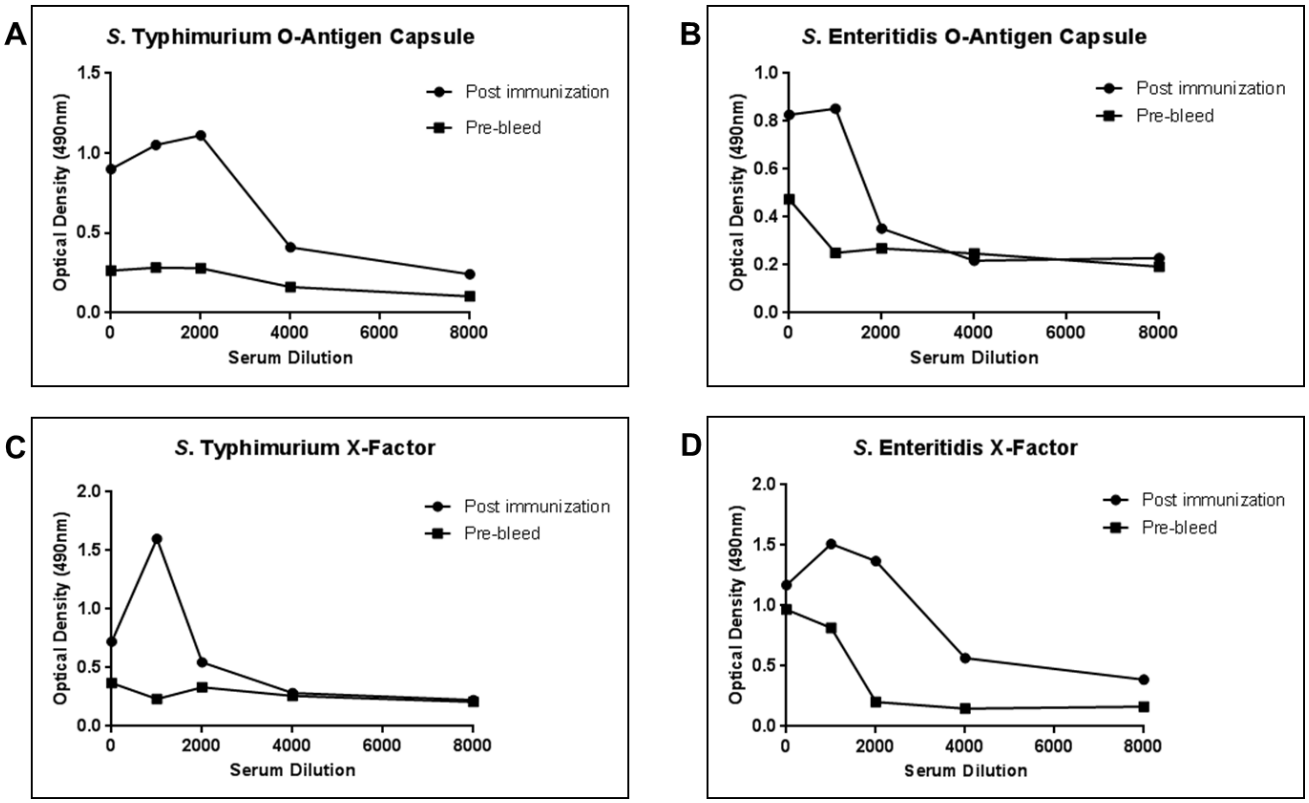


Figure 4.2.1(b): Antibody titers of rabbits that received O-Antigen capsule and X-factor immunizations. ELISA following 3rd immunization with the respective antigen. 30% Emulsigen was used as the adjuvant. Serum dilution 0 is direct serum without any dilutions. Graphs represent the antibody titers for the following antigens: (A) *S.* Typhimurium O-Antigen capsule at a titer of 46340, (B) *S.* Enteritidis O-Antigen capsule at a titer of 2046, (C) *S.* Typhimurium X-factor at a titer of 3140, and (D) *S.* Enteritidis X-factor at a titer of 61121.

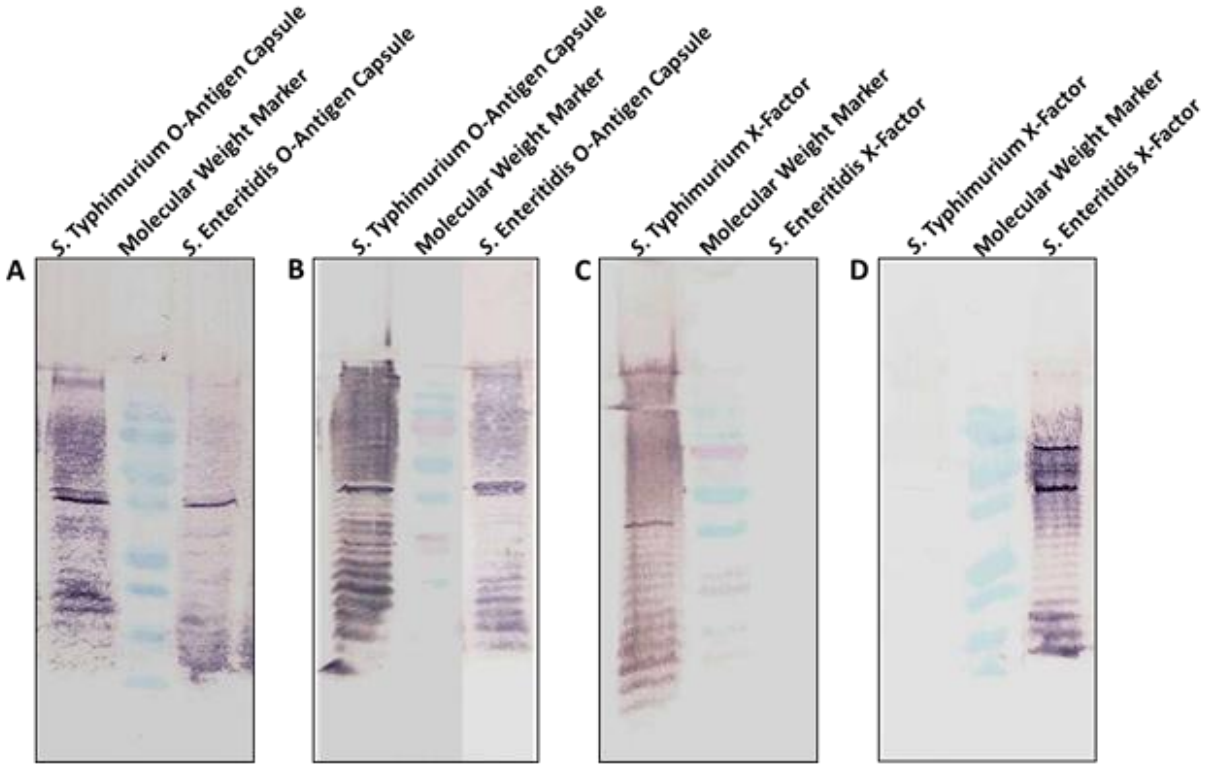


Figure 4.2.1(c): O-Antigen capsule and X-factor recognition ability of its respective antiserum: (A) Rabbit polyclonal anti-*S*. Typhimurium O-Antigen capsule serum, (B) Rabbit polyclonal anti-*S*. Enteritidis O-Antigen capsule serum, (C) Rabbit polyclonal anti-*S*. Typhimurium X-factor serum, and (D) Rabbit polyclonal anti-*S*. Enteritidis X-factor serum. The high molecular weight material corresponds to the O-Antigen capsule, while the low molecular weight material corresponds to LPS.

4.2.2 Dosage Trial

Immunization experiments were carried out to determine the dosage of O- Antigen capsule required to obtain the best possible immune response in mice. Seven groups of mice (n=6) were immunized intramuscularly (i.m.) with different concentrations of purified *S*. Typhimurium O-Antigen capsule, with and without adjuvant (Figure 4.2.2(a)). The adjuvant used was the "triple-combo" formulation developed at VIDO-InterVac, that include polyphosphazenes as a delivery vehicle, CPG ODN (oligo-deoxynucleotides) to stimulate the innate and adaptive immune systems, and Poly I:C, a cationic host defence peptide, to activate antigen presenting cells and other immune cells (Kovacs-Nolan et al., 2009). The positive control group of mice received 50ug of *S*. Typhimurium O-Antigen capsule with 30% Emulsigen as the adjuvant. There was no difference in immune response between treatment groups that received O-Antigen capsule alone

or in combination with the adjuvant (Figure 4.2.2(b)). In addition, the average post-immunization titer was the same as the pre-immunization (pre-bleed) immune response, indicating that immunization with O-Antigen capsule did not generate a detectable level of capsule specific antibody.

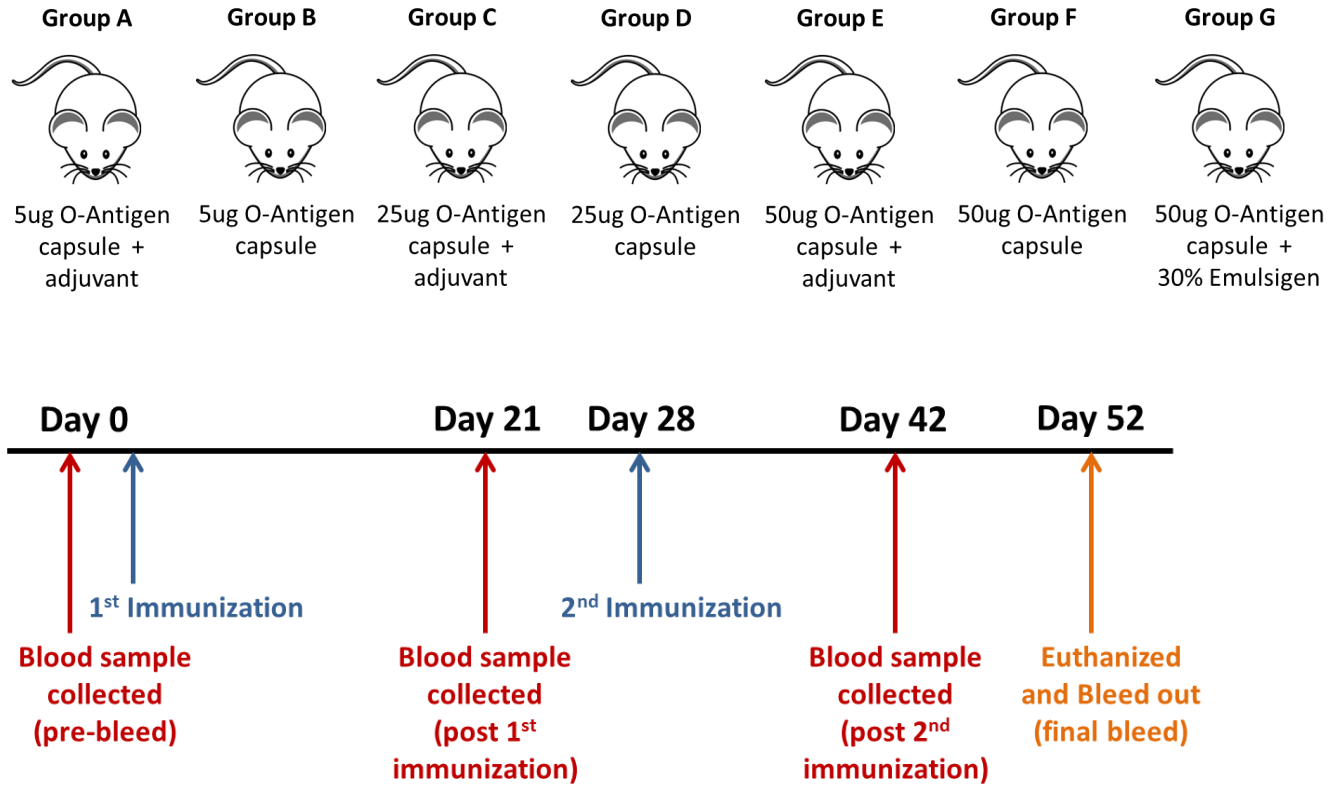


Figure 4.2.2(a): Dosage Trial - Antigen used and the immunization schedule. Top figure: concentration of O-Antigen capsule and adjuvant used to immunize mice in each treatment group (n=6). Bottom figure: the immunization and blood sample collection timeline.

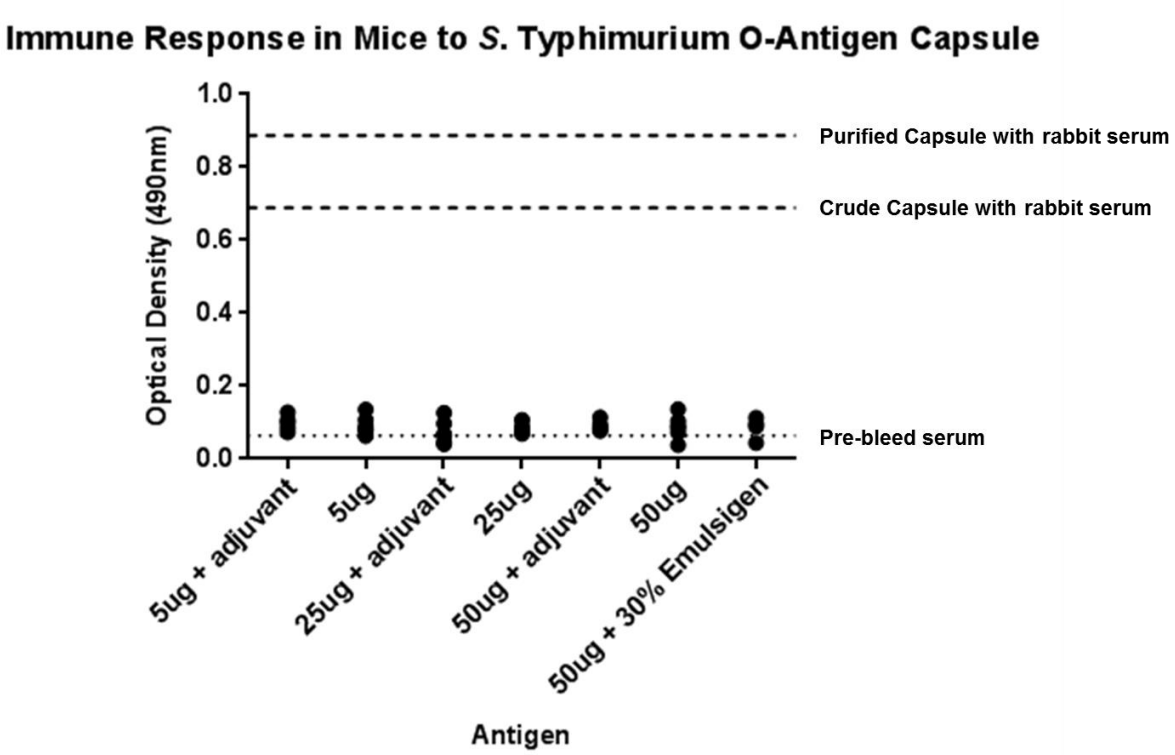


Figure 4.2.2(b): Immune response in mice immunized with purified O-Antigen capsule. Each group (n=6) received a different concentration of O-Antigen capsule with or without adjuvant. A positive control group received 50ug antigen with 30% Emulsigen. Murine serum dilution was at 1:50, and rabbit serum dilution was at 1:1000. This was a controlled, randomized, double-blinded trial.

4.3 Gene Expression Studies

4.3.1 Luciferase Assays and Repressor YihW

Since purification of the O-antigen capsule was a difficult, inefficient and time-consuming process, we wanted to determine if genetic manipulation could be used to increase the amount of O-Antigen capsule produced by *Salmonella*. The genes responsible for O-Antigen capsule production in *Salmonella* are distributed into two divergently transcribed operons: *yihUTSRQPO* and *yihVW* (Figure 4.3.1(a)). YihU is a putative oxidoreductase, similar to *E. coli* hydroxybutyrate dehydrogenase for alternative succinic semialdehyde metabolism (Saito et al., 2009; NCBI-blastn 2015). YihT is a putative aldolase and is involved in glycolysis among many other activities (Gorman et al., 2004; BioCyc 2015; NCBI-blastn 2015). YihS is a putative isomerase and is involved in the interconversion of monosaccharides such as mannose, fructose, glucose, lyxose, and xylulose (Itoh et al., 2008; BioCyc 2015; NCBI-blastn 2015). YihR is a putative aldolase-1-epimerase involved in sucrose, galactose, and trehalose degradation (Herzberg et al., 2006; BioCyc 2015; NCBI-blastn 2015). YihQ has homology to an α-glucosidase that hydrolyses α-glucosyl fluoride, in addition to breakdown of glycogen (Okuyama et al., 2004; BioCyc 2015; NCBI-blastn 2015). In *Salmonella*, YihQ is involved in O-Antigen capsule assembly (Gibson et al., 2006). YihP is a GPH family transport protein similar to *E. coli* putative permease and is involved in transport of galactosides, pentoses, hexuronides (Herzberg et al., 2006; NCBI-blastn 2015). YihO is also a GPH family membrane transport protein and in *Salmonella*, is involved in O-Antigen capsule translocation from inside to the outside of the cell (Herzberg et al., 2006; NCBI-blastn 2015; Giboson et al., 2006). In the divergent operon, YihV is a putative sugar kinase with an unknown regulatory role (NCBI-blastn 2015; BioCyc 2015). YihW is a putative glycerol-3-phosphate regulon repressor similar to *E. coli* putative DeoR-type transcriptional regulator. YihW is predicted to encode a DNA binding protein – a transcriptional repressor (NCBI-blastn 2015; BioCyc 2015; Gibson et al., 2006).

To examine the influence of YihV and YihW on O-Antigen capsule biosynthesis, a Δ*yihVW* strain was constructed using the λ–red recombination system (Datsenko and Wanner 2000). The Δ*yihVW* strain yielded lower amounts of crude O-Antigen capsule compared to *S.* Typhimurium Δ*bcsA* strain (discussed in 4.1 "Purifications"). One possibility is that YihV and YihW were acting as positive regulators of *yihUTSRQPO* operon. To analyze this further, a

pBR322 plasmid construct containing *yihVW* was generated and transformed to both *S.* Typhimurium Δ*bcsA* and *S.* Typhimurium Δ*bcsA* Δ*yihVW* strains. Chromosomal deletion and plasmid-based over expression of *yihVW* was designed to indicate the influence of YihVW on the expression of *yihUTSRQPO* operon.

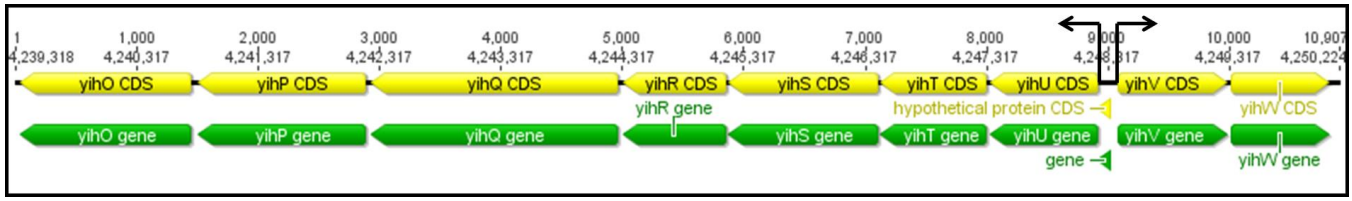


Figure 4.3.1(a): The genes responsible for O-Antigen capsule production in *Salmonella*. The divergent *yihUTSRQPO* operon and the *yihVW* operon are present in all subspecies of *S. enterica*.

To gain information about the real time expression of *yihUTSRQPO* and *yihVW* capsule operons at a cellular level, the promoter (P*yihUTSRQPO* or P*yihVW*) sequences were cloned into a luciferase reporter vector (pCS26-Pac), designed to measure gene expression via light production (Bjarnason et al., 2003), P*yihUTSRQPO* or P*yihVW* was cloned in front of the bacterial luciferase operon (*luxCDABE*) from *Photorhabdus luminescens*. Activation of transcription from the inserted promoter would lead to the production of light (Figure 4.3.1(b)). Thus the intensity of luciferase produced was a clear indication of the promoter activity. Reporter strains were generated with a pCS26-Pac plasmid containing either P*yihUTSRQPO* or P*yihVW*.

In all four strains tested: *S.* Typhimurium Δ*bcsA*, *S.* Typhimurium Δ*bcsA* pBR322-*yihVW*, *S.* Typhimurium Δ*bcsA* Δ*yihVW*, and *S.* Typhimurium Δ*bcsA* Δ*yihVW* pBR322-*yihVW* promoter activity of *yihUTSRQPO* was higher than that of *yihVW* under biofilm inducing conditions (i.e., 30^{o}C in 1% Tryptone). The highest P*yihUTSRQPO* activity was measured in the *S.* Typhimurium Δ*bcsA* Δ*yihVW* strain, which was 100 times higher than that of the *S.* Typhimurium WT or *S.* Typhimurium Δ*bcsA* strains (Figure 4.3.1(c)). The *S.* Typhimurium WT and *S.* Typhimurium Δ*bcsA* strains had comparable levels of P*yihUTSRQPO* activity. The fact that the *S.* Typhimurium Δ*bcsA* Δ*yihVW* strain had the highest P*yihUTSRQPO* activity indicated that YihVW were potential negative regulators of P*yihUTSRQPO* transcription. In addition, *S.* Typhimurium strains containing the pBR322-*yihVW* had only basal levels of P*yihUTSRQPO* activity, indicating that over-production of YihVW decreased the promoter activity of *yihUTSRQPO*. This further supported the observation that YihV and YihW alone or together act

as repressors of the *yihUTSRQPO* operon. Thus, based on gene expression levels observed in this assay, *S.* Typhimurium Δ*bcsA* Δ*yihVW* strain was expected to produce more O-Antigen capsule.

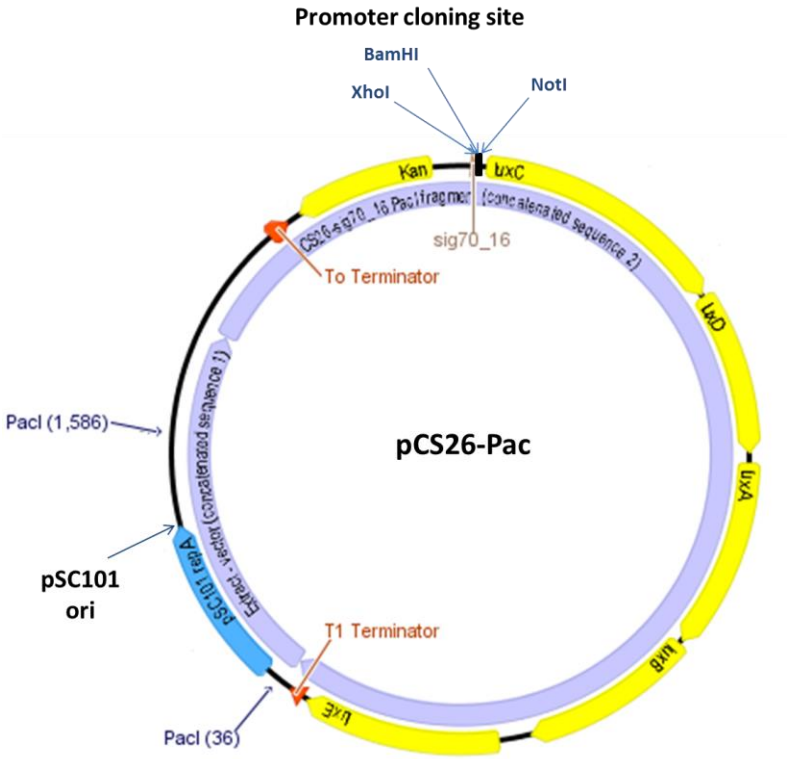


Figure 4.3.1(b): The reporter plasmid pCS26-Pac. This is a low copy number plasmid containing *luxCDABE* of *Photorhabdus luminescens* (Bjarnason et al., 2003).

As the purification protocol involved growing cells on agar surfaces, next it was determined whether the gene expression observed in liquid culture in luciferase assay was also reflected during growth on agar. Light production by individual colonies was observed for *S.* Typhimurium *ΔbcsA*, *S.* Typhimurium *ΔbcsA* pBR322-*yihVW*, *S.* Typhimurium *ΔbcsA* Δ*yihVW*, and *S.* Typhimurium *ΔbcsA* Δ*yihVW* pBR322-*yihVW* strains. As observed in liquid culture, the highest P*yihUTSRQPO* expression occured in the *S.* Typhimurium *ΔbcsA* Δ*yihVW* strain – approximately 100 times more P*yihUTSRQPO* expression as compared to *S.* Typhimurium *ΔbcsA* (Figure 4.3.1(d)). In contrast, the *S.* Typhimurium *ΔbcsA* pBR322-*yihVW* and *S.* Typhimurium *ΔbcsA* Δ*yihVW* pBR322-*yihVW* strains had lowered light production. Therefore,

when comparing among the strains, growing cells on agar or liquid had no clear difference in P*yihUTSRQPO* activity.

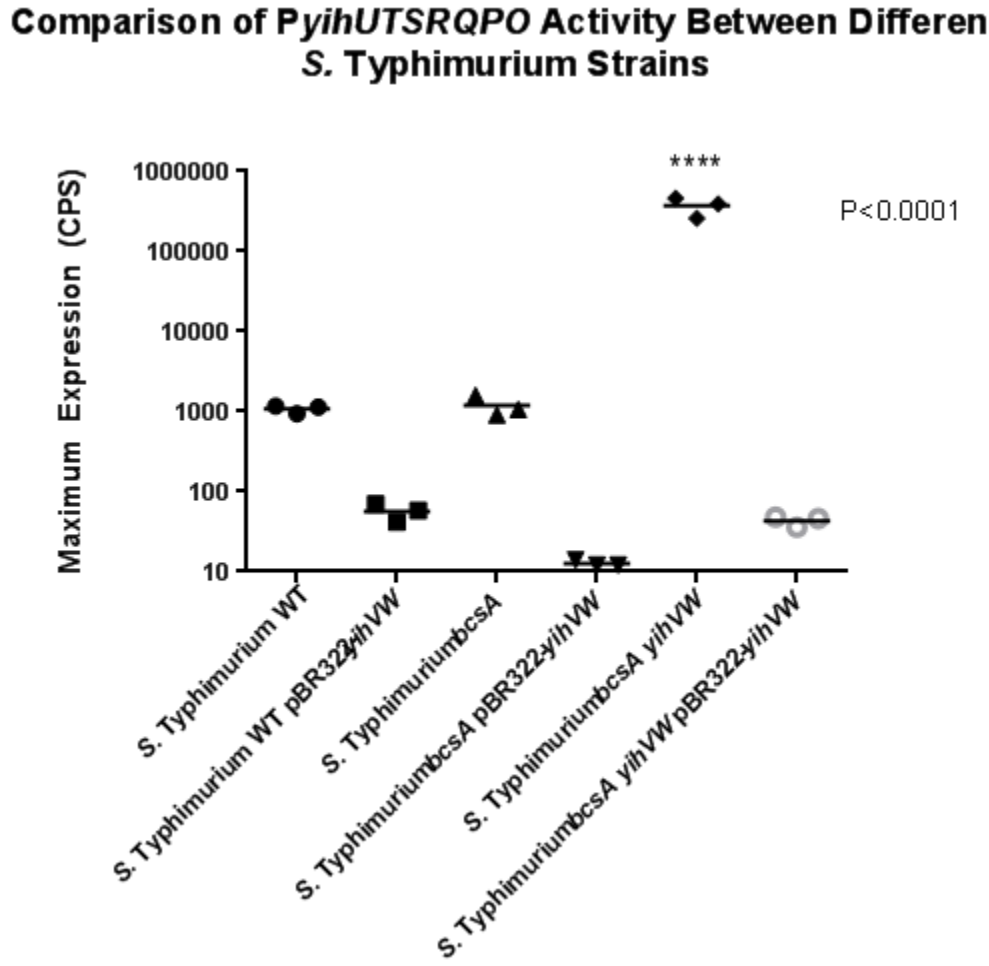


Figure 4.3.1(c): P*yihUTSRQPO* activity of different *S*. Typhimurium strains. Promoter activity of *yihUTSRQPO* in *S*. Typhimurium WT, *S*. Typhimurium Δ*bcsA*, and *S*. Typhimirum Δ*bcsA* Δ*yihVW* strains with and without pBR322-*yihVW* construct grown at 30ºC in 1% Tryptone. Statistics on Graph Pad Prism 6.0 Ordinary one-way ANOVA: P<0.0001. Each strain has a significant difference (****) compared to *S*. Typhimirum Δ*bcsA* Δ*yihVW* strain.

During crude capsule purifications neither the *yihVW* deleted strain (*S*. Typhimirum Δ*bcsA* Δ*yihVW)* nor the *yihVW* over-expressed strain (*S*. Typhimurium Δ*bcsA* pBR322-*yihVW)* produced more capsule than the parent strain (*S*. Typhimurium Δ*bcsA*) (discussed in 4.1 "Purifications"). Thus, it was considered that maybe YihV, being a putative sugar kinase, had a regulatory role in capsule production, and that deletion of *yihV* would adversely affect the amount of capsule produced. Therefore, a *yihW* deletion strain was generated using the λ–red

recombination system to determine whether YihW alone can provide repression and whether YihV was necessary for capsule production. Expression of P*yihUTSRQPO* was measured by luciferase assays.

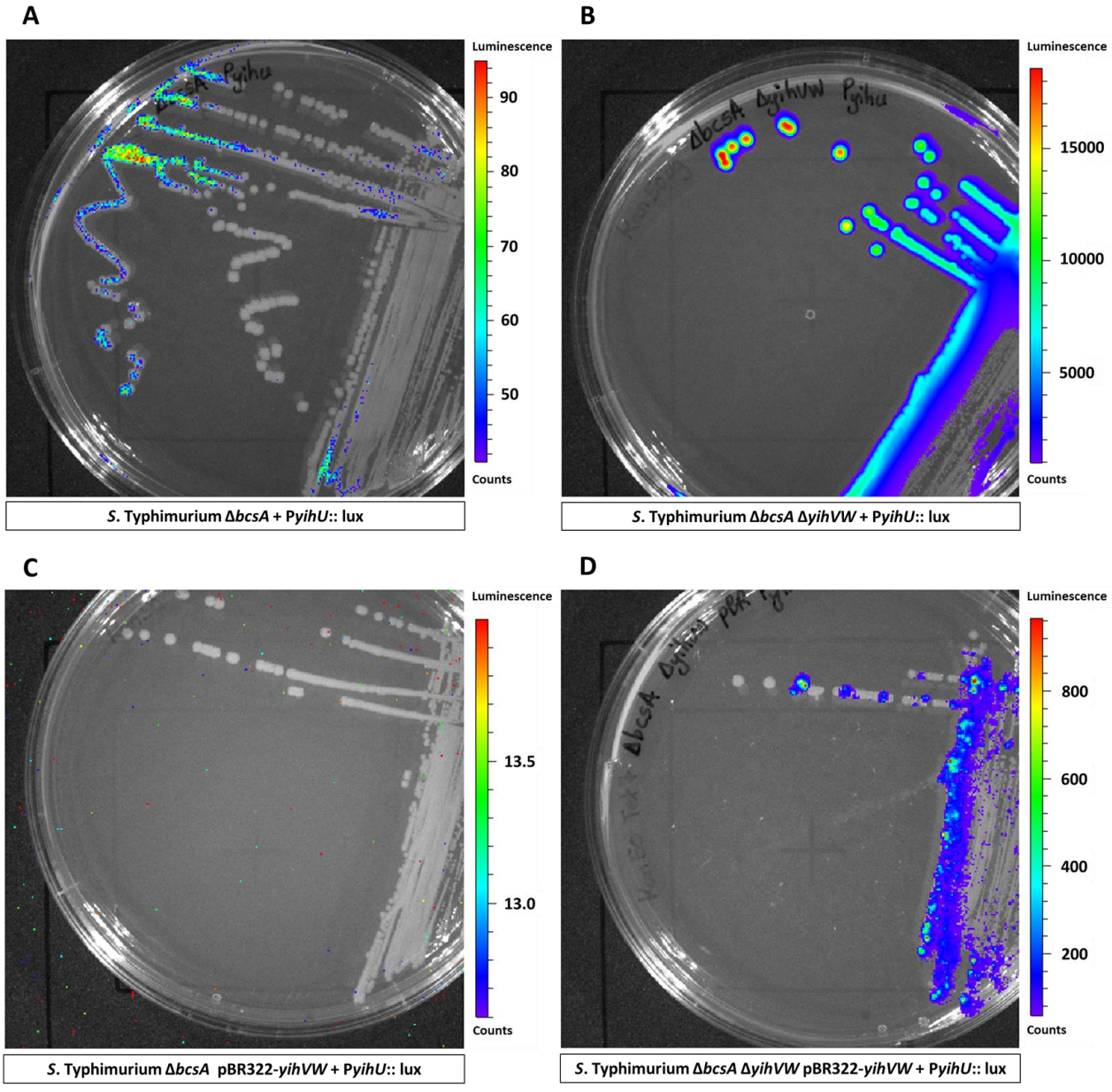


Figure 4.3.1(d): Bioluminescence images of P*yihUTSRQPO* activity of *S*. Typhimurium cells grown on LB agar. P*yihUTSRQPO* activity is measured by the level of luciferase production: (A) *S*. Typhimurium *ΔbcsA*, (B) *S*. Typhimurium *ΔbcsA* Δ*yihVW*, (C) *S*. Typhimurium *ΔbcsA* pBR322-*yihVW*, and (D) *S*. Typhimurium *ΔbcsA* Δ*yihVW* pBR322-*yihVW*

The *S*. Typhimurium Δ*bcsA* Δ*yihW* strain had a slightly higher P*yihUTSRQPO* activity than *S*. Typhimurium Δ*bcsA* Δ*yihVW* strain, but the difference was not statistically significant

(Figure 4.3.1(e)). This indicated that YihW alone was acting as the repressor of *yihUTSRQPO* transcription. Deletion of *yihW* significantly increased P*yihUTSRQPO* activity, and over-expression of *yihVW* from plasmids reduced P*yihUTSRQPO* activity to background levels. However, once again, this increase in promoter activity in the Δ*yihW* strain was not reflected in the capsule yield (discussed in 4.1 "Purifications"). These results suggested that YihV may not have an important positive regulatory role in O-Antigen capsule production. Thus further testing is required to determine the nature of YihV regulation on O-Antigen capsule production.

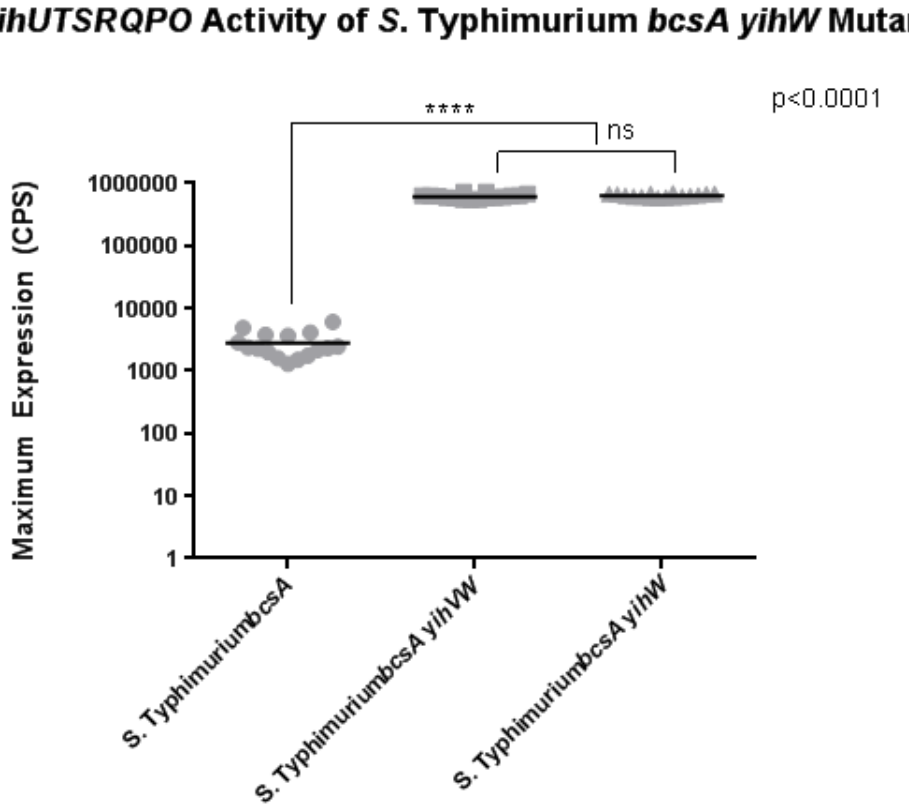


Figure 4.3.1(e): Comparison of P*yihUTSRQPO* activity in S. Typhimurium Δ*bcsA*, Δ*bcsA* Δ*yihVW* and Δ*bcsA* Δ*yihW* strains. Promoter activity of *yihUTSRQPO* in *S.* Typhimurium Δ*bcsA*, *S.* Typhimurium Δ*bcsA* Δ*yihVW*, and *S.* Typhimurium Δ*bcsA* Δ*yihW* strains in 1% Tryptone grown at 30ºC. Statistics on Graph Pad Prism 6.0 Kruskal-Wallis test: P<0.0001. *S.* Typhimurium Δ*bcsA* and *S.* Typhimurium Δ*bcsA* Δ*yihVW* strains significant (****); *S.* Typhimurium Δ*bcsA* and *S.* Typhimurium Δ*bcsA* Δ*yihW* strains significant (****); *S.* Typhimurium Δ*bcsA* Δ*yihVW* and *S.* Typhimurium Δ*bcsA* Δ*yihW* strains not significant.

In order to externally control capsule production, native promoters (P*yihUTSRQPO* and P*yihVW*) were replaced with inducible promoters P*bad* or P*tac* (P*bad-yihUTSRQPO* and P*tac-yihVW* or P*tac-yihUTSRQPO* and P*bad-yihVW*) using lambda red recombinase system (Strozen

et al., 2012). Thus, the addition of arabinose (P*bad*) or IPTG (P*tac*) to the growth medium would lead to the activation of one operon and repression of the other, thereby controlling the capsule production. However, cloning of this promoter construct was not successful.

4.3.2 Effect of growth Conditions on P*yihUTSRQPO* Activity

Different growth parameters were assessed to determine the optimal condition(s) for maximum expression of the *yihUTSRQPO* capsule operon. It was hypothesized that this condition would also lead to increased yield of O-Antigen capsule. First, cells were grown in three different media: EPS media, EPS media supplemented with 40uM of 2, 2'-dipyridyl (an iron chelator), and 1% Tryptone. The P*yihUTSRQPO* activity was measured as light production during growth. *S.* Typhimurium Δ*bcsA*, and *S.* Typhimurium Δ*bcsA* Δ*yihVW* strains both had the highest P*yihUTSRQPO* activity in 1% Tryptone, and the lowest activity in EPS media containing 2,2'-dipyridyl (Figure 4.3.2(a)). *S.* Typhimurium Δ*bcsA* Δ*yihVW* strain had five times greater P*yihUTSRQPO* expression in 1% Tryptone than in EPS. Thus, growth in 1% Tryptone was considered a better alternative to EPS media. Most of the luciferase assays were performed with *S.* Typhimurium Δ*bcsA* Δ*yihVW* strain prior to construction of *S.* Typhimurium Δ*bcsA* Δ*yihW* strain. As the difference in P*yihUTSRQPO* activity between the two strains was not statistically significant, results from *S.* Typhimurium Δ*bcsA* Δ*yihVW* strain was not replaced with *S.* Typhimurium Δ*bcsA* Δ*yihW* strain

Furthermore, *yihUTSRQPO* promoter activity of *S.* Typhimurium Δ*bcsA* and Δ*bcsA* Δ*yihW* strains grown in 1% Tryptone media supplemented with glucose, galactose, rhamnose, and/or mannose was tested (Figure 4.3.2(b)). For *S.* Typhimurium Δ*bcsA* Δ*yihW*, addition of each sugar alone or all four sugars combined did not cause a significant increase in P*yihUTSRQPO* activity, as compared to the base media without sugar added. However for *S.* Typhimurium Δ*bcsA*, addition of galactose, rhamnose and mannose significantly increased P*yihUTSRQPO* activity. Yet, this increase in P*yihUTSRQPO* activity was notably lower than the base level expression by *S.* Typhimurium Δ*bcsA* Δ*yihW*.

For standard O-Antigen capsule purifications, the cells were grown at 30^{o}C under biofilm inducing conditions. To determine if a change in temperature to physiological level would affect P*yihUTSRQPO* expression, luciferase assays were conducted at 30^{o}C and 37^{o}C and

P*yihUTSRQPO* activity was measured. Both *S.* Typhimurium Δ*bcsA* and *S.* Typhimurium Δ*bcsA* Δ*yihW* strains had higher P*yihUTSRQPO* expression at 30ºC (Figure 4.3.2(c)). The *S.* Typhimurium Δ*bcsA* Δ*yihW* strain had 1.2 times more activity at 30ºC, while *S.* Typhimurium Δ*bcsA* strain had 9.7 times more activity at 30ºC than at 37ºC. Therefore, growth at 30ºC was optimal for P*yihUTSRQPO* expression.

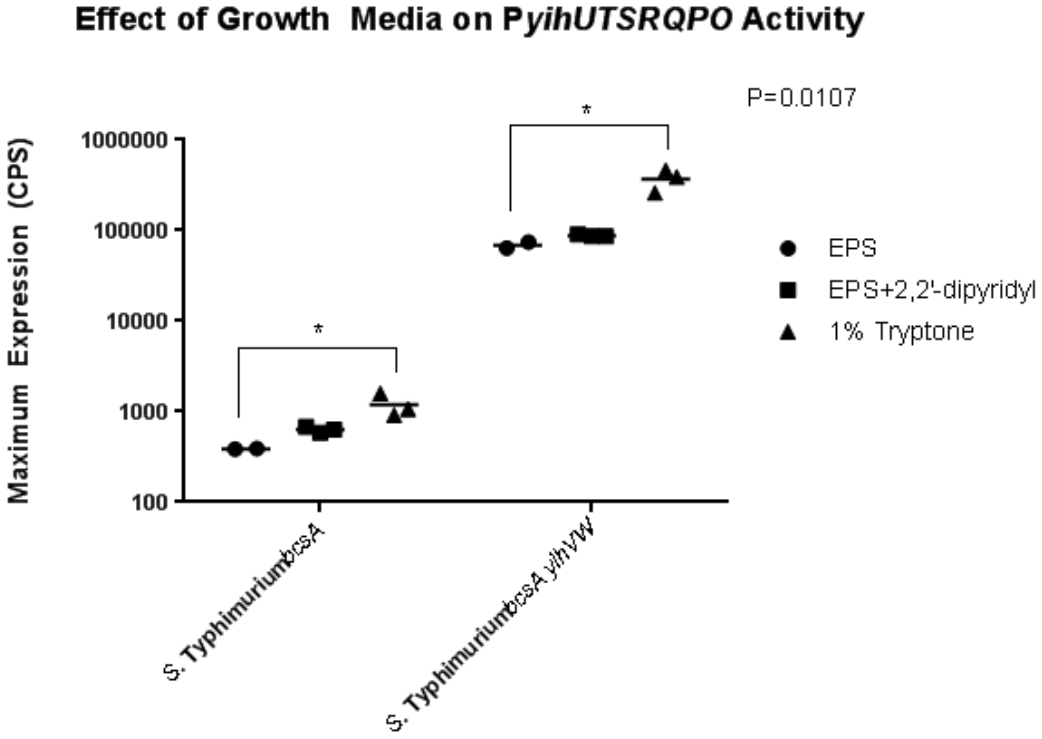


Figure 4.3.2(a): Effect of growth media on P*yihUTSRQPO* activity. Promoter activity of *yihUTSRQPO* when cells were cultured at 30ºC on: EPS, EPS+2,2'-dipyridyl, or 1% Tryptone media. Statistics on Graph Pad Prism 6.0 Kruskal-Wallis test: P=0.0107. For both *S.* Typhimurium Δ*bcsA* and Δ*bcsA* Δ*yihVW* strains, the difference between EPS and 1% Tryptone media is significant, but the differences between the EPS or 1% Tryptone media and EPS+2,2'-dipyridyl media are not significant.

It was decided to determine whether O/N growth conditions would have an impact on P*yihUTSRQPO* expression during the standard O-Antigen capsule purification protocol, because cells were grown O/N in liquid media before inoculating onto agar. During this analysis, luciferase assays were performed under standard conditions: cells diluted in 1% Tryptone and incubated at 30ºC (Figure 4.3.2(d)). The *S.* Typhimurium Δ*bcsA* O/N culture grown in LB and at 37ºC resulted in slightly higher P*yihUTSRQPO* expression than cells grown in 1% Tyrptone and at 30ºC. For *S.* Typhimurium Δ*bcsA* Δ*yihW* there was no significant difference in

P*yihUTSRQPO* expression if the overnight cultures were grown in 1% Tryptone or LB. However, O/N cultures grown at 30°C had increased P*yihUTSRQPO* activity than cultures grown at 37°C. Based on these results, the optimal P*yihUTSRQPO* expression was expected by culturing *S.* Typhimurium Δ*bcsA* Δ*yihW* strain O/N in LB at 30°C.

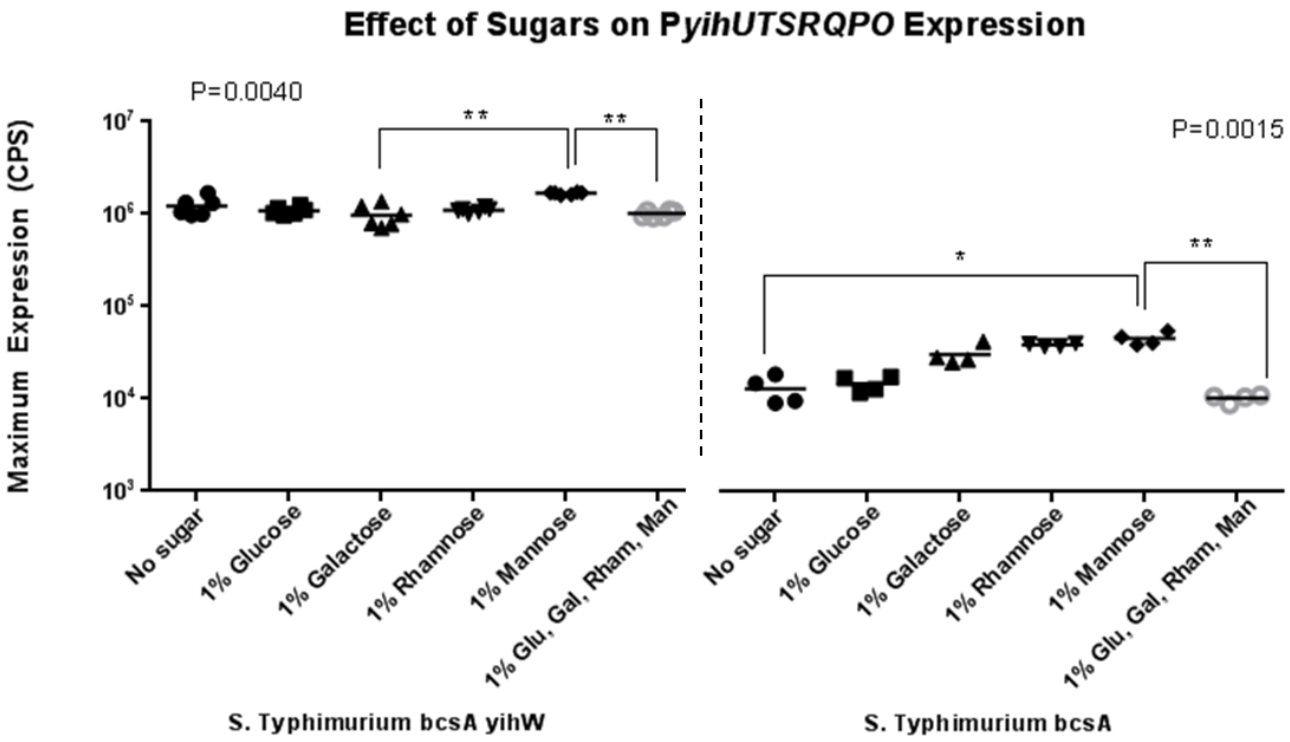


Figure 4.3.2(b): Effect of different sugar precursors on P*yihUTSRQPO* activity. The *S.* Typhimurium Δ*bcsA* and *S.* Typhimurium Δ*bcsA* Δ*yihW* strains were grown at 30°C on 1% Tryptone media with or without the addition of 1% sugar(s): glucose, galactose, rhamnose, mannose, or all four sugars. Statistics on Graph Pad Prism 6.0 Kruskal-Wallis test: For *S.* Typhimurium Δ*bcsA* strain P=0.0015; the difference between No sugar and 1% mannose (*) and between 1% Mannose and all four sugars (**) are significant. For *S.* Typhimurium Δ*bcsA* Δ*yihW* strain P=0.0040; the difference between 1% Galactose and 1% Mannose (**) and between 1% Mannose and all four sugars (**) are significant.

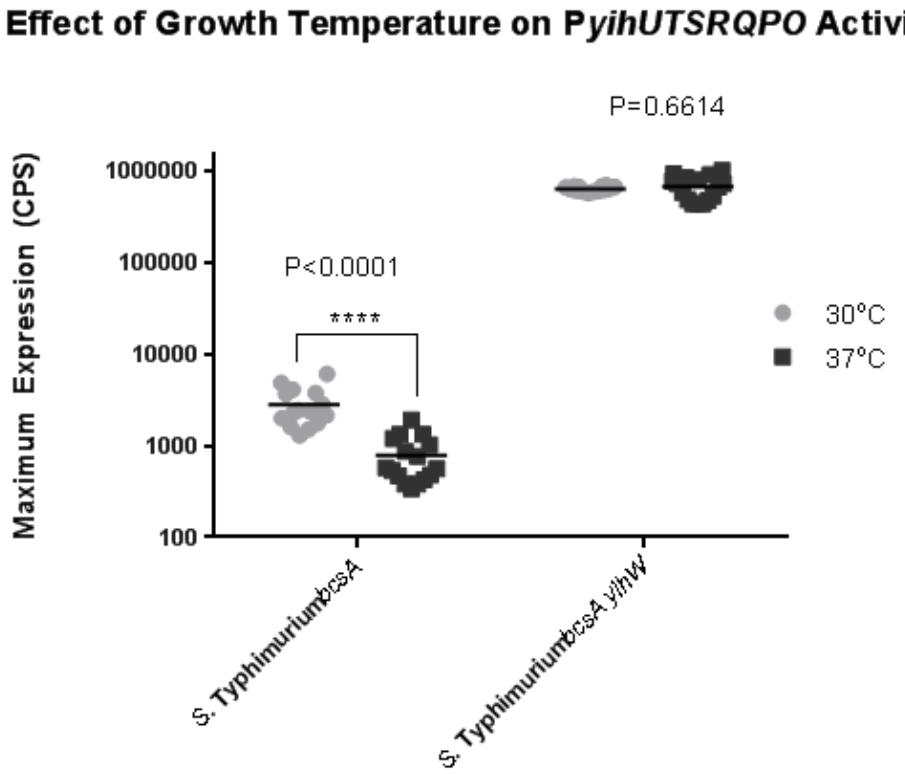


Figure 4.3.2(c): Expression of P*yihUTSRQPO* under different growth temperatures. Comparison of P*yihUTSRQPO* expression during growth at 30ºC and at 37ºC in 1% Tryptone for *S.* Typhimurium Δ*bcsA* and *S.* Typhimurium Δ*bcsA* Δ*yihW*. Statistics on Graph Pad Prism 6.0 Mann-Whitney test: For *S.* Typhimurium Δ*bcsA* strain P<0.0001 (****); the difference between 30ºC and 37ºC growth conditions is significant. For *S.* Typhimurium Δ*bcsA* Δ*yihW* strain P=0.0366 (*) and the difference is not significant.

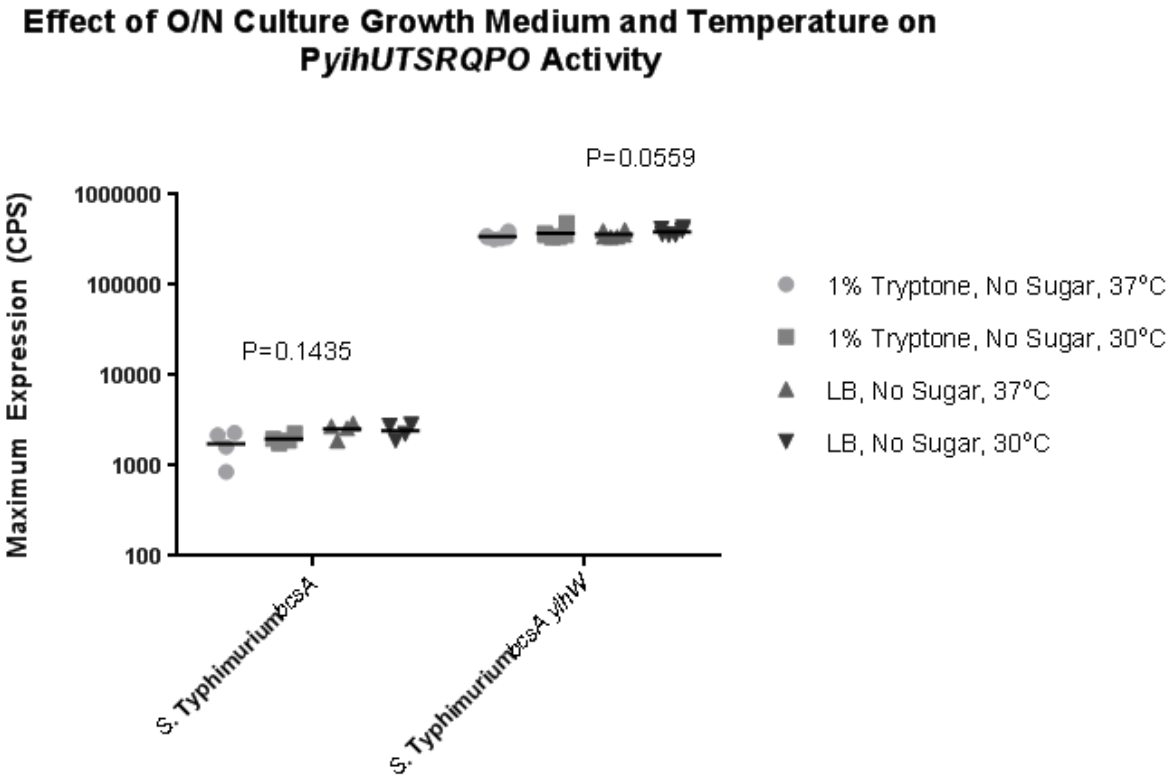


Figure 4.3.2(d): Effect of O/N culture conditions on P*yihUTSRQPO* activity. Effect of O/N culture growth medium and temperature on P*yihUTSRQPO* expression during subsequent luciferase assays carried out under standard conditions: 1% Tryptone at 30ºC. Statistics on Graph Pad Prism 6.0 Kruskal-Wallis test. For *S.* Typhimurium Δ*bcsA* strain P=0.1435 (ns). For *S.* Typhimurium Δ*bcsA* Δ*yihW* strain P=0.0559 (ns).

5.0 DISCUSSION

5.1 Concluding Remarks

The O-Antigen capsule of Non-Typhoidal *Salmonella* is a ubiquitous surface polysaccharide that may become a promising vaccine candidate for immunization against NTS associated gastroenteritis. O-Antigen capsule biosynthesis is controlled by the divergently transcribed operons *yihUTSRQPO* and *yihVW*. For the purpose of this study, O-Antigen capsule was purified from *S*. Typhimurium Δ*bcsA*, *S*. Typhimurium Δ*bcsA* Δ*yihVW* and *S*. Typhimurium Δ*bcsA* Δ*yihW* strains. Comparison of crude polysaccharide yield between the three strains revealed that deletion of *yihVW* resulted in inability to extract any polysaccharides, but over expression of *yihVW* from plasmids in *S*. Typhimurium Δ*bcsA* only produced half as much crude polysaccharides compared to the *S*. Typhimurium Δ*bcsA* parent strain. However, deletion of Δ*yihW* alone resulted in 2.5x more crude polysaccharide production compared to the *S*. Typhimurium Δ*bcsA* strain. This indicates that in the absence of repression by YihW, more crude polysaccharides are produced, and the over expression of the YihVW negatively affect the crude polysaccharide yield. As the *yihVW* mutant strain was unable to produce any capsule, YihV, being a sugar kinase, may have a positive regulatory role in O-Antigen capsule production. However, more experiments are needed to determine the specific regulatory nature of YihV.

A luciferase based reporter system was used to observe the P*yihUTSRQPO* and P*yihVW* activity of *S*. Typhimurium Δ*bcsA*, *S*. Typhimurium Δ*bcsA* pBR322-*yihVW*, *S*. Typhimurium Δ*bcsA* Δ*yihVW*, and *S*. Typhimurium Δ*bcsA* Δ*yihVW* pBR322-*yihVW* strains. In all conditions tested, P*yihUTSRQPO* had higher expression than P*yihVW*. The *S*. Typhimurium Δ*bcsA* Δ*yihVW* strain had 100 times higher P*yihUTSRQPO* activity compared to *S*. Typhimurium WT and *S*. Typhimurium Δ*bcsA* strains. In addition, strains containing the pBR322-*yihVW* plasmid had only basal levels of P*yihUTSRQPO* activity, indicating that over production of YihVW decreased the promoter activity of P*yihUTSRQPO*. Comparison of P*yihUTSRQPO* activity between *S*. Typhimurium Δ*bcsA* Δ*yihVW* and *S*. Typhimurium Δ*bcsA* Δ*yihW* strains showed that there was no significant difference in expression between the two strains. Thus the deletion of *yihW* significantly increased P*yihUTSRQPO* activity, and over expression of *yihVW* from plasmids

reduced P*yihUTSRQPO* expression to background levels, indicating that YihW alone acts as a repressor of the *yihUTSRQPO* operon.

However, the lack of crude capsule production, even when the P*yihUTSRQPO* expression increased by 100 times compared to the parent strain indicates that there may be other regulatory mechanisms governing O-Antigen capsule production, such as (a) secondary RNA structures preventing translation of the *yihUTSRQPO* operon, (b) mRNA stability, (C) post-translational modifications, (d) or the cell simply cannot support large amounts of capsule production, especially if the O-Antigen capsule machinery takes precursor subunits from the LPS assembly pathway. Therefore further research, such as phosphoproteome studies, are needed to understand the discrepancy between promoter activity and capsule production.

Upon assessment of the influence of growth conditions on the P*yihUTSRQPO* activity and capsule production, it was observed that *S.* Typhimurium Δ*bcsA* Δ*yihVW* strain grown on 1% Tryptone media resulted in the highest P*yihUTSRQPO* activity, at 5X more activity than when grown in EPS. Thus to increase capsule production, growth in 1% Tryptone was considered a better alternative to EPS media. In support of this, *S.* Typhimurium *ΔbcsA* Δ*yihVW* grown on 1% Tryptone agar produced ~2.3X more crude polysaccharide compared to growth on EPS agar.

In addition, single colonies of *S.* Typhimurium *ΔbcsA* Δ*yihVW* grown on 100 ug/mL Congo red + 1% Tryptone agar in the presence of 1% glucose or 1% galactose produced dry, flat, maroon/brown colonies, while in the presence of 1% rhamnose, 1% mannose, or no additional sugars colonies were shiny, raised, and red/orange in colour. During capsule purification, the presence of 1% glucose and 1% galactose in the media reduced the amount of crude capsule obtained by half. However, luciferase assays showed that for *S.* Typhimurium Δ*bcsA* Δ*yihW* strain, addition of either sugar alone or combined all four sugars have no significant difference in P*yihUTSRQPO* activity compared to the No Sugar condition (1% Tryptone). Among the sugars tested, the highest activity was observed in 1% Mannose and lowest activity was observed in the presence of all four sugars. Nonetheless, there were no differences observed on the chromatograms or Western blots between crude polysaccharides extracted from cells grown in the presence or absence of sugar.

Previously it had been shown that *yihUTSRQPO* operon is carbon catabolite repressed in *Salmonella*, and cAMP receptor protein (CRP) binding on two sites in the regulatory region of the *yihUTSRQPO* operon is required for its transcriptional activation (Villarreal et al., 2011). The

implication of this is that genes that allow the use of secondary carbon sources are repressed in the presence of glucose or other preferred carbon sources (Bruckner and Titgemeyer 2002; Gorke and Stulke 2008). In *Salmonella*, cAMP acts as a sensory signal responding to the availability of glucose, and in turn affects the expression of many catabolic pathways (Botsford and Harman 1992; Ullmann and Monod 1968). Villarreal et al., demonstrated that growth in the presence of glucose had a suppressive effect on the expression of P*yihUTSRQPO* compared to growth in glycerol (2011). This observation is supported by the notion that cAMP positively regulates *yihUTSRQPO* operon leading to CRP binding and transcriptional activation, while glucose in the medium supresses the expression of *yihUTSRQPO* operon (Villarreal et al., 2011).

As growing cells on agar or liquid culture resulted in no clear difference in P*yihUTSRQPO* activity, capsule purification was carried out by growing cells on agar surfaces. Both *S.* Typhimurium Δ*bcsA* and *S.* Typhimurium Δ*bcsA* Δ*yihW* strains had the strongest P*yihUTSRQPO* expression when they were grown at 30^{o}C. The *S.* Typhimurium Δ*bcsA* strain had 9.7X greater activity at 30^{o}C as compared at 37^{o}C, whereas the *S.* Typhimurium Δ*bcsA* Δ*yihW* strain had 1.2X more activity at 30^{o}C, mainly because P*yihUTSRQPO* expression was already maximal in this strain. The culture conditions that the strains were grown in O/N, prior to inoculating the agar plates, did not pappear to influence subsequent growth or capsule production.

The effects of the growth conditions on the colony morphology and capsule production were also assessed. Colony morphology, capsule staining, and microscopic imaging provided valuable information about the characteristics of different strains, but did not reveal any unique distinguishing features between the strains tested. Light microscopy at a higher magnification or visualization with an electron microscope would be able to provide more concrete information as to how deletion of specific genes affects the appearance and texture of the O-Antigen capsule.

Silver staining and Western blots, using *S.* Typhimurium LPS specific serum, confirmed that crudely purified O-Antigen capsule was associated with endotoxin (LPS) even after anion exchange and size exclusion chromatography. Since a LPS-dominated immune response could prove problematic for furture vaccine development, Triton X-114 was used to remove LPS associated with the crude O-Antigen capsule. Although Triton extraction removed a significant proportion of LPS, it also reduce the concentration of O-Antigen capsule. Even though the final purified O-Antigen capsule had LPS associated with it ($2.5*10^3$ EU/ml as measured by the LAL

assay, Section 4.1.3 “Endotoxin Removal”), this was within the biologically safe limit to administer to mice (LD_{50} was $4.55*10^6$ EU/ml - Beutler et al., 1985).

In addition to the O-Antigen capsule, a second extracellular polysaccharide called X-factor was also purified and tested on Western blots. X-factor was retained in the anion exchange column during the elution of other extra cellular matrix components (including the O-Antigen capsule) and only eluted at the end when the column was ‘cleaned’ with NaCl. Furthermore, serum specific for *S.* Typhimurium O-Antigen capsule was able to recognize *S.* Typhimurium X-factor, and also importantly, serum specific for *S.* Typhimurium X-factor was able to recognize *S.* Typhimurium O-Antigen capsule. However, in contrast to the O-Antigen capsule serum, X-factor serum did not cross recognize between serovars, such that *S.* Typhimurium X-factor serum did not recognize *S.* Enteritidis X-factor and vice versa. The lack of cross reactivity in the X-factor serum between serovars could mean the immune response is strain-specific as would be expected if it is generated against LPS. Therefore either the X-factor serum is primarily recognizing LPS specific for the given serovar or the X-factor is also strain-specific. However, this also indicates that O-Antigen capsule specific serum, being cross reactive, is not specific for LPS, or has a very low reactivity with LPS. Serum was generated by immunizing rabbits with purified O-Antigen capsule or X-factor from *S.* Enteritidis and *S.* Typhimurium.

Purified O-Antigen capsule was administerd at different doses with and without adjuvant to mice in an effort to determine the proper dosage required to generate a robust antibody response. However, none of the treatment groups yielded a statistically significant immune response to the capsule (i.e immunization with O-Antigen capsule did not generate a detectable level of capsule-specific antibody). This could be due to O-Antigen capsule not being a strong immunogen. In addition, the antibody response could be mainly IgM driven, as would be expected for a pure polysaccharide antigen as the O-Antigen capsule. However, the alkaline phonsphatase conjugated goat anti-mouse secondary antibody was specific for both heavy and light chains of immunoglobulins, and thus the secondary antibody was able to recognize IgG as well as IgM antibodies (Thermo Scientific 2015). Therefore if O-Antigen capsule specific IgM was present in the murine serum this would have been detected during ELISA.

Waite and March (2002) showed that when BALB/c mice were immunized with *Mycoplasma mycoides* subsp. *mycoides* small colony biotype vaccine candidates (i.e. capsular polysaccharide conjugated to ovalbumin and whole inactivated ultrasonically disrupted *M.*

mycoides [WID]), only mice immunized with the conjugate vaccine generated a capsule-specific antibody response. However, upon challenge with *M. mycoides*, mice immunized with the conjugate vaccine did not develop a protective response, while mice immunized with WID vaccine were completely protected and exhibited a significant reduction in the bacterial load despite the lack of detectable antibody production (Waite and March 2002). This indicates that the apparent antibody response is not necessarily a direct indication of immunity and protection.

In another study (Svenson and Lindberg 1981) it was shown that rabbits immunized with *S.* Typhimurium O-antigen (O4 and O12) conjugated to a hapten carrier suspended in Freund's complete adjuvant promptly responded with antibody titers as high as those observed after immunization with heat-killed bacteria. However, in mice, the same antigens generated a 50-fold lower response as was seen with immunizations with heat-killed bacteria. Nevertheless, passively transferred rabbit antibodies against the polysaccharide-hapten conjugate was able to protect mice against challenges of 100 times the 50% lethal dose of *S.* Typhimurium (Svenson and Lindberg 1981). It had also been shown that even in the absence of a detectable primary antibody response, some children were able to produce a detectable memory B cell response towards *H. influenzae* type b (McVernon et al., 2003).

Furthermore, Waite and March showed that in contrast to mouse antisera which had high antibody titers but no protective antibodies, rabbit serum was able to inhibit *in vitro* growth of *M. mycoides*. Thus it was proposed that either the mouse antibodies to *M. mycoides* were not active *in vitro* or immunity to *M. mycoides* in mice was through cell-mediated immunity rather than through humoral immunity. Alternatively, it was also suggested that the mechanism of antibody-mediated protection may differ between the antibodies of rabbits and mice (Waite and March 2002). These observations can be applied to explain the lack of antibody production by mice, but not rabbits, towards the O-Antigen capsule. In addition to the possibility of O-Antigen capsule being a poor immunogen, rabbits and mice may respond differently towards polysaccharide antigens, specifically the O-Antigen capsule.

5.2 Future Directions

It would be valuable to consider different LPS extraction methods, such that it will be easier to purify more O-Antigen capsule with less LPS contamination. In addition, deletion of

specific LPS biosynthesis genes or mutation of genes coding for proteins involved in LPS export pathway could potentially positively influence the yield of the O-Antigen capsule. The impact of the absence of LPS on *Salmonella* growth has to be investigated along with whether the LPS export pathway is also involved in O-Antigen capsule export to the cell surface.

To better understand the discrepancy between the increased *yihUTSRQPO* promoter activity and the lack of a corresponding increase in capsule production, a closer look at the stability of the mRNA molecules and quantification of the amount of each protein (YihUTSRQPO) produced would be valuable. This could help identifying the rate-limiting step in the capsule biosynthesis pathway. Another possible experiment would be to perform transposon-mutagenesis in the *S.* Typhimurium Δ*bcsA* Δ*yihW* strain to determine if disruption of secondary genetic factors could lead to an increase in capsule production.

A better understanding of the antigenic epitopes of the O-Antigen capsule would serve valuable, such that the O-Antigen capsule can be better formulated to fully expose the antigenic epitopes, making it easier for the immune cells to recognize the capsule. One approach would be to infect mice with *Salmonella* and use the serum from these mice to screen NTS produced extracellular proteins and polysaccharides (including the O-Antigen capsule) to identify whether O-Antigen capsule is recognized by immune serum and also what other antigens are being recognized by this serum that is likely to contain antibody against these extracellular antigens of *Salmonella*.

Going forward, it is my belief that a conjugate vaccine approach chemically coupling the O-Antigen capsule to flagella or outer membrane proteins from *Salmonella* would be the most promising approach for development of a NTS vaccine. If the O-Antigen capsule is found to be non-cross protective *in vivo*, then a multivalent vaccine approach, as with the pneumococcus PPSV23 vaccine (Shapiro et al., 1991), can be used containing capsule antigens from the most prevalent *Salmonella* serovars.

6.0 REFERENCES

1. Adam O., Vercellone A., Paul F., Monsan P.F., Puzo G. (1995). A Nondegradative Route for the Removal of Endotoxin from Exopolysaccharides. *Analytical Biochemistry*. 225:321-327.

2. Adams W.G., Deaver K.A., Cochi S.L., Plikaytis B.D., Zell E.R., Broome C.V., and Wenger J.D. (1993). Decline of Childhood *Haemophilus influenzae* Type b (Hib) Disease in the Hib Vaccine Era. *Journal of the American Medical Association*. 269(2):221-226.

3. Amor P.A., and Whitfield C. (1997). Molecular and Functional Analysis of Genes Required for Expression of Group 1B K Antigens in *Escherichia coli*: Characterization of the *his*-region Containing Gene Clusters for Multiple Cell-Surface Polysaccharides. *Molecular Microbiology*. 26(1):145-161.

4. Andrews-Polymenis H.L., Baumler A.J., McCormick B.A., and Fang F.C. (2010). Taming the Elephant: *Salmonella* Biology, Pathogenesis and Prevention. *Infection and Immunity*. 78(6):2356-2369.

5. Auckland C., Gray S., Borrow R., Andrews N., Goldblatt D., Ramsay M., and Miller E. (2006). Clinical and Immunologic Risk Factors for Meningococcal C Conjugate Vaccine Failure in the United Kingdom. *The Journal of Infectious Disease*. 194:1745-1752.

6. Barak J.D., Jahn C.E., Gibson D.L., and Charkowski A.O. (2007). The Role of Cellulose and O-Antigen Capsule in the Colonization of Plants by *Salmonella enterica*. *Molecular Plant-Microbe Interactions*. 20(9):1083-1091.

7. Barrow P.A. (2007). *Salmonella* Infections: Immune and Non-Immune Protection with Vaccines. *Avian Pathology*. 36(1):1-13.

8. Bellet M.M., Deriu E., Liu J.Z., Grimaldi B., Blaschitz C., Zeller M., Edwards R.A., Sahar S., Dandekar S., Baldi P., George M.D., Raffatellu M., and Sassone-Corsi P. (2011). Circadian Clock Regulates the Host Response to *Salmonella*. *Proceedings of the National Academy of Sciences*. 10(24):9897-9902.

9. Berry A., DeVault J.D., and Chakrabarty A.M. (1989). High Osmolarity is a Signal for Enhanced *algD* Transcription in Mucoid and Nonmucoid *Pseudomonas aeruginosa* Strains. *Journal of Bacteriology*. 171(5):2312-2317.

10. Beutler B., Milsark I.W., and Cerami A.C. (1985). Passive Immunization against Cachectin/Tumor Necrosis Factor Protects mice from Lethal Effect of Endotoxin. *Science*. 229(4716):869-871.

11. Bjarnason J., Southward C.M., and Surette M.G. (2003). Genomic Profiling of Iron-Responsive Genes in *Salmonella enterica* serovar Typhimurium by High Throughput Screening of a Random Promoter Library. *Journal of Bacteriology*. 185(16):4973-4982.

12. Blanchard-Rohner G., Snape M.D., Kelly D.F., John T., Morant A., Yu L.-M., Borkowski A., Ceddia F., Borrow R., Siegrist C.-A., and Pollard A.J. (2008). The Magnitude of the Antibody and Memory B Cell Responses During Priming with a Protein-Polysaccharide Conjugate Vaccine in Human Infants is Associated with the Persistence of Antibody and the Intensity of Booster Response. *The Journal of Immunology*. 180(4):2165-2173.

13. Blanchard-Rohner G., and Pollard A. (2011). Long-Term Protection after Immunization with Protein-Polysaccharide Conjugate Vaccines in Infancy. *Expert Review of Vaccines*. 10(5):673-684.

14. Blanden R.V., Mackaness G.B., and F.M. Collins. (1966). Mechanisms of Acquired Resistance in Mouse Typhoid. *The Journal of Experimental Medicine*. 124(4):585-600.

15. Bobat S., Flores-Langarica-Flores A., Hitchcock J., Marshall J.L., Kingsley R.A., Goodall M., Gil-Cruz C., Serre K., Leyton D.L., Letran S.E., Gaspal F., Chester R., Chamberlain J.L., Dougan G., Lopez-Macias C., Henderson I.R., Alexander J., MacLennan I.C.M., and Cunningham A.F. (2011). Soluble Flagellin, FliC, Induces an Ag-Specific Th2 Response, Yet Promotes T-Bet-Regulated Th1 Clearance of *Salmonella Typhimurium* Infection. *The Journal of Immunology*. 41:1606-1618.

16. Bordier C. (1981). Phase Separation of Integral Membrane Proteins in Triton X-114 Solution. *The Journal of Biological Chemistry*. 256(4):1604-1607.

17. Borrow R., Andrews N., Findlow H., Waight P., Southern J., Crowley-Luke A., Stapley L., England A., Findlow J., and Miller E. (2010). Kinetics of Antibody Persistence Following Administration of a Combination Meningococcal Serogroup C and *Haemophilus influenzae* Type b Conjugate Vaccine in Healthy Infants in the United Kingdom Primed with a Monovalent Meningococcal Serogroup C Vaccine. *Clinical and Vaccine Immunology*. 17(1):154-159.

18. Botsford J.L., and Harman J.G. (1992). Cyclic AMP in Prokaryotes. *Microbiological Reviews*. 56(1):100-122.

19. Brisabois A., Cazin I., Breuil J., and Collatz E. (1997). Surveillance of Antibiotic Resistance in Salmonella. Eurosurveillance. 2(3):181.

20. Bruckner R., and Titgemeyer F. (2002). Carbon Catabolite Repression in Bacteria: Choice of the Carbon Source and Autoregulatory Limitation of Sugar Utilization. *FEMS Microbiology Letters*. 209:141-148.

21. "Canadian Integrated Surveillance Report: Salmonella, Campylobacter, verotoxigenic *E. coli* and *Shigella*, from 2000 to 2004". Public Health Agency of Canada. 2009. Web. Mar 2015. < http://www.phac-aspc.gc.ca/publicat/ccdr-rmtc/09vol35/35s3/index-eng.php>.

22. Carlin N.I.A., Svenson S.B., and Lindberg A.A. (1987). Role of Monoclonal O-Antigen Antibody Epitope specificity and Isotype in Protection against Experimental Mouse Typhoid. *Microbial Pathogenesis*. 2:171-183.

23. Centers for Disease Control and Prevention. (2011). Incidence and Trends of Infection with Pathogens Transmitted Commonly Thorugh Food – Foodborne Diseases Active Surveillance Network, 10 U.S. Sites, 1996-2012. *Morbidity and Mortality Weekly Report*. 62(15):283-287.

24. Chaudhuri R.R., Morgan E., Peters S.E., Pleasance S.J., Hudson D.L., Davies H.M., Wang J., van Diemen M., Buckley A.M., Bowen A.J., Pullinger G.D., Turner D.J., Langridge G.C., Turner A.K., Parkhill J., Charles I.G., Maskell D.J., and Stevens M.P. (2013). Comprehensive Assignment of Roles for *Salmonella* Typhimurium Genes in Intestinal Colonization of Food- Producing Animals. *PLOS Genetics*. 9(4):e1003456.

25. Cheminay C., Schoen M., Hensel M., Wandersee-Steinhauser A., Ritter U., Korner H., Rollinghoff M., Hein J. (2002). Migration of *Salmonella typhimurium*—harboring bone marrow-derived dendritic cells towards the chemokines CCL119 and CCL21. Microbial Pathogenesis. 32:207-218.

26. Claesson B.A., Trolfors B., Lagergard T., Taranger J., Bryla D., Otterman G., Cramton T., Yang Y., Reimer C.B., Robbins J.B., and Schneerson R. (1988). Clinical and Immunologic Responses to Capsular Polysaccharide of *Haemophilus influenzae* type b Alone or Conjugated to Tetanus Toxoid in 18- to 23-Month-Old Children. *The Journal of Pediatrics*. 112:695-702.

27. Cohen D., Orr N., Robin G., Slepon R., Ashkenazi S., Ashkenazi I., and Shemer J. (1996). Detection of Antibodies to *Shigella* Lipopolysaccharide in Urine after Natural *Shigella* Infection or Vaccination. *Clinical and Diagnostic Laboratory Immunology*. 3(4):451-455.

28. Cohen D., Ashkenazi S., Green M.S., Gdalevich M., Robin G., Slepon R., Yavzori M., Orr N., Block C., Asshkenazi I., Shemer J., Taylor D.N., Hale T., Sadoff J.C., Pavliakova D., Schneerson R., and Robbins J.B. (1997). Double-Blinded Vaccine-Controlled Randomised Efficacy Trial of an Investigational *Shigella sonnei* Conjugate Vaccine in Young Adults. *The Lancet*. 349:155-159.

29. "Community Immunity ("Herd Immunity")". Vaccines.gov. 2015. Web. July 2015. <http://www.vaccines.gov/basics/protection/>

30. Coombes B.K., Coburn B.A., Potter A.A., Gomis S., Mirakhur K., Li Y., and Finlay B.B. (2005). Analysis of the Contribution of *Salmonella* Pathogenicity Islands 1 and 2 to Enteric

Disease Progression Using a Novel Bovine Ileal Loop Model and a Murine Model of Infectious Enterocolitis. *Infection and Immunity*. 73(11):7161-7169.

31. Copeland S., Warren H.S., Lowry S.F., Calvano S.E., Remick D., and the Inflammation and the Host Response to Injury Investigators. (2005). Acute Inflammatory Response to Endotoxin in Mice and Humans. *Clinical and Diagnostic Laboratory Immunology*. 12(1):60-67.

32. Costerton J.W., Irvin R.T., and Cheng K.-J., (1981). The Bacterial Glycocalyx in Nature and Disease. *Annual Review of Microbiology*. 35:299-324.

33. Costerton J.W., Cheng K.-J., Geesey G.G., Ladd T.I., Nickel J.C., Dasgupta M., Marrie T.J. (1987). Bacterial Biofilms in Nature and Disease. *Annual Reviews of Microbiology*. 41:435-464.

34. Cryz Jr S.J., Vanprapar N., Thisyakorn U., Olanratmanee T., Losonsky G., Levine M.M., and Chearskul S. (1993). Safety and Immunogenicity of *Salmonella typhi* Ty21a Vaccine in Young Thai Children. Infection and Immunity. 61(3):1149-1151.

35. Cunningham A.F., Khan M., Ball J., Toellner K.-M., Serre K., Mohr E., and MacLennan I.C.M. (2004). Responses to the Soluble Flagellar Protein FliC are Th2, while Those to FliC on *Salmonella* are Th2. *The Journal of Immunology*. 34:2986-2995.

36. Dagan R., Melamed R., Muallem M., Piglansky L., Greenberg D., Abramson O., Mendelman P.M., Bohidar N., and Yagupsky P. (1996). Reduction of Nasopharyngeal Carriage of Pneumococci during the Second Year of Life by a Heptavalent Conjugate Pneumococcal Vaccine. *The Journal of Infectious Diseases*. 174:1271-1278.

37. Datsenko K.A., and Wanner B.L. (2000). One-Step Inactivation of Chromosomal Genes in *Escherichia coli* K-12 Using PCR Products. *Proceedings of the National Academy of Sciences*. 97(12):6640-6645.

38. De Jong H.K., Parry C.M., van der Poll T., and Wiersinga W.J. (2012). Host-Pathogen Interaction in Invasive Salmonellosis. *PLOS Pathogens*. 8(10):e1002933.

39. De Rezende C.E., Anriany Y., Carr L.E., Joseph S.W., and Weiner R.M. (2005). Capsular Polysaccharide Surrounds Smooth and Rugose Types of *Salmonella enterica* serovar Typhimurium DT104. *Applied and Environmental Microbiology*. 71(11):7345-7351.

40. DeShazer D., Waag D.M., Fritz D., and Woods D.E. (2001). Identification of a *Burkholderia mallei* Polysaccharide Gene Cluster by Subtractive Hybridization and Demonstration that the Encoded Capsule is an Essential Virulence Determinant. Microbial Pathogenesis. *Microbial Pathogenesis*. 30:253-269.

41. Delves P.J., Martin S.J., Burton D.R., and Roitt I.M. *Essential Immunology*. New Jersey: Wiley-Blackwell, 2011. Print.

42. Desin T.S., Koster W., and Potter A.A. (2013). *Salmonella* Vaccines in Poultry: Past, Present and Future. *Expert Review of Vaccines*. 12(1):87-96.

43. El-Gazzar F.E., and Marth E.H. (1992). *Salmonellae,* Salmonellosis, and Dairy Foods: A Review. *Journal of Dairy Science*. 75:2327-2343.

44. Engels E.A., Falagas M.E., Lau J., and Bennish M.L. (1998). Typhoid Fever Vaccines: a Meta-Analysis of Studies on Efficacy and Toxicity. *British Medical Journal*. 316:110-116.

45. Eom J.S., Kim J.S., Jang J.I., Kim B.-H., Yoo S.Y., Choi J.H., Bang L.-S., Lee I.S., and Park Y.K. (2013). Enhancement of Host Immune Responses by Oral Vaccination to *Salmonella enterica* serovar Typhimurium Harboring Both FliC and FljB Flagella. *PLOS One*. 8(9):e74850.

46. "Executive Summary for the National Enteric Surveillance Program 2012 Annual Report". Public Health Agency of Canada. 2014. Web. Mar 2015. <https://www.nml-lnm.gc.ca/NESP-PNSME/surveillance-2012-eng.html>.

47. "Fact Sheet on Salmonella." Canadian Meat Council. Web. Mar. 2015. <www.cmc-cvc.com/sites/default/files/files/FactSheetonSalmonella.pdf>.

48. Farzan A., and Friendship R.M. (2010). A Clinical Field Trial to Evaluate the Efficacy of Vaccination in Controlling *Salmonella* Infection and the Association of *Salmonella*-Shedding and Weight Gain in Pigs. *The Canadian Journal of Veterinary Research*. 74:258-263.

49. Fattom A.I., Sarwar J., Basham L., Ennifar S., and Naso R. (1998). Antigenic Determinants of *Staphylococcus aureus* Type 5 and type 8 Capsular Polysaccharide Vaccines. *Infection and Immunity*. 66(10):4588-4592.

50. Feasey N.A., Archer B.N., Heyderman R.S., Sooka A., Dennis B., Gordon M.A., and Keddy K.H. (2010). Typhoid Fever and Invasive Nontyphoid Salmonellosis, Malawi and South Africa. *Emerging Infectious Disease*. 16(9):1448-1451.

51. Ferreccio C., Prado V., Ojeda A., Cayyazo M., Abrego P., Guers L., and Levine M.M. (1991). Epidemiologic Patterns of Acute Diarrhea and Endemic *Shigella* Infections in Children in a Poor Periurban Setting in Santiago, Chile. *American Journal of Epidemiology*. 134(6):614-627.

52. Fink S.L., and Cookson B.T. (2005). Apoptosis, Pyroptosis, and Necrosis: Mechanistic Description of Dead and Dying Eukaryotic Cells. *Infection and Immunity*. 73(4):1907-1916.

53. Fookes M., Schroeder G.N., Langridge G.C., Blondel C.J., Mammina C., Connor T.R., Seth-Smith H., Vernikos G.S., Robinson K.S., Sanders M., Petty N.K., Kingsley R.A.,

Baumler A.J., Nuccio S.-P., Contreras I., Santiviago C.A., Maskell D., Barrow P., Humphrey T., Nastasi A., Roberts M., Frankel G., Parkhill J., Dougan G., and Thomson N.R. (2011). *Salmonella bongori* Provides Insights into the Evolution of the Salmonellae. *PLOS Pathogens.* 7(8):e1002191.

54. Fournier J.-M., Vann W.F., and Karakawa W.W. (1984). Purification and Characterization of *Staphylococcus aureus* Type 8 Capsular Polysaccharide. *Infection and Immunity*. 45(1):87-93.

55. Fraser A., Paul M., Goldberg E., Acosta C.J., and Leibovici L. (2007). Typhoid Fever Vaccines: Systematic Review and Meta-Analysis of Randomised Controlled Trials. *Vaccine*. 25:7848-7857.

56. Galan J.E. (1999). Interaction of *Salmonella* with Host Cells through the Centisome 63 Type III Secretion System. *Current Opinion in Microbiology*. 2:46-50.

57. Gast R.K. (2007). Serotype-Specific and Serotype-Independent Strategies for Preharvest Control of Food-Borne *Salmonella* in Poultry. *Avian Diseases*. 51(4):817-828.

58. Germanier R., and Furer E. (1975). Isolation and Characterization of *Gal* E Mutant Ty 21a of *Salmonella typhi*: A Candidate Strain for a Live, Oral Typhoid Vaccine. *The Journal of Infectious Diseases*. 131(5):553-558.

59. Gibson D.L. Salmonella Enteritidis This Aggregative Fimbriae and the Extracellular Matrix. Doctor of Philosophy Thesis. University of Victoria, 2000.

60. Gibson D.L., White A.P., Snyder S.D., Martin S., Heiss C., Azadi P., Surette M., and Kay W.W. (2006). *Salmonella* Produces an O-Antigen Capsule Regulated by AgfD and Important for Environmental Persistence. *Journal of Bacteriology*. 188(22):7722-7730.

61. Gil-Cruz C., Bobat S., Marshall J.L., Kingsley R.A., Ross E.A., Henderson I.R., Leyton D.L., Coughlan R.E., Khan M., Jensen K.T., Buckley C.D., Dougan G., MacLennan I.C.M., Lopez-Macias C., and Cunningham A.F. (2009). The Porin OmpD From Nontyphoidal *Salmonella* is a Key Target for a Protective B1b Cell Antibody Response. *Proceedings of the National Academy of Sciences*. 106(24):9803-9808.

62. Goldman R.C., White D., Orskov F., Orskov I., Rick P.D., Lewis M.S., Bhattacharjee A.K., and Leive L. (1982). A Surface Polysaccharide of *Escherichia coli* O111 Contains O-Antigen and Inhibits Agglutination of Cells by O-Antiserum. *Journal of Bacteriology*. 151(3):1210-1221.

63. Gondwe E.N., Molyneux M.E., Goodall M., Graham S.M., Mastroeni P., Drayson M.T., and MacLennan C.A. (2010). Importance of Antibody and Complement for Oxidative Burst and Killing of Invasive Nontyphoidal *Salmonella* by Blood Cells in Africans. *Proceedings of the National Academy of Sciences*. 107(7):3070-3075.

64. Gordon M.A., Banda H.T., Gondwe M., Gordon S.B., Boeree M.J., Walsh A.L., Corkill J.E. Hart A., Gilks C.F., and Molyneux M.E. (2002). Non-Typhoidal Salmonella Bacteraemia Among HIV-Infected Malawian Adults: High Mortality and Frequent Recrudescence. *AIDS*. 16:1633-1641.

65. Gorke B., and Stulke J. (2008). Carbon Catabolite Repression in Bacteria: Many Ways to Make the Most Out of Nutrients. *Nature Reviews Microbiology*. 6:613-624.

66. Gorman J., Shapiro L., and Burley S.K. (2004). 1TO3: Structure of YihT from *Salmonella typhimurium*. New York SGX Research Center for Structural Genomics. Worldwide Protein Data Bank. <http://dx.doi.org.cyber.usask.ca/10.2210/pdb1to3/pdb>.

67. Grados O., and Ewing W.H. (1970). Antigenic Relationship between *Escherichia coli* and *Neisseria meningitidis*. *The Journal of Infectious Diseases*. 122(1&2):100-103.

68. Gray J.T., and Fedorka-Cray P.J. (2001). Long-Term Survival and Infectivity of *Salmonella choleraesuis*. *Berliner und Munchener Tierarztliche Wochenschrift*. 1114(9-10):370-374.

69. Grimont P.A.D., and Weill F.-X. (2007). "Antigenic Formulae of the *Salmonella* Serovars". WHO Collaborating Centre for Reference and Research on *Salmonella*. 9th Edition.Web. March. 2015. https://www.pasteur.fr/ip/portal/action/WebdriveActionEvent/oid/01s -000036-089

70. Gupta R.K., Taylor D.N., Bryla D.A., Robbins J.B., and Szu S.C. (1998). Phase 1 Evaluation of *Vibrio cholera* O1, Serotype Inaba, Polysaccharide-Cholera Toxin Conjugates in Adult Volunteers. *Infection and Immunity*. 66(7):3095-3099.

71. Halloran M.E., Haber M., Longini Jr. I.M., and Struchiner C.J. (1991). Direct and Indirect Effects in Vaccine Efficacy and Effectiveness. *American Journal of Epidemiology*. 133:323-331.

72. Hannemann S., Gao B., and Galan J.E. (2013). *Salmonella* Modulation of Host Cell Gene Expression Promotes Its Intracellular Growth. *PLOS Pathogens*. 9(10):e1003668.

73. Haraga A., Ohlson M.B., and Miller S.I. (2008). *Salmonellae* Interplay with Host Cells. *Nature Reviews Microbiology*. 6:53-66.

74. Hawley P.R., Simmons J.S. (1934). The Effectiveness of Vaccines Used for the Prevention of Typhoid Fever in the United States Army and Navy. *American Journal of Public Health*. 24(7):689-709.

75. Heath P.T., and McVernon J. (2002). The UK Hib Vaccine Experience. *Archives of Disease in Childhood*. 86:396-399.

76. Herzberg M., Kaye I.K., Peti W., and Wood T.K. (2006). YdgG (TqsA) Controls Biofilm Formation in *Escherichia coli* K-12 through Autoinducer 2 Transport. *Journal of Bacteriology*. 188(2):587-598.

77. Hirose K., Ezaki T., Miyake M., Li t., Khan A.Q., Kawamura Y., Yokoyama H., Takami T. (1997). Survival of Vi-Capsulated and Vi-Deleted *Salmonella typhi* Strains in Cultured Macrophage Expressing Different Levels of CD14 Antigen. *FEMS Microbiology Letters*. 147:259-265.

78. Hurley D., McCusker M.P., Fanning S., and Martins M. (2014). *Salmonella*-Host Interactions – Modulation of the Host Innate Immune System. *Frontiers in Immunology*. 5,481; doi:10.3389fimmu.2014.00481.

79. Ibarra J.A., and Steele-Mortimer O. (2009). *Salmonella* – the Ultimate Insider. Salmonella Virulence Factors That Modulate Intracellular Survival. *Cellular Microbiology*. 11(11):1579-1586.

80. Itoh T., Mikami B., Hashimoto W., and Murata K. (2008). Crystal Structure of YihS in Complex with D-Mannose: Structural Annotation of *Escherichia coli* and *Salmonella enterica yihS*-encoded Proteins to an Aldose-Ketose Isomerase. Journal of Molecular Biology. 377:1443-1459.

81. Ivanoff B., Levine M.M., and Lambert P.H. (1994). Vaccination against Typhoid Fever: Present Status. *Bulletin of the World Health Organization*. 72(6):957-971.

82. Jokhdar H., Borrow R., Sultan A., Adi M., Riley C., Fuller E., and Baxter D. (2004). Immunologic Hyporesponsiveness to Serogroup C but Not Serogroup A Following Repeated Meningococcal A/C Polysaccharide Vaccination in Saudi Arabia. *Clinical and Diagnostic Laboratory Immunology*. 11(1):83-88.

83. Jones B.D., Ghori N., and Falkow S. (1994). *Salmonella Typhimurium* Initiates Murine Infection by Penetrating and Destroying the Specialized Epithelial M Cells of the Peyer's Patches. *The Journal of Experimental Medicine*. 180:15-23.

84. Jones B.D. (1997). Host Responses to Pathogenic *Salmonella* Infection. *Genes and Development*. 11:679-687.

85. Kaiser P. (2010). Advances in Avian Immunology—Prospects for Disease Control: A Review. *Avian Pathology*. 39(5):309-324.

86. Kalka-Moll W.M., Tzianabos A.O., Bryant P.W., Niemeyer M., Ploegh H.L., and Kasper D.L. (2002). Zwitterionic Polysaccharides Stimulate T Cells by MHC Class II-Dependent Interactions. *The Journal of Immunology*. 169:6149-6153.

87. Kantele A., Pakkanen S.H., Siitonen A., Karttunen R., and Kantele J.M. (2010). Live Oral Typhoid *Salmonella* Typhi Ty21a – A Surrogate Vaccine Against Non-Typhoid *Salmonella*? *Vaccine*. 30:7238-7245.

88. Karlyshev A.V., and Wren B.W. (2001). Detection and Initial Characterization of Novel Capsular Polysaccharide Among Diverse *Campylobacter jejuni* Strains Using Alcian Blue Dye. *Journal of Clinical Microbiology*. 39(1):279-284.

89. Kasper D.L., Onderdonk A.B., Crabb J., and Bartlett J.G. (1979). Protective Efficacy of Immunization with Capsular Antigen against Experimental Infection with *Bacteroides fragilis*. *The Journal of Infectious Diseases*. 140(5):724-731.

90. Kawai T., and Akira S. (2010). The Role of Pattern-Recognition Receptors in Innate Immunity: Update on Toll-Like Receptors. *Nature Immunology*. 11:373-384.

91. Kenzel S., and Henneke P. (2006). The Innate Immune System and its Relevance to Neonatal Sepsis. 19:264-270.

92. Kingsley R.A., Msefula C.L., Thomson N.R., Kariuki S., Holt K.E., Gordon M.A., Harris D., Clarke L., Whitehead S., Sangal V., Marsh K., Achtman M., Molyneus M.E., Cormican M., Parkhill J., MacLennan C.A., Heyderman R.S., and Dougan G. (2009). Epidemic Multiple Drug Resistant *Salmonella* Typhimurium Causing Invasive Disease in Sub-Saharan Africa Have a Distinct Genotype. *Genome Research*. 19:2279-2287.

93. Kovacs-Nolan J., Latimer L., Landi A., Jenssen H., Hancock R.E.W., Babiuk L.A., van Drunen Little-van den Hurk S. (2009). The Novel Adjuvant Combination of CpG ODN, Indolicidin and Polyphosphazene Induces Potent Antibody- and Cell-Mediated Immune Responses in Mice. *Vaccine*. 27:2055-2064.

94. Kropinski A.M., Berry D., and Greenberg E.P. (1986).The Basic of Silver Staining of Bacterial Lipopolysaccharides in Polyacrylamide Gels. *Current Microbiology*. 13:29-31.

95. Kruetzmann S., Rosado M.M., Weber H., Germing U., Tournilhac O., Peter H.-H., Berner R., Peters A., Boehm T., Plebani A., Quinti I., Carsetti R. (2003). Human Immunoglobulin M Memory B Cells Controlling *Streptococcus pneumoniae* Infections Are Generated in the Spleen. *The Journal of Experimental Medicine*. 197(7):939-945.

96. Kruschinski C., Zidan M., Debertin A.S., Von Horsten S., and Pabst R. (2004). Age-Dependent Development of the Splenic Marginal Zone in Human Infants is Associated With Different Causes of Death. Human Pathology. 35(1):113-121.

97. Laxalt K.A., and Kozel T.R. (1979). Chemotaxigenesis and Activation of the Alternative Complement Pathway by Encapsulated and Non-Encapsulated *Cryptococcus neoformans*. *Infection and Immunity*. 26(2):435-440.

98. Lazarus G.M., and Neu H.C. (1975). Agents Responsible for Infection in Chronic Granulomatous Disease of Childhood. *The Journal of Pediatrics*. 86(3):415-417.

99. Ledeboer N.A., and Jones B.D. (2005). Exopolysaccharide Sugars Contribute to Biofilm Formation by *Salmonella enterica* Serovar Typhimurium on Hep-2 Cells and Chicken Intestinal Epithelium. *Journal of Bacteriology*. 187(9):3214-3226.

100. Lesinski G.B., and Westerink M.A.J. (2001). Novel Vaccine Strategies to T-Independent Antigens. *Journal of Microbiological Methods*. 47:135-149.

101. Lin F.Y.C., ho V.A., Khiem H.B., Trach D.D., Bay P.V., Thanh T.C., Kossaczka Z., Bryla D.A., Shiloach J., Robbins J.B., Schneerson R., and Szu S.C. (2001). The Efficacy of a *Salmonella Typhi* Vi Conjugate Vaccine in Two-to-Five-Year-Old Children. *The New England Journal of Medicine*. 344(17):1263-1269.

102. Mackaness G.B., Blanden R.V., and Collins F.M. (1966). Host-Parasite Relations in Mouse Typhoid. *The Journal of Experimental Medicine*. 124(4):573-583.

103. MacLennan C.A., Gondwe E.N., Msefula C.L., Kingsley R.A., Thomson N.R., White S.A., Goodall M., Pickard D.J., Graham S.M., Dougan G., Hart A., Molyneus M.E., and Drayson M.T. (2008). The Neglected Role of Antibody in Protection Against Bacteremia Caused by Nontyphoidal Strains of *Salmonella* in African Children. *The Journal of Clinical Investigation*. 118(4):1553-1562.

104. MacLennan C.A. (2013). Vaccines for Low-Income Countries. *Seminars in Immunology*. 25:114-123.

105. MacLennan C.A., Martin L.B., and Micoli F. (2014). Vaccines against Invasive *Salmonella* Disease. *Human Vaccines and Immunotherapeutics*. 10(6):1478-1493.

106. Magalhaes P.O., Lopes A.M., Mazzola P.G., Rangel-Yagui C., Penna T.C.V., and Pessoa Jr. A. (2007). Methods of Endotoxin Removal from Biological Preparations: A Review. *Journal of Pharmacy and Pharmaceutical Sciences*. 10(3):388-404.

107. Majowicz S.E., Musto J., Scallan E., Angulo F.J., Kirk M., O'Brien S.J., Jones T.F., Fazil A., and Hoekstra R.M. (2010). The Global Burden of Nontyphoidal *Salmonella* Gastroenteritis. *Clinical Infectious Diseases*. 50:882-889.

108. Makela P.H., and Kayhty H. (2002). Evolution of Conjugate Vaccines. *Expert Review of Vaccines*. 1(3):399-410.

109. Maloy S.R. "Phage P22". *Experimental Techniques in Bacterial Genetics*. Boston, M: Jones and Bartlett Publishers, Inc., 1990. Print.

110. Mastroeni P., Villarreal-Ramos B., and Hormaeche C.E. (1993). Adoptive Transfer of Immunity to Oral Challenge with Virulent *Salmonellae* in Innately Susceptible BALB/c

Mice Requires Both Immune Serum and T Cells. *Infection and Immunity*. 61(9):3981-3984.

111. Mastroeni P., Chabalgoity J.A., Dunstan S.J., Maskell D.J., and Dougan G. (2001). *Salmonella*: Immune Responses and Vaccines. *The Veterinary Journal*. 161:132-164.

112. Mastroeni P., Grant A., Restif O., and Maskell D. (2009). A Dynamic View of the Spread and Intracellular Distribution of *Salmonella enterica*. *Nature Reviews Microbiology*. 7:73-80.

113. Mazmanian S.K., Kasper D.L., (2006). The Love-Hate Relationship between Bacterial Polysaccharides and the Host Immune System. *Nature Reviews Immunology*. 6:849-858.

114. McCormick B.A., Colgan S.P., Delp-Archer C., Miller S.I., and Madara J.L. (1993). *Salmonella Typhimurium* attachment to Human Intestinal Epithelial Monolyaers: Transcellular Signalling to Subepithelial Neutrophils. *The Journal of Cell Biology*. 123(4):895-907.

115. McQuiston J.R., Waters R.J., Dinsmore, B.A., Mikoleit ML., and Fields, P.I. (2011). Molecular Determination of H Antigens of *Salmonella* by Use of a Microsphere-Based Liquid Array. *Journal of Clinical Microbiology*. 49(2):565-573.

116. McSorley S.J., and Jenkins M.K. (2000). Antibody is Required for Protection against Virulent but Not Attenuated *Salmonella enterica* Serovar Typhimurium. *Infection and Immunity*. 68(6):3344-3348.

117. McVernon J., Johnson P.D.R., Pollard A.J., Slack M.P.E., and Moxon E.R. (2003). Immunologic Memory in *Haemophilus influenzae* Type b Conjugate Vaccine Failure. *Archives of Disease in Childhood*. 88:379-383.

118. Mittrucker H.-W., and Kaufmann S.H.E. (2000). Immune Response to Infection with *Salmonella Typhimurium* in Mice. *Journal of Leukocyte Biology*. 67:457-463.

119. Mutwiri G., Gerdts V., Van Drunen Little-van den Hurk S., Auray G., Eng N., Garlapati S., Babiuk L.A., and Potter A. (2011). Combination Adjuvants: The Next Generation of Adjuvants? *Expert Review of Vaccines*. 10(1):95-107.

120. Nair M. (2012). Protein Conjugate Polysaccharide Vaccines: Challenges in Development and Global Implementation. *Indian Journal of Community Medicine*. 37(2):79-82.

121. Nath T.R., Malaviya A.N., Kumar R., Balakrishnan K., and Singh B.P. (1977). A Study of the Efficacy of Typhoid Vaccine in Inducing Humoral and Cell-Mediated Immune Responses in Human Volunteers. *Clinical and Experimental Immunology*. 30:38-43.

122. Nelson A.L., Roche A.M., Gould J.M., Chim K., Ratner A.J., Weiser J.N. (2007). Capsule Enhances Pneumococcal Colonization by Limiting Mucus-Mediated Clearance. *Infection and Immunity*. 75(1):83-90.

123. Nuccio S-P., and Baumler A.J. (2014). Comparative Analysis of *Salmonella* genomes Identifies a Metabolic Network for Escalating Growth in the Inflamed Gut. *mBio*. 5(2):e00929-14.

124. Ohtake S., Martin R., Saxena A., Pham B., Chiueh G., Osorio M., Kopecko D., Xu D.Q., Lechuga-Ballesteros D., and Truong-Le. V. (2011). Room Temperature Stabilization of Oral, Live Attenuated *Salmonella enterica* Serovar Typhi-Vectored Vaccines. *Vaccine*. 29:2761-2771.

125. Okuyama M., Mori H., Chiba S., and Kimura A. (2004). Overexpression and Characterization of Two Unknown Proteins, YicI and YihQ, Originated from *Escherichia coli*. *Protein Expression and Purification*. 37:170-179.

126. Ophir T., and Gutnick D.L. (1994). A Role for Exopolysaccharides in the Protection of Microorganisms from Desiccation. *Applied and Environmental Microbiology*. 60(2):740-745.

127. Opinion of the Scientific Panel on Biological Hazards on a request from the Commission related to the use of vaccines for the control of Salmonella in poultry. (2004). *The EFSA Journal*. 114:1-74.

128. Orskov F., and Orskov I. (1992). *Escherichia coli* Serotyping and Disease in Man and Animals. *Canadian Journal of Microbiology*. 38(7):699-704.

129. "Overview of Secondary Antibodies". Thermo Scientific. 2015. Web. July 2015 <http://www.pierce-antibodies.com/products/secondary-antibodies/>

130. Pakkanen S.H., Kantele J.M., and Kantele A. (2012). Cross-Reactive Gut-Directed immune Response Against *Salmonella enterica* serovar Paratyphi A and B in Typhoid Fever and After Oral Ty21 a typhoid Vaccination. *Vaccine*. 30:6047-6053.

131. Pakkanen S.H., Kantele J.M., Herzog C., and Kantele A. (2014). Cross-Reactive Immune Response Elicited by Parenteral Vi Polysaccharide Typhoid Vaccine Against Non-Typhoid *Salmonellae*. *Vaccine*. 32:544-551.

132. Parke J.C., Schneerson R., Relmer C., Black C., Welfare S., Bryla D., Levi L., Pavliakova D., Cramton T., Schulz D., Cadoz M., and Robbins J.B. (1991). Clinical and Immunologic Reponses to *Haemophilus influenzae* Type b-Tetanus Toxoid Conjugate Vaccine in Infants Injected at 3,5,7 and 18 Months of Age. *The Journal of Pediatrics*. 118(2):184-190.

133. Passwell J.H., Harlev E., Ashkenazi S., Chu C., Miron D., Ramon R., Farzan N., Shiloach J., Bryla D.A., Majadly F., Roberson R., Robbins J.B., and Schneerson R. (2001). Safety

and Immunogenicity of Improved *Shigella* O-Specific Polysaccharide-Protein Conjugate Vaccines in Adults in Israel. *Infection and Immunity*. 69(3):1351-1357.

134. Pickard D., Li J., Roberts m., Maskell D., Hone D., Levine M., Dougan G., and Chatfield S. (1994). Characterization of Defined *ompR* Mutants of *Salmonella typhi*: *ompR* is involved in the Regulation of Vi Polysaccharide Expression. *Infection and Immunity*. 62(9):3984-3993.

135. Pietila T.W., Veckman V., Kyllonen P., Lahteenmaki K., Korhonen T.K. (2005). Activation, cytokine production, and intracellular survival of bacteria in *Salmonella*-infeted human monocyte-derived macrophages and dendritic cells. *Journal of Leukocyte Biology*. 78:909-920.

136. Plotkin S.A. (2003). Vaccines, Vaccination, and Vaccinology. *The Journal of Infectious Diseases*. 187:1349-1359.

137. Podda A., Saul A., Arora R., Bhutta Z., Sinha A., Gaind R., Singhal T., Saha S., Brooks A., Martin L.B., Amdekar Y., Chitkara A.J., Shieh M., Kapur A.N., and Chugh T.D. (2010). Conjugated Vaccines for Enteric Fever: Proceedings of a Meeting Organized in New Delphi, India in 2009. *The Journal of Infection in developing Countries*. 4(6):404-411.

138. Podolak R., Enache E., Stone W., Black D.G., and Elliott P.H. (2010). Sources and Risk Factors for Contamination, Survival, Persistence, and Heat Resistance of *Salmonella* in Low-Moisture Foods. *Journal of Food Protection*. 73(10):1919-1936.

139. Pollard A.J., Perrett K.P., and Beverley P.C. (2009). Maintaining Protection against Invasive Bacteria with Protein-Polysaccharide Conjugate vaccines. *Nature Reviews Immunology*. 9:213-220.

140. Portillo F.G.-D., and Finaly B. (1994). *Salmonella* Invasion of Nonphatocytic Cells Induces Formation of Macropinosomes in the Host Cell. *Infection and Immunity*. 62(10):4641-4645.

141. Quandt J., and M.F. Hynes. (1993). Versatile Suicide Vectors Which Allow Direct Selection for Gene Replacement in Gram-Negative Bacteria. *Gene*. 127(1):15-21.

142. Quintero E., and Weiner R.M. (1995). Evidence for the Adhesive Function of the Exopolysaccharide of *Hyphomonas* Strain MHS-3 in Its Attachment to Surfaces. *Applied and Environmental Microbiology*. 61(5):1897-1903.

143. Raffatellu M., Chessa D., Wilson R.P., Tukel C., Akcelik M., and Baumler A.J. (2006). Capsule-Mediated Immune Evasion: A New Hypothesis Explaining Aspects of Typhoid Fever Pathogenesis. *Infection and Immunity*. 74(1):19-27.

144. Rajagopalan P., Kumar R., and Malaviya A.N. (1982). A Study of Humoral and Cell-Mediated immune Response Following Typhoid Vaccination in Human volunteers. *Clinical and Experimental Immunology*. 47:275-282.

145. Ramarathinam L., Shaban R.A., Niesel D.W., and Klimpel G.R. (1991). Interferon Gamma (IFN-γ) Production by Gut-Associated Lymphoid Tissue and Spleen Following Oral *Salmonella Typhimurium* Challenge. *Microbial Pathogenesis*. 11(347-356).

146. Ramsay M.E., McVernon J., Andrews N.J., Heath P.T., and Slack M.P. (2003-a). Estimating *Haemophilus influenzae* Type b Vaccine Effectiveness in England and Wales by Use of the Screening Method. *The Journal of Infectious Diseases*. 188:481-485.

147. Ramsay M.E., Andrews N.J., Trotter C.L., Kaczmarski E.B., and Miller E. (2003-b). Herd Immunity form Meningococcal Serogroup C Conjugate Vaccination in England: Database Analysis. *British Medical Journal*. 326(7385):365-366.

148. Raupach B., Peuschel S.-K., Monack D.M., Zychlinsky A. (2006). Caspase-1-Mediated Activation of Interleukin-1β (IL-1β) and IL-18 Contributes to Innate Immune Defenses Against *Salmonella enterica* Serovar Typhimurium Infectoin. *Infection and Immunity*. 74(8):4922-4926.

149. Richmond P., Kaczmarski E., Borrow R., Findlow J., Clark S., McCann R., Hill J., Barker M., and Miller E. (2000). Meningococcal C Polysaccharide Vaccine Induces Immunologic Hyporesponsiveness in Adults That is Overcome by Meningococcal C Conjugate Vaccine. The Journal of Infectious Diseases. 181:761-764.

150. Robbins J.B., Chu C., and Schneerson R. (1992). Hypothesis for Vaccine Development: Protective Immunity to Enteric Diseases Caused by Nontyphoidal *Salmonella* and *Shigellae* May be Conferred by Serum igG Antibodies to the O-Specific Polysaccharide of Their Lipopolysaccharides. *Clinical Infectious Diseases*. 15:346-361.

151. Roberts I.S. (1996). The Biochemistry and Genetics of Capsular Polysaccharide Production in Bacteria. *Annual Review of Microbiology*. 50:285-315.

152. Rodrigues L.P., Schneerson R., and Robbins J.B. (1971). Immunity to *Hemophilus influenzae* Type b. I. The Isolation, and Some Physicochemical, Serologic and Biologic Properties of the Capsular Polysaccharide of *Hemophilus influenzae* Type b. *The Journal of Immunology*. 107(4):1071-1080.

153. Rondini S., Lanzilao L., Necchi F., O'Shaughnessy C.M., Micoli F., Saul A., MacLennan C.A. (2013). Invasive African *Salmonella* Typhimurium Induces Bactericidal Antibodies Against O-Antigens. *Microbial Pathogenesis*. 63:19-23.

154. Saito N., Robert M., Kochi H., Matsuo G., Kakazu Y., Soga T., and Tomita M. (2009). Metabolite Profiling Reveals YihU as a Novel Hydroxybutyrate Dehydrogenase for

Alternative Succinic Semialdehyde Metabolism in *Escherichia coli*. *The Journal of Biological Chemistry*. 284(24):16442-16451.

155. Salazar-Gonzalez R.M., Maldonado-Bernal C., Ramirez-Cruz N.E., Rios-Sarabia N., Beltran-Nava J., Castanon-Gonzalez J., Castillo-Torres N., Palma-Aguirre J.A., Carrera-Camargo M., Lopez-Macias C., and Isibasi A. (2004). Induction of Cellular Immune Response and Anti-*Salmoneall enterica* Serovar *Typhi* Bactericidal Antibodies in Healthy Volunteers by Immunization with a Vaccine Candidate Against Typhoid Fever. *Immunology Letters*. 93:115-122.

156. Salerno-Goncalves R., Pasetti M.F., and Sztein M.B. (2002). Characterization of CD8+ Effector T Cell Responses in Volunteers Immunized with *Salmonella enterica* Serovar Typhi Strain Ty21a Typhoid Vaccine. *The Journal of Immunology*. 169:2196-2203.

157. "*Salmonella Enterica* Spp". Public Health Agency of Canada. 2011. Web. Mar 2015. <http://www.phac-aspc.gc.ca/lab-bio/res/psds-ftss/salmonella-ent-eng.php>.

158. "*Salmonella* Heidelberg Ceftiofur – Related Resistance in Human and Retail Chicken Isolates". Public Health Agency of Canada. 2007. Web. Mar 2015. <http://www.phac-aspc.gc.ca/cipars-picra/heidelberg/heidelberg-eng.php>.

159. "*Salmonella* (non-typhoidal)". World Health Organization. 2013. Web. Mar 2015. <http://www.who.int/mediacentre/factsheets/fs139/en/>.

160. "*Salmonella* Outbreak Investigations: Timeline for Reporting Cases". Centers for Disease Control and Prevention. 2010. Web. Mar 2015. http://www.cdc.gov/salmonella/reporting timeline.html>.

161. "Salmonellosis". Alberta Health and Wellness. Government of Alberta, 2011. Web. Mar. 2015. <www.health.alberta.ca/documents/Guidelines-Salmonellosis-2014.pdf>.

162. Sandal I., Inzana T.J., Molinaro A., De Castro C., Shao J.Q., Apicella M.A., Cox A.D., St Michael F., and Berg G. (2011). Identification, Structure, and Characterization of an Exopolysaccharide Produced by *Histophilus somni* during biofilm formation. *BioMed Central Microbiology*. 11(186); doi:10.1186/1471-2180-11-186.

163. Schneerson R., Robbins J.B., Parke Jr J.C., Bell C., Schlesselman J.J., Sutton A., Wang Z., Schiffman G., Karpas A., and Shiloach J. (1986). Quantitative and Qualitative Analyses of Serum Antibodies Elicited in Adults by *Haemophilus influenzae* Type b Pneumococcus Type 6A Capsular Polysaccharide-Tetanus Toxoid Conjugates. *Infection and immunity*. 52(2):519-528.

164. Schoendorf K.C., Adams W.G., Kiely J.L, and Wenger J.D. (1994). National Trends in *Haemophilus influenzae* Meningitis Mortality and Hospitalization among Children, 1980 through 1991. *Pediatrics*. 93(4):663-668.

165. Secundino I., Lopez-Macias C., Cervantes-Barragan L., Gil-Cruz C., Rios-Sarabia N., Pastelin-Palacios R., Villasis-Keever M.A., Becker I., Puente J.L., Calva E., and Isibasi A. (2005).*Salmonella* Porins Induce a Sustained, Lifelong Specific Bactericidal Antibody Memory Response. *Immunology*. 117:59-70.

166. Sette A., and Rappuoli R. (2010). Reverse Vaccinology: Developing Vaccines in the Era of Genomics. *Immunity*. 33:530-541.

167. Shapiro E.D., Berg A.T., Austrian R., Schroeder D., Parcells V., Margolis A., Adair R.K., and Clemens J.D. (1991). The Protective Efficacy of Polyvalent Pneumococcal Polysaccharide Vaccine. *The New England Journal of Medicine*. 325(21):1453-1460.

168. Simon R., Tennant S. M., Wang J.Y., Schmidlein P.J., Lees A., Ernst R.K., Pasetti M.F., Galen J.E., and Levine M.M. (2011). *Salmonella enterica* Serovar Enteritidis Core O Polysaccharide Conjugated to H:g,m Flagellin as a Candidate Vaccine for Protection against Invasive Infection with *S.* Enteritidis. *Infection and Immunity*. 79(10):4240-4249.

169. Simon R., and Levine M.M. (2012). Glycoconjugate Vaccine Strategies for Protection against Invasive *Salmonella* Infections. *Human Vaccines and Immunotherapeutics*. 8(4):494-498.

170. Simon R., Wang J.Y., Boyd M.A., Tulapurkar M.E., Ramachandran G., Tennant S.M., Pasetti M., Galen J.E., Levine M.M. (2013). Sustained Protection in Mice Immunized with Fractional Doses of *Salmonella* Enteritidis Core and O Polysaccharide-Flagellin Glycoconjugates. *PLOS One*. 8(5):e64680.

171. Singh B.R. (2009). *Salmonella* Vaccines for Animals and Birds and Their Future Perspective. *The Open Vaccine Journal*. 2:100-112.

172. Singh S.P., Williams Y.U., Benjamin W.H., Klebba P.E., and Boyd D. (1996). Immunoprotection by Monoclonal Antibodies to the Porins and Lipopolysaccharide of *Salmonella typhimurium*. *Microbial Pathogenesis*. 21:249-263.

173. Sinha K., Mastroeni P., Harrison J., De Hormaeche R.D., and Hormaeche C.E. (1997). *Salmonella typhimurium aroA*, *htrA*, and *aroD htrA* Mutants Cause Progressive Infections in Athymic (*nu*/*nu*) BALB/c Mice. *Infection and Immunity*. 65(4):1566-1569.

174. Slauch J., Taylor R., and Maloy S. (1997). Survival in a Cruel World: How *Vibrio cholera* and *Salmonella* Respond to an Unwilling Host. *Genes and Development*. 11:1761-1774.

175. Smith D.H., Peter G., Ingram D.L., Harding A.L., and Anderson P. (1973). Responses of Children Immunized with the Capsular Polysaccharide of *Hemophilus influenzae* Type B. *Pediatrics*. 52(5):637-644.

176. Smith G.W., Smith F., Zuidhof S., and Foster D.M. (2015). Short Communication: Characterization of the Serologic Response Induced by Vaccination of Late-Gestation Cows with a *Salmonella* Dublin Vaccine. *Journal of Dairy Science*. 98(4):2529-2532.

177. Snape M.D., and Pollard A.J. (2005). Meningococcal Polysaccharide-Protein Conjugate vaccines. *The Lancet Infectious Diseases*. 5:21-30.

178. Snape M.D., Kelly D.F., Salt P., Green S., Snowden C., Diggle L., Borkowski A., Yu L.-M. Moxon R., and Pollard A.J. (2006). Serogroup C Meningococcal Glycoconjugate Vaccine in Adolescents: Persistence of Bactericidal Antibodies and Kinetics of the Immune Reponse to a Booster vaccine More Than 3 Years after Immunization. *Clinical Infectious Diseases*. 43:1387-1394.

179. Snape M.D., Kelley D.F., Lewis S., Banner C., Kibwana L., Moore C.E., Diggle L., John T., Yu L.-M. Borrow R., Borkowski A., Nau C., and Pollard A.J. (2008). Seroprotection Against Serogroup C Meningococcal Disease in Adolescents in the United Kingdom: Observational Study. *British Medical Journal*. 336(7659):1487-1491.

180. Snyder D.S., Gibson D.L., Heiss C., Kay W., and Azadi P. (2006). Structure of a Capsular Polysaccharide Isolated From *Salmonella Enteritidis*. *Carbohydrate Research*. 341:2388-2397.

181. Solano C., Garcia B., Valle J., Berasain C., Ghigo J.-M., Gamazo C., and Lasa I. (2002). Genetic Analysis of *Salmonella Enteritidis* Biofilm Formation: Critical Role of Cellulose. *Molecular Microbiology*. 43(3):793-808.

182. Sood R.K., and Fattom A. (1998). Capsular Polysaccharide-Protein Conjugate Vaccines and Intravenous Immunoglobulins. *Expert Opinion on Investigational Drugs*. 7(3):333-347.

183. "Standard Nucleotide BLAST". National Center for Biotechnology Information. 2015. Web. April 2015. < http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome>.

184. Strozen T.G., Li G., and Howard S.P. (2012). YghG ($GspS_{\beta}$) is a Novel Pilot Protein Required for Localization of the $GspS_{\beta}$ Type II Secretion System Secretin of Enterotoxigenic *Escherichia coli*. *Infection and Immunity*. 80(8):2608-2622.

185. Sukupolvi-Petty S., Grass S., and St. Geme III J.W. (2006). The *Haemophilus influenzae* Type B *hcsA* and *hcsB* Gene Products Facilitate Transport of Capsular Polysaccharide across the Outer Membrane and Are Essential for Virulence. *Journal of Bacteriology*. 188(11):3870-3877.

186. "Summary of Salmonella enterica, Subspecies enterica, Strain serovar Typhimurium str. 14028S, version 19.0". BioCyc Database Collection. 2015. Web. April 2015. <http://biocyc.org/organism-summary?object=SENT588858>.

187. Svenson S.B., Nurminen M., and Lindberg A.A. (1979). Artificial *Salmonella* Vaccines: O-Antigenic Oligosaccharide-Protein Conjugates Induce Protection Against Infection with *Salmonella typhimurium*. *Infection and Immunity*. 25(3):863-872.

188. Svenson S.B., and Lindberg A.A. (1981). Artificial *Salmonella* Vaccines: *Salmonella typhimurium* O-Antigen-Specific Oligosaccharide-Protein Conjugates Elicit Protective Antibodies in Rabbits and Mice. *Infection and Immunity*. 32(2):490-496.

189. Tacket C.O., Pasetti M.F., Sztein M.B., Livio S., and Levine M.M. (2004). Immune Responses to an Oral Typhoid Vaccine Strain that is modified to Constitutively Express Vi Capsular Polysaccharide. *The Journal of Infectious Diseases*. 190:565-570.

190. Tan S., Gyles C.L., and Wilkie B.N. (1997).Evaluation of an *aro*A Mutant *Salmonella typhimurium* Vaccine in Chickens Using Modified Semisolid Rappaport Vassiliadis Medium to Monitor Faecal Shedding. *Veterinary Microbiology*. 54(3-4):247-254.

191. Thiem V.D., Lin F.-Y., Canh D.G., Son N.H., Anh D.D., Mao N.D., Chu C., Hunt S.W., Robbins J.B., Schneerson R., and Szu S. (2011). The Vi Conjugate Typhoid Vaccine is Safe, Elicits Protective Levels of IgG Anti-Vi, and is Compatible with Routine Infant Vaccines. *Clinical and Vaccine Immunology*. 18(5):730-735.

192. Tolle L.B., and Standiford T.J. (2013). Danger-Associated Molecular Pattersn (DAMPs) in Acute Lung Injury. *Journal of Pathology*. 229:145-156.

193. Trotter C.L., Andrews N.J., Kaczmarski E.B., Miller E., and Ramsay M.E. (2004). Effectiveness of meningococcal Serogroup C Conjugate Vaccine 4 Years after Introduction. *The Lancet*. 364:365-367.

194. Troy F.A., Frerman F.E., and Heath E.C. (1971). The Biosynthesis of Capsular Polysaccharide in *Aerobacter aerogenes*. *The Journal of Biological Chemistry*. 246(1):118-133.

195. Ullmann A., and Monod J. (1968). Cyclic AMP as an Antagonist of Catabolite Repression in *Escherichia coli*. *FEBS Letters*. 2(1):57-60.

196. Upreti R.K., Kumar M., and Shankar V. (2003). Bacterial Glycoproteins: Functions, Biosynthesis and Applications. *Proteomics*. 3:363-379.

197. Uzzau S., Brown D.J., Wallis T., Rubino S., Leori G., Bernard S., Casadesus J., Platt D.J., and Olsen J.E. (2000). Host Adapted Serotypes of *Salmonella enterica*. *Epidemiology and Infection*. 125:229-255.

198. Vann W.F., Soderstrom T., Egan W., Tsui F.-P., Schneerson R., Orskov I., and Orskov F. (1983). Serological, Chemical, and Structural Analyses of the *Escherichia coli* Cross-

Reactive Capsular Polysaccharides K13, K20, and K23. *Infection and Immunity*. 39(2):623-629.

199. Villarreal J.M., Hernandez-Lucas I., Gil F., Calderon I.L., Calva E., and Saavedra C.P. (2011). cAMP Receptor Protein (CRP) Positively Regulates the *yihU-yshA* Operon in *Salmonella enterica* serovar Typhi. *Microbiology*. 157:636-647.

200. Voetsch A.C., Van Gilder T.J., Angulo F.J., Farley M.M., Shallow S., Marcus R., Cieslak P.R., Deneen V.C., and Tauxe R.V. (2004). FoodNet Estimates of the Burden of Illness Caused by Nontyphoidal *Salmonella* Infections in the United States. *Clinical Infectious Diseases*. 38(Suppl 3):S127-134.

201. Wahdan M.H., Sippel J.E., Mikhail I.A., Rahka A.E., Anderson E.S., Sparks H.A., and Cvjetanovic B. (1975). Controlled Field Trial of a Typhoid Vaccine Prepared with a Nonmotile Mutant of *Salmonella typhi Ty2*. *Bulletin of the World Health Organization*. 52(1):69-73.

202. Wahid R., Simon R., Zafar S.J., Levine M.M., and Sztein M.B. (2012). Live Oral Typhoid Vaccine Ty21a Induces Cross-Reactive Humoral Immune Responses Against *Salmonella enterica* Serovar Paratyphi A and S. Paratyphi B in Humans. *Clinical and Vaccine Immunology*. 19(6):825-834.

203. Waite E.R., and March J.B. (2002). Capsular Polysaccharide Conjugate Vaccines against Contagious Bovine Pleuropneumonia: Immune Responses and Protection in Mice. *Journal of Comparative Pathology*. 126:171-182.

204. Waldner L.L., MacKenzie K.D., Koster W., and White A.P. (2012). From Exit to Entry: Long-Term Survival and Transmission of *Salmonella*. *Pathogens*. 1:128-155; doi:10.3390/pathogens1020128.

205. Wang L., and Coppel R.L. "Triton X-114 Phase Partitioning for Antigen Characterization". *Malaria Methods and Protocols: Methods and Protocols*. Vol. 72. Humana, 2002. 581-585. Print.

206. Wangdi T., Lee C.-Y., Spees A.M., Yu C., Kingsbury D.D., Winter S.E., Hastey C.J., Wilson R.P., Heinrich V., Baumler AJ. (2014). The Vi Capsular Polysaccharide Enables *Salmonella enterica* Serovar Typhi to Evade Microbe-Guided Neutrophil Chemotaxis. *PLOS Pathogens*. 10(8):e1004306.

207. Watson D.C., Robbins J.B., and Szu S.C. (1992). Protection of Mice against *Salmonella typhimurium* with an O-Specific Polysaccharide-Protein Conjugate Vaccine. Infection and Immunity. 60(11):4679-4686.

208. Weiss D.S., Brotcke A., Henry T., Margolis J.J., Chan K., and Monack D.M. (2007). *In vivo* Negative Selection Screen Identifies Genes Required for *Francisella* Virulence. *Proceedings of the National Academy of Sciences*. 104(14):6037-6042.

209. White A.P., Gibson D.L., Collinson S.K., Banser P.A., and Kay W.W. (2003). Extracellular Polysaccharides Associated with Thin Aggregative Fimbriae of *Salmonella enterica* Serovar Enteritidis. *Journal of Bacteriology*. 185(18):5398-5407.

210. White A.P., Allen-Vercoe E., Jones B.W., DeVinney R., Kay W.W., and Surette M.G. (2007). An Efficient System for Markerless Gene Replacement Applicable in a Wide Variety of Enterobacterial Species. Canadian Journal of Microbiology. 53:56-62.

211. White A.P., Gibson D.L., Grassl G.A., Kay W.W., Finaly B.B., Vallance B.A., and Surette M.G. (2008). Aggregation via the Red, Dry, and Rough Morphotype is not a Virulence Adaptation in Salmonella enterica serovar Typhimurium. *Infeciton and Immunity*. 76(3):1048-1058.

212. Whitfield C., Amor P.A., and Koplin R. (1997). Modulation of the Surface Architecture of Gram-negative Bacteria by the Action of Surface Polymer:Lipid A-Core Ligase and by Determinants of Polymer Chain Length. *Molecular Microbiology*. 23(4): 629-638.

213. Whitfield C., and Roberts I.S. (1999). Structure, Assembly and Regulation of Expression of Capsules in *Escherichia coli*. *Molecular Microbiology*. 31(5):1307-1319.

214. Whitfield C. (2006). Biosynthesis and Assembly of Capsular Polysaccharides in *Escherichia coli*. *Annual Review of Biochemistry*. 75:39-68.

215. "WHO Product Development for vaccines Advisory Committee Meeting". World Health Organization. 2014. Web. Mar 2015. <http://www.who.int/immunization/research/meetings_workshops/pdvac/en/>.

216. Wilder-Smith A. (2009). Meningococcal Vaccines: A neglected Topic in Travel Medicine? *Expert Reviews Vaccines*. 8(10):1343-1350.

217. Wilson R.P., Winter S.E., Spees A.M., Winter M.G., Nishimori J.H., Sanchez J.F., Nuccio S-P., Crawford R.W., Tukel C., and Baumler A.J. (2011). The Vi Capsular Polysaccharide Prevents Complement Receptor 3-Mediated Clearance of *Salmonella enterica* Serotype Typhi. *Infection and Immunity*. 79(2):830-837.

218. Zogaj X., Nimtz M., Rohde M., Bokranz W., and Romling U. (2001). The Multicellular Morphotypes of *Salmonella* typhimurium and *Escherichia coli* Produce Cellulose as the Second Component of the Extracellular Matrix. *Molecular Microbiology*. 39(6):1452-1463.