Chronic Carriage of *Salmonella*: Biofilms and Gallstones

Dissertation

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Abstract

*Salmonella enterica* serovar Typhi can establish a chronic, asymptomatic infection of the gallbladder, suggesting that this bacterium utilizes novel mechanisms to mediate enhanced colonization and persistence in a bile-rich environment. The typhoid carrier state is a significant risk factor for hepatobiliary carcinomas, frequently associated with the presence of gallstones, and typically unresolved by regimens of clinically administered antibiotics. This description led us to hypothesize that serovar Typhi biofilm formation on gallstones facilitates gallbladder carriage, and we have demonstrated previously that salmonellae form bile-mediated biofilms on human gallstones in vitro.

To eliminate dependence on human gallstone samples and to allow gallstone constituents to be analyzed individually for their abilities to interact with salmonellae, siliconized Eppendorf tubes were coated with cholesterol and shown to support *Salmonella* biofilm formation in a new Tube Biofilm Assay (TBA). Random transposon mutants of *S. enterica* serovar Typhimurium were screened for impaired adherence to and biofilm formation on cholesterol in the TBA, but not on glass and plastic surfaces, to elucidate mechanisms of attachment initiating biofilm formation specifically on cholesterol surfaces. We identified 49 mutants with this phenotype. Upon sequence analysis of the transposon insertion sites, two unique insertions were identified in both *fimW* and *ompC*, one in *sseI*, and five in various flagellar genes. Focusing on the role of
flagella, subsequent analysis in the TBA suggested that the presence of the flagellar filament and functional chemotaxis enhanced binding/biofilm formation in the presence of bile, while flagellar motility was unimportant. Purified *Salmonella* flagellar proteins used in a modified TBA ELISA showed that FliC was the critical subunit mediating binding to cholesterol.

A serovar Typhimurium mutant deficient in biosynthesis of colanic acid and cellulose formed a mature biofilm on gallstones and in the TBA, suggesting the presence of an unidentified exopolysaccharide necessary for mature biofilm development. Our experiments indicate that the *Salmonella* O-antigen capsule is a crucial determinant in gallstone and cholesterol biofilms but that expression of this exopolysaccharide is not necessary for binding to glass or plastic. Growth in bile resulted in upregulation of the O-antigen capsule-encoding operon in an *agfD*-independent manner and enhanced capsule expression in biofilms on gallstone/cholesterol-coated surfaces.

Naturally resistant 129X1/SvJ mice fed a lithogenic diet developed cholesterol gallstones and, as a result, demonstrated enhanced colonization of gallbladder tissue and bile. These mice also showed pronounced fecal shedding during persistent serovar Typhimurium infection. Furthermore, cholesterol gallstones were shown to associate with significant amounts of salmonellae that increased with time post infection. In a human study conducted in typhoid endemic Mexico City, individuals seeking cholecystectomy due to gallstones were analyzed for asymptomatic serovar Typhi carriage. Results
indicate that 5% of patients with cholelithiasis carried serovar Typhi and that bacterial biofilms could be visualized on gallstones from 3 of 4 of these carriers. Gallstone biofilm formation was not observed in patients positive for other bacteria, suggesting that gallstone biofilms are a typhoid specific phenomenon.

These findings offer direct evidence that gallstone biofilms occur in humans and mice carrying salmonellae asymptotically in their gallbladders, which facilitates gallbladder colonization and shedding. In addition, this work brings forward new therapeutic targets against typhoid fever transmission and a novel murine model for their evaluation.
Dedication

To those reading this dissertation:
You have positively influenced this journey, and I am truly grateful.
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Fields of Study

Major Field: Microbial Pathogenesis
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Chapter 1

Introduction

1.1 *Salmonella*, a pathogen of global concern

1.1.1 Historical perspective and nomenclature

Salmonellae are rod-shaped pathogens that infect a broad array of hosts, causing significant morbidity and mortality in impoverished and developed nations worldwide. Members of this genus have been preeminent research models in laboratories for over a century and belong to the family Enterobacteriaceae, a large group of clinically relevant gram-negative, facultative anaerobic commensal and invasive bacilli (Schaechter, 1987). *Salmonella* has minimal nutritional requirements, does not ferment lactose, resists the caustic nature of bile salts, and, as such, can be differentiated on selective growth media that take advantage of these traits (2002).

The namesake of the *Salmonella* genus is Daniel Elmer Salmon, the first American citizen to be granted the Doctor of Veterinary Medicine degree (Howell et al., 2008). Dr. Salmon presided over the United States Department of Agriculture during the late nineteenth century when *Salmonella* was discovered, a feat attributed to the epidemiologist and pathologist Theobald Smith (Howell et al., 2008). *Salmonella* nomenclature has a complex, dynamic evolution and remains a topic of debate today.
Initially, members of the genus were differentiated based on simplified antigenic formulae proposed by Kauffman and White. In this one serotype-one species scheme, variations in the somatic O polysaccharides and the flagellar H proteins designated names of new species (Brenner et al., 2000). Due in large part to DNA hybridization experiments performed in 1973 by the laboratory of Stan Falkow that demonstrated species level relationships amongst different serotypes and subgenera (Crosa et al., 1973), only two species are currently referenced in *Salmonella* taxonomy, *S. enterica* and *S. bongori*.

The World Health Organization (WHO) Collaborating Centre for Reference and Research on *Salmonella* is charged with defining and maintaining nomenclature standards, and new serotypes (serovars) are introduced in annual updates of the Kauffman-White scheme (Brenner et al., 2000). The Centers for Disease Control and Prevention (CDC) reports that *S. enterica* is comprised of six subspecies and over 2,400 serotypes (serovars) that are generally classified by locations of initial isolation (Popoff et al., 2000). Over the past 25 years, the National *Salmonella* Surveillance System at the Robert Koch Institute has monitored human salmonellosis and geographical trends in specific serotypes (Brenner et al., 2000). The complete 4,857-kilobase (kb) chromosome and 94-kb virulence plasmid of *S. enterica* serovar Typhimurium were sequenced and published in 2001, revolutionizing the scientific investigation of this microorganism (McClelland et al., 2001).

*S. enterica* serovar Typhi caused typhoid fever long before the discovery of etiologic causality by German bacteriologist Karl Eberth. In 1882, Eberth examined intestinal tissue from an infected individual under a microscope and made a hand drawn
woodcut of his observations (Coats, 1882). He noticed that bacilli existed as aggregates or isolated rods, and that there was a tropism for clumps toward lymphatic cells (Fig. 1.1). It is believed that typhoid fever ravaged societies during the Parliamentary Wars of 1642-48, and one of the first descriptions of this illness was catalogued by English physician Thomas Willis in 1684, who wrote that ‘the first assault is for the most part accompanied with a shivering or horror (Paterson, 1949). Widal and Sicard identified specific agglutinins for O and H antigens in the serum of typhoid patients in 1896, a diagnostic technique still used in clinics today (Paterson, 1949). Because other Salmonella serovars and members of the Enterobacteriacae family share these antigens, the sensitivity, specificity, and predictive values of the Widal test are controversial (Parry et al., 2002).

1.1.2 Burdens to public health world-wide

An estimated 1.3 billion cases of disease are mediated by S. enterica serovars across the globe each year (Coburn et al., 2007). The two most common clinical manifestations are gastroenteritis and enteric (typhoid) fever, both of which result in large financial burdens to hospitals and patients. The severity and outcome of illness depend on host susceptibility, including age and immune function, and the infectious S. enterica serovar (2002). S. Typhi is exquisitely adapted to induce disease strictly in humans, while non-typhoidal serovars are ubiquitous in nature and can colonize poultry, reptiles, livestock, rodents, birds, water, and soil.

Approximately 1.4 million incidences of enterocolitis due to non-typhoidal salmonellae occur in the U.S. each year and over 200 million more outside North
America, estimates that lack statistical consistency due to diagnostic and reporting problems (Coburn et al., 2007). In humans, the onset of symptoms occurs between 6 and 72 hours after consumption of food or water contaminated with greater than $10^5$ bacteria, and is typically characterized by abdominal pain, diarrhea, nausea, and vomiting (Coburn et al., 2007). Infections are commonly limited to the gastrointestinal tract and resolve between 5-7 days with oral or intravenous administration of fluids. The pathological hallmarks of salmonellosis caused by *S. enterica* serovars Enteritidis or Typhimurium differ from those of enteric fever and include intestinal neutrophilia, localized inflammatory diarrhea, the loss of electrolytes, serum protein and fluid, as well as sepsis-induced death in immunocompromised individuals such as patients with acquired immunodeficiency syndrome (AIDS) (Tsolis et al., 2008; Dharmana et al., 2002; Fleisher, 1991; Santos et al., 2002). Models for examining *Salmonella* enteropathogenesis include inoculations of bovine ileal loops, tissue cultured epithelial cells, and, recently, pretreatment of mice with streptomycin (Barthel et al., 2003).

*S. Typhi* is the etiologic agent of typhoid fever, a debilitating illness characterized by interstitial inflammation, a gradual onset of symptoms, and, ultimately, bacterial dissemination to systemic sites (Tsolis et al., 2008). *S. enterica* serovars Paratyphi A, Schottmuelleri, and Hirschfeldii induce a mild form of enteric fever called paratyphoid fever. The CDC estimates that at least 22 million illnesses and 600,000 deaths worldwide are attributed to typhoid fever and person-to-person spread of *S. Typhi* is enhanced by its low infectious dose (Butler et al., 1978; Crump et al., 2004; Parry et al., 2002). Symptoms manifest 10 to 14 days post inoculation, vary with geography, and usually include generalized fever, myalgias, and malaise. In adults, typhoid fever can be
accompanied by constipation and bradycardia whereas children and immunosuppressed patients experience intense diarrhea (Tsolis et al., 2008). Blood antibody titers are highest during the first week, but delayed onset or absence altogether of overt symptoms in many patients makes the clinical diagnosis of typhoid fever challenging (Parry et al., 2002). *S. Typhi* is transmitted via the fecal-oral route, yet stool cultures are positive in only 30% of symptomatic typhoid patients (Parry et al., 2002). A regimen of fluoroquinolones such as ciprofloxacin and bed rest is used to treat most cases of enteric fever, but the introduction of multi-drug resistant strains in endemic areas is making management of the disease particularly problematic (Fig. 1.2). Approximately 3-5% of patients suffering from typhoid fever develop an asymptomatic chronic carrier state thought to account for much of the human-to-human transmission of *S. Typhi* (Levine et al., 1982). This disease manifestation will be discussed in detail later.

1.1.3 Mechanisms of pathogenesis

Interestingly, much of what is known about the systemic pathogenesis of *S. Typhi* during typhoid fever has been extrapolated from infections of inbred mouse strains lacking *Nramp1* (e.g. BALB/c and C57BL/6) with non-typhoidal serovar Typhimurium. *Nramp1* is a macrophage phagosome transmembrane protein hypothesized to affect transport of cations (Skamene et al., 1998). In this model, *Salmonella* pathogenicity island (SPI)-1 facilitates translocation of the intestinal epithelium (bacterial mediated endocytosis) at microfold (M) cells overlying the lymphoid follicles of Peyer’s patches (Wolf et al., 1981), and is followed by a primary bacteraemia in the blood and uncontrolled replication within macrophages of the liver, spleen, and bone marrow
leading to death at 7 to 10 days post infection (Jepson and Clark, 2001; Jones and Falkow, 1996; Galan and Curtiss, 1991; Richter-Dahlfors et al., 1997; Salcedo et al., 2001). Alternatively, salmonellae can disseminate from the gastrointestinal tract to the blood independently of SPI-1 in CD18-expressing phagocytes (Vazquez-Torres et al., 1999).

Collectively, these results demonstrate that salmonellae express an arsenal of virulence factors that are highly regulated in space and time to enhance replication, colonization, and transmission when confronted by innate and adaptive host immune responses. SPIs are critically important to bacterial fitness during infection and may have been acquired by horizontal gene transfer. These regions are characterized by a lower content of guanine and cytosine relative to the overall genome, and are flanked by transposon insertion sites (Groisman and Ochman, 1996). Although several SPIs exist and more are currently being elucidated, the best characterized are SPI-1, a mediator of *Salmonella* invasion and induction of host inflammatory pathways, and SPI-2, required for intracellular evasion of phagocytic oxidases and systemic infection in mice (Galan and Curtiss, 1989; Fass and Groisman, 2009). SPI-1 and SPI-2 regulate two distinct type III secretion systems (TTSS) that deliver specialized effector proteins into eukaryotic host cells at specific stages of pathogenesis (McGhie et al., 2009).

Over 30 secreted effectors mediated by SPI-1 and SPI-2 TTSSs have been shown to influence the host biochemistry and cell physiology. For example, SPI-1 proteins SipA, SipC, SopB, SopD, SopE, and SopE2 trigger bacterial internalization into *Salmonella*-containing vacuoles (SCVs) of host cells by a coordinated process of actin polymerization and cytoskeletal rearrangements termed membrane ruffling (reviewed in
SPI-1 effectors also induce inflammation. Specifically, SipA stimulates interleukin (IL)-8 production and neutrophil recruitment across cultured epithelial monolayers (McCormick et al., 1998; Lee et al., 2000b), SopE activates mitogen-associated protein kinases (MAPKs) and thus nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling (Hardt et al., 1998), and SipB binds to caspase-1, increasing IL-1β and IL-18-mediated proinflammatory cell death (pyroptosis) (Lara-Tejero et al., 2006).

SPI-2 facilitates replication and survival in host cells and is positively or negatively regulated by Salmonella factors including but not limited to nucleoid-associated proteins (NAPs) IHF, Fis, SlyA, and HilD, and the two-component systems PhoP/PhoQ and OmpR/EnvZ (Fass and Groisman, 2009). SPI-2 encodes two-component system SsrA/SsrB, whose signaling is dependant on external factors such as osmolarity and pH (Worley et al., 2000). SsrA/B are directly regulated in turn by transcription factors SlyA and OmpR (Feng et al., 2004; Lee et al., 2000a; Feng et al., 2003).

Two-component regulatory systems allow bacteria to respond to conditions in the external milieu and are comprised of a membrane-bound sensor histidine kinase and a response regulator that, when phosphorylated at a conserved aspartic acid, activates or represses gene transcription (Gunn et al., 1996). More than 4,000 two-component signal transduction systems have been identified in 145 sequenced bacterial genomes accounting for diverse response regulation including nutrient acquisition, energy metabolism and virulence (Mohapatra et al., 2007). One such pathway, required for phenotypes including antimicrobial-peptide resistance, survival within macrophages and virulence in animal models and human volunteers, is the PhoP-PhoQ (PhoPQ) two-
component system (TCS) (Groisman, 2001; Hohmann et al., 1996). PhoPQ post-
transcriptionally activates another TCS, PmrA-PmrB (PmrAB), via the PmrD protein
(Kato and Groisman, 2004). The PmrAB TCS has orthologs in other bacterial pathogens
(Mohapatra et al., 2007), responds directly to environmental signals including high iron
and aluminum concentrations and low pH, and modifies lipopolysaccharide (LPS) to
mediate resistance to killing by ions or innate immune factors (reviewed in (Gunn,
2008)).

Pathogen-associated molecular patterns (PAMPs) are factors recognized by
specific pattern recognition receptors (PRRs) of the innate immune system. Multiple pro-
and anti-inflammatory cytokines and chemokines synthesized by macrophages, epithelial
cells, and Dendritic cells in response to PAMPs, including interferon (IFN)-γ, IL-12,
tumor necrosis factor (TNF)-α, IL-18, transforming growth factor-β, IL-10 MCP-1,
CCL2, CCL20 and CCL3, play pivotal roles during Salmonella infections (Coburn et al.,
2007).

The Vi-capsular polysaccharide antigen of serovar Typhi is a PAMP encoded by
SPI-7, a large mosaic, genetic island that is genetically unstable, can be lost upon
laboratory passage, and is absent from the serovar Typhimurium chromosome altogether
(Nair et al., 2004). The Vi-capsule has been shown to prevent Toll-like receptor (TLR) 4
recognition (Wilson et al., 2008), and its loss in serovar Typhi isolates increased TNF-α
production or IL-8 secretion production in macrophage or epithelial cell lines,
respectively (Hirose et al., 1997; Sharma and Qadri, 2004). Human enterocolitis induces
massive neutrophilia whereas typhoid fever is characterized by mononuclear infiltrate,
suggesting that different PAMPs stimulate varying responses in association with specific PRRs (reviewed in reference (Tukel et al., 2006)).

The major components of the Salmonella outer membrane have been studied extensively and include lipopolysaccharide (LPS), enterobacterial common antigen (ECA), porins, and flagellin, the monomeric subunits comprising peritrichous flagella. LPS is composed of a hydrophobic lipid A membrane anchor, the distal, hydrophilic O-antigen protrusion, and a core region of polysaccharide repeat units that connect the two (Muhlradt, 1976; Nikaido, 1987). O-antigen capsule mutants are attenuated for virulence and highly sensitive to hydrophobic chemicals including bile salts (described later), whereas strains with incomplete assembly of lipid A are conditionally lethal (Jansson et al., 1981; Nikaido, 1976). TLR4 recognition of bacterial LPS by macrophages is required for the host to generate complete inflammatory responses to invading pathogens (MacVittie et al., 1982; Vazquez-Torres et al., 2004; Royle et al., 2003). Another LPS receptor, CD14, is not expressed by intestinal epithelial cells, suggesting that these cells may not directly generate LPS-mediated host responses (Coburn et al., 2007).

The ECA is an acidic polysaccharide containing N-acetyl-D-glucosamine, N-acetyl-D-mannosaminuronic acid, and 4-acetamido-4,6-dideoxy-D-galactose covalently linked to the external leaflet of the outer membrane in all species of the family Enterobacteriaceae (Lugowski and Romanowska, 1991). S. Typhimurium strains carrying mutations in the ECA biosynthesis and assembly genes wecD and wecA were highly attenuated in oral infections in vivo compared to wild-type (Valtonen et al., 1976; Ramos-Morales et al., 2003) and susceptible to bile salts in vitro (described later).
One of the most abundant proteins in *S. enterica* are the porins, which form channels that facilitate passive diffusion of hydrophilic molecules across the outer membrane (Nikaido, 1987). The three major *S. Typhimurium* porins vary in size and are encoded by the *ompC*, *ompD*, and *ompF* genes (Norioka et al., 1986). OmpR positively regulates expression of OmpC and OmpF, and is necessary for virulence in murine models, whereas individual mutations in *ompC*, *ompD*, or *ompF* do not affect disease outcome (Dorman et al., 1989). Expression of OmpS1 and OmpS2 porins occurs at low levels, but is required for lethality in mice (Rodriguez-Morales et al., 2006).

The *Salmonella* flagellum is encoded by a highly ordered transcriptional hierarchy comprised of more than 60 genes and is comprised of two antigenically distinct subunit proteins, FliC (H:i) and FljB (H:1,2) (Frye et al., 2006). Flagellin is detected by mammalian cells via surface-expressed TLR-5 and cytosolic Nod-like receptors (Rochon and Romling, 2006; Simon and Samuel, 2007). Secretion of IL-1 beta and IL-18 by mouse cell lines is activated comparably by the FliC and FljB flagellins (Simon and Samuel, 2008), but memory CD4+-T-cells preferentially recognize FliC during adaptive immunity and *Salmonella* committed to expressing only FljB is attenuated in vivo (Bergman et al., 2005; Ikeda et al., 2001). *S. Typhimurium*-induced colitis in streptomycin-pretreated mice required flagella production and recent experiments have demonstrated that SPI-2 translocates flagellin to the basolateral surface of the intestinal epithelium (Stecher et al., 2004; Lyons et al., 2004).
1.2 Bacteria and Bile, a Dynamic Duo during Disease

1.2.1 Bile composition and characteristics

Normal physiology of healthy individuals requires emulsification and solubilization of lipids as well as elimination of substances that cannot be efficiently excreted in urine. This is accomplished by bile, a complex digestive secretion produced by the liver at nearly one liter per day that in general is lipid rich and protein poor (Hofmann, 1998). Bile acids are the primary constituents of bile, act as detergents due to their surface active, amphipathic properties, and comprise 12% of the weight and approximately 50% of the organics in bile. In addition to aiding the dispersal of lipids, bile acids serve as potent antimicrobial agents. Thus, bile contributes to the harsh environment of the gastrointestinal tract that also includes variations in pH and oxygen tension, the presence of commensal bacteria, and limitations in nutrients. These and other factors combine to be a significant barrier for invading enteric pathogens (Begley et al., 2005a). Gastrointestinal commensals and pathogens, including salmonellae, have developed mechanisms to resist the deleterious effects of bile and subsequently colonize human intestines, and, as such, the manner in which microbes sense and respond to bile has been a subject of recent interest (Fig. 1.3).

Along with bile acids, other lipid-containing molecules found in bile include cholesterol and phospholipids (e.g., phosphatidylcholine). One of the major protein constituents is immunoglobulin, while the pigment biliverdin provides bile its characteristic yellowish green color (Hofmann, 1999). IgA and mucus in bile contribute to making it a sterile compound by preventing bacterial growth and adhesion to the biliary and small intestine epithelium (McPhee, 1987). Synthesis of bile occurs in
pericentral hepatocytes of the liver and is followed by secretion into bile canaliculi and drainage into bile ducts that merge to form hepatic ducts. The common hepatic duct meets the cystic duct from the gallbladder, and the two channels merge into the common bile duct to provide an exit route for bile from the liver. From there, the sphincter of Oddi regulates the flow of bile into the duodenum (Begley et al., 2005a).

Bile is concentrated up to 10-fold by removal of water and electrolytes and can be acidified by ion exchange. This process occurs in the gallbladder, the storage organ for bile between meals (Hofmann, 1999; Johnson, 1998). About half the hepatic bile produced is diverted to the gallbladder until fats stimulate the enteric hormones secretin and cholecystokinin to induce bile secretion and flow, causing the gallbladder to contract and the sphincter of Oddi to relax so that bile may reach the duodenum and aid digestion.

Bile acids are derived from cholesterol in the liver through a multienzyme process that includes an important oxidative step which converts the molecule from an eight carbon to a five carbon acid moiety. This mechanism permits conjugation of bile acids through a peptide linkage with either glycine or taurine in a 3:1 ratio depending on diet (Hardison, 1978; Sjovall, 1959). Cholic acid and chenodeoxycholic acid are the primary bile salts of human bile. In their unconjugated form, bile acids are only slightly soluble at physiologically relevant pH. Therefore, amidation of bile acids is critical because it lowers their pK_a to provide solubility over a wide range of ionic strengths, calcium concentrations and pH values (Hofmann and Roda, 1984; Hofmann and Mysels, 1992).

Additionally, conjugation of bile acids discourages passive absorption in the biliary tract and small intestine so that bile acids are maintained between 0.2 to 2% in the intestine and approximately 8% in the gallbladder (Hofmann, 1998). Fasting and
malnourishment have been shown to decrease the amount of bile in the intestine and, consequently, leave such individuals vulnerable to bacterial pathogens (Opleta et al., 1988; Reed et al., 1996). As mentioned above, bile acids are amphipathic and as such self-associate in water to form polymolecular micelles that solubilize other lipids. It has been suggested that these aggregates decrease the toxicity individual bile salts exude upon biliary epithelial cells (Hofmann, 1999).

De novo synthesis of bile acids from cholesterol is not always required because up to 95% of the bile acid pool is recycled on a daily basis in a process called enterohepatic circulation (Begley et al., 2005a). Absorption of conjugated and unconjugated bile acids occurs along the entire length of the gut mucosa to facilitate transfer back to the liver for reconjucation and delivery into bile (Hofmann, 1998). The constant flow of bile through the gastrointestinal tract is critical for bile homeostasis. Excess intraluminal bile acids can cause diarrhea whereas suboptimal levels may result in cholesterol supersaturated bile responsible for gallstone formation (Paumgartner and Sauerbruch, 1991).

1.2.2 Bile and the normal flora

The resident, bile resistant microbial flora of the caecum and colon can alter the properties of bile acids that were bypassed for enterohepatic circulation. Intestinal microbiota, primarily anaerobes, deconjugate bile acids and stimulate mechanisms that convert primary bile acids cholate and chenodeoxycholate into secondary bile acids deoxycholate and lithocholate, respectively (Franklund et al., 1993). As outlined by Bortolini et al., the categories of microbial induced biotransformations of bile acids
include epimerization, oxidation, hydroxylation, reduction and dehydroxylation (Bortolini et al., 1997). Some of these natural reactions are pharmaceutically relevant, such as ursodeoxycholic acid, the 7-hydroxy epimer of chenodeoxycholic acid that can be synthesized industrially and prescribed as a treatment for cholestatic liver diseases (28).

Deconjugation cleaves amino acid side chains and is an important mechanism used by bacteria to impact the properties of bile. This reaction may be carried out by intracellular bile salt hydrolases (BSHs) belonging to the choloylglycine hydrolase family of enzymes (Begley et al., 2005a). Species of the intestinal microflora expressing BSH activity include Clostridium, Bifidobacterium and Enterococcus (Coleman and Hudson, 1995; Grill et al., 2000b; Knarreborg et al., 2002). Although the physiological significance of BSH activity remains uncharacterized, key studies have advanced our understanding of their proposed roles in enteric microorganisms. Van Eldere et al. demonstrated that Clostridia energetics utilize taurine released from bile acids, suggesting that deconjugation by hydrolytic strains provides nutritional sources of carbon and nitrogen (Van Eldere et al., 1996). It has also been postulated that BSHs increase tensile strength of bacterial membranes residing in the intestine and serve as a detoxification system mediating bile tolerance (Boggs, 1987; Grill et al., 2000a).

The inherent cost of altering the properties of bile acids to cultivate a healthy microflora may be at the expense of the human host. Unconjugated bile acids emulsify dietary lipids less effectively than their conjugated counterparts, leaving lipid digestion and fatty acid removal impaired (De Smet et al., 1998). The products of dehydroxylation reactions are potentially toxic and have been associated with increased risk of colon cancer (Mower et al., 1979). On the other hand, probiotic researchers encourage
consumption of BSH active bacteria. They suggest that subsequent limitations to
enterohepatic circulation in the presence of unconjugated bile acids causes enhanced
faecal loss of bile salts. The result may be manifested in lower serum cholesterol levels
due to an increased demand by the liver for cholesterol for de novo bile acid synthesis
(De Smet et al., 1998).

1.2.3 Interactions of bile with pathogenic bacteria and viruses

Bile affects the expression of important \textit{Salmonella} proteins necessary for bile
tolerance, some of which are thought to be regulated by the two-component system,
PhoPQ. Van Velkinburgh and Gunn observed that mutant strains constitutively
expressing this regulon survived prolonged incubation with bile at concentrations
exceeding 60%, whereas a \textit{phoP}-null strain was susceptible to significantly lower bile
concentrations than wild type strains. However, no PhoPQ regulated genes responsible
for bile resistance where identified (van Velkinburgh and Gunn, 1999). Therefore,
PhoPQ-mediated bile resistance may occur via genes that overlap this regulon.

As mentioned above, LPS protects cells from damaging agents like amphipathic
bile acids. Loss of the O-antigen component of LPS, which protrudes from the
membrane to serve as its main barrier, creates a rough colony phenotype resulting in
decreased resistance to bile (Kaper et al., 1995; Prouty et al., 2002b). Interestingly, LPS
modifications (aminoarabinose addition to the lipid A anchor portion of LPS and
ethanolamine changes to the core and lipid A regions) did not alter the high tolerance of
\textit{Salmonella} to bile, eliminating the possibility that electrostatic interactions between bile
and \textit{S. Typhimurium} contributed to resistance (van Velkinburgh and Gunn, 1999).
Furthermore, activation of the PmrAB system, which mediates these modifications, actually sensitizes both *E. coli* and *Salmonella* to killing by bile salts (J. Gunn unpublished observation), genes other than those mediating LPS alteration are likely to be possible.

One of the most well characterized mechanisms through which bacteria exhibit bile tolerance is efflux pump activity. In addition to mediating resistance to a variety of antibiotics and oxidative stress agents, these systems expel out of the cell bile salts that have traversed the inner membrane. *Salmonellae* express the bile-activated AcrAB efflux pump, which has a proton motive force-dependent drug efflux transporter, AcrB, and an element to bridge the inner and outer membranes, AcrA (Ma et al., 1994; Zgurskaya and Nikaido, 1999). The *acrAB* efflux pump genes are absolutely required for growth of *S. Typhimurium* in the presence of bile. Similarly, the *marRAB* operon of *S. Typhimurium* is involved in resistance to structurally unrelated antimicrobials including chloramphenicol, tetracycline and bile. DNA microarray and transcriptional assays performed by Prouty et al. showed activation of *marRAB* in the presence of bile. Deoxycholate, but not other bile acids or a non-ionic detergent, interacted directly with the *marRAB* repressor MarR to interfere with its ability to bind to the *mar* operator. Interestingly, growth in sublethal amounts of bile enhanced chloramphenicol resistance by both *mar*-dependent and *mar*-independent pathways (Prouty et al., 2004a).

Tol proteins function in the uptake of colicins and filamentous phage DNA and many gram-negative transport systems require a Tol homolog at the outer membrane to serve as a pore (Gunn, 2000). In this regard, TolC contributes to the AcrAB efflux pump system and is an essential component of bile resistance. Several bile-sensitive mutations
in *S. Typhimurium* isolated from a MudJ transposon experiment were located in the *tolQRA* region (Prouty et al., 2002b). Mutations in these genes compromise membrane integrity, possibly allowing an increase in bile acid traffic into the cell. The *S. Typhimurium* ECA also provides bile protection. Strains carrying mutations in the ECA biosynthesis and assembly genes *wecD* and *wecA* were 40-fold more sensitive to deoxycholate *in vitro* and highly attenuated in oral infections *in vivo* compared to wild-type (Valtonen et al., 1976; Ramos-Morales et al., 2003).

As described above, the SPI-1 encoded TTSS mediates endocytosis of *S. Typhimurium* into nonphagocytic eukaryotic cells. Invasion gene regulators including *sirA* and *invF* are transcriptionally repressed by bile, leading to repression of protein secretion by the SPI-1 TTSS and a dramatic reduction in epithelial cell invasion (Prouty and Gunn, 2000; Prouty et al., 2004b). The presence of bile also reduced expression of flagellar biosynthesis genes and motility. Therefore, successful host invasion may rely on temporal sensing of bile by salmonellae. High concentrations of bile encountered in the intestinal lumen may halt invasive properties until bacteria traverse the epithelial mucus layer to the underlying surface. It is there, where bile is likely in low concentration and invasion is needed for disease progression, that SPI-1 TSS repression is alleviated.

The ubiquitous, gram-negative bacterium *Escherichia coli* facilitate waste processing, vitamin K production and food absorption in a healthy functioning gastrointestinal microenvironment. However, it is in this same niche that commensal *E. coli* plays destructive roles implicated in gastroenteritis, urinary tract infections and peritonitis. Despite advances in sterilization techniques aimed at killing active bacteria,
*E. coli* also remains an important foodborne pathogen. Recent outbreaks of contaminated beef and vegetables have caused numerous cases of severe diarrhea (Jeong et al., 2007).

Like *Salmonella* and other gram-negative bacteria, *E. coli* actively removes bile acids that have traversed the membranes into the cytoplasm as a mechanism of bile resistance. Thanassi et al. demonstrated that bile acids in the unconjugated, lipophilic form entered the cell through the OmpF porin of the outer membrane and were subsequently expelled via energy-dependent efflux (Thanassi et al., 1997). The *mdtABCD* multidrug resistance cluster encodes three different transporter proteins and has been linked to enhanced resistance to deoxycholate (Baranova and Nikaido, 2002). *E. coli* possesses two additional efflux systems, AcrAB and EmrAB, required for tolerance to antibiotics such as tetracycline and chloramphenicol and toxic stressors such as bile. As mentioned previously, the constituents of AcrAB are well characterized whereas EmrAB is not as well described outside of its negative regulator EmrR (Lomovskaya and Lewis, 1992). *E. coli* strains carrying *acrA emrB* double mutations demonstrated growth inhibition in the presence of bile and intracellular accumulation of chenodeoxycholate.

Enteropathogenic *E. coli* (EPEC) are significant causes of infantile diarrhea. Virulence factors encoded by the chromosomal locus of enterocyte effacement (LEE) region and adherence factors allow EPEC attachment to the intestinal mucosa (Trabulsi et al., 2002). The ability of bile to mediate EPEC adhesion was demonstrated by deJesus et al. in experiments showing that bile acids including chenodeoxycholate and deoxycholate indeed increased adherence to HEp2 cells in tissue culture (de Jesus et al., 2005). Although specific EPEC adhesins responsible for the bile acid stress induced adherence
were not elucidated, virulence factors intimin and flagella were ruled out. This question
was similarly addressed by Torres et al. using strains of atypical enteropathogenic E. coli
(aEPEC). Bile acids enhanced aEPEC adhesion to in vitro cultured HeLa cells by
regulating expression of the afimbrial locus for diffuse adherence (LDA) adhesion
(Torres et al., 2007). Other detergents tested could not achieve the same LDA induction,
suggesting a specific mechanism by which bile promotes disease manifestation by
increasing the binding of aEPEC to intestinal epithelial cells.

*Shigella flexneri*, closely related to enteroinvasive E. coli (EIEC), is a facultative
intracellular pathogen and the etiologic agent of bacillary dysentery (Hale, 1991).
Invasion of colonic epithelial cells is mediated by a type III secretion system and triggers
a strong inflammatory response. Disease onset is characterized by diarrhea, abdominal
cramps and fever (Blocker et al., 2003). Interestingly, natural infections in humans
require only 10 to 100 organisms of *S. flexneri* whereas simulated infection of tissue
cultured cells is relatively inefficient and needs a centrifugation step to initiate contact
between bacteria and target cells. This observation led to the hypothesis that bile acids
encountered in vivo act as specific environmental factors that enhance *Shigella* invasion
and disease progression (Pope et al., 1995a).

Indeed, Pope et al. demonstrated that exposure to physiological concentrations of
deoxycholate and chenodeoxycholate increased attachment and entry of *Shigella* species
to HeLa cells in an in vitro model system (Pope et al., 1995a). Interestingly, this
phenomenon did not occur in EIEC. The presence of bile upregulates type III machinery
and invasion in *Shigella* but has the opposite effect in *Salmonella*, further suggesting a
unique mechanism contributing to bile-signaling and pathogenesis. This group also
observed the ability of bile acids to enhance secretion of Ipa proteins released during type three-dependent cell entry. Olive et al. elaborated upon this finding, reporting that deoxycholate stimulated recruitment of IpaB to the *S. flexneri* surface. Once there, IpaB colocalized with IpaD at the tip of the type III secretion needle. The authors speculate that IpaD is an environmental sensor capable of changing conformation in the presence of bile acids to permit type III mediated secretion (Olive et al., 2007).

Since the discovery in the late 1970s that *Campylobacter jejuni* could induce human illness, this gram-negative pathogen has become the leading bacterial cause of enteritis in the United States (Slutsker et al., 1998). A small percentage of patients develop Guillain-Barre syndrome, an autoimmune disorder of the peripheral nervous system characterized by weak respiratory muscles, neurologic deficits or death (Nachamkin et al., 1998). Effective control and prevention of *Campylobacter* infection remains elusive as the pathogen becomes increasingly resistant to fluoroquinolone treatment and innate host defense (Tauxe, 2002). Bile salts present an important antimicrobial barrier for *Campylobacter* in the intestine. Accordingly, the pathogen expresses a multidrug resistance (MDR) efflux pump called CmeABC which is essential for adaptation to the harsh gastrointestinal environment (Luo et al., 2003). This resistance-nodulation-division (RND)-type multidrug efflux system is capable of mediating the bile resistant phenotype *C. jejuni* requires for successful host colonization (Grkovic et al., 2002).

CmeABC is encoded by a three gene operon and is constitutively expressed at moderate levels in wild type *Campylobacter* strains cultured in conventional media (Lin et al., 2005b). Lin et al. showed expression of *cmeABC* to be controlled by the
transcriptional repressor CmeR which, when inactivated, leads to overexpression of the pump (Lin et al., 2005a). Researchers have also demonstrated direct interaction of bile salts with CmeR much like that described previously for Salmonella MarR (Lin et al., 2005b). Both conjugated and unconjugated bile salts increased expression of the CmeABC efflux pump while other antimicrobials, including chloramphenicol, ethidium bromide and erythromycin, did not affect transcription of cmeABC. Surface plasmon resonance provided evidence that bile salts were capable of inhibiting binding of CmeR to the cmeABC promoter, leading to increased pump expression and elevated bile resistance. Interestingly, the presence of bile salts in culture media enhanced resistance of Campylobacter to multiple antibiotics including cefotaxime, novobiocin and fusidic acid (Lin et al., 2005b). Mutations in cmeABC drastically reduced resistance to bile salts, as inactivation of the CmeABC pump led to impaired growth in the presence of 2 mM choleate culture media whereas wild type bacteria displayed no growth defect under the same conditions (Lin et al., 2003).

Bile salts are proposed to act as a stimulatory signal for synthesis of virulence-associated Campylobacter invasion antigens (Cia) present upon coculture with epithelial cells (Rivera-Amill et al., 2001). These invasion-related proteins were not induced by altering the pH, calcium concentration, osmolarity or temperature of the culture media, suggesting a specific effect for bile salts sodium deoxycholate, cholate and chenodeoxycholate. The flagellum of C. jejuni is another important virulence determinant whose expression is known to be enhanced by bile. Experiments done by Allen and Griffiths showed that bile positively induced the flagellar flaA σ^{28} promoter
and that subsequent chemotaxis led to colonization of the mucus layer and pathogenesis (Allen and Griffiths, 2001).

The presence of bile causes pleiotropic responses that affect production of virulence factors, motility and other phenotypes in pathogenic, non-invasive *Vibrio cholerae*. The diarrheal disease cholera is acquired following consumption of food or water contaminated with *V. cholerae*, and developing countries are particularly susceptible to devastating outbreaks where mortality rates may exceed 20% in affected populations (Chatterjee et al., 2007). Production of cholera toxin (CT) and toxin-coregulated pilus (TCP) are typically required for colonization of the intestinal epithelium (Kaper et al., 1995). Expression of these virulence factors, as well as others, is controlled by a cascade of regulatory proteins comprising the ToxR regulon. ToxR and TcpP are inner membrane DNA binding proteins that activate ToxT, an important transcriptional regulator that controls a variety of factors necessary for colonization and disease in the host, including *ctxAB* and *tcpA* (Chatterjee et al., 2007).

As evidenced by several pieces of conflicting data, the relationship between *Vibrio* and bile is quite complex. It was initially reported that the production of CT and TCP is dramatically diminished when *V. cholerae* is grown in the presence of crude ox bile extract (Gupta and Chowdhury, 1997). This finding was extended by Schumacher and Klose who used a plasmid-based heterologous P_{lac} promoter to show that sodium choleate addition resulted in a significantly reduced ability of ToxT to activate transcription of regulated genes. In sum, these results suggested that ToxT interacts directly with bile or with a factor induced in the presence of bile to alter its ability to regulate target genes (Schumacher and Klose, 1999).
Furthering the studies of the Klose laboratory, Chatterjee et al. examined bile-regulated virulence factor expression by elaborately fractioning crude bile to identify the components mediating virulence gene repression and enhancement of motility in *V. cholerae* (Chatterjee et al., 2007). They isolated the unsaturated fatty acids arachidonic, linoleic and oleic acids from bile and showed them to repress expression of the *ctxAB* and *tcpA* genes independent of ToxT. These fatty acids also increased expression of *flrA*, the first gene in a cascade controlling motility. It was proposed that the ToxR-ToxT system that negatively regulates motility is nonfunctional in the presence of unsaturated fatty acids. The resulting increase in motility was also observed upon addition of cholesterol. However, Hung and Mekalanos later reported that purified bile acids, unlike crude bile extracts, do not inhibit CT or TcpA expression. Interestingly, this work shows that bile acids can activate CT expression in a ToxR-dependent, ToxT-independent manner (Hung and Mekalanos, 2005).

*Vibrio cholerae* also express outer membrane proteins that are affected by bile. ToxR activity, independent of ToxT, leads to reciprocal expression of OmpU and OmpT, two general diffusion porins that affect bile resistance (Chakrabarti et al., 1996; Champion et al., 1997). Gupta and Chowdhury reported that crude ox bile extracts increased *V. cholerae* motility in a ToxR-independent manner but had no effect on OmpU/OmpT expression (Schuhmacher and Klose, 1999). However, Provenzano et al. later demonstrated that bile increased OmpU expression in a ToxR-dependent manner to confer enhanced bile resistance for *V. cholerae* (Duret and Delcour, 2006; Provenzano et al., 2000; Provenzano and Klose, 2000).
Similar to other enteric gram-negative pathogens, *V. cholerae* expresses an efflux system contributing to antimicrobial resistance. Bina et al. recently characterized two RND-family efflux systems called *vexAB* and *vexCD* that provide *V. cholerae* resistance to bile (Bina et al., 2006). Microarrays were used previously to show that the two open reading frames, now known to encode *vexAB*, were upregulated in stool of cholera patients and in rabbit ileal loops (Bina et al., 2003; Merrell et al., 2002; Xu et al., 2003). Experiments by Bina et al. showed that expression of the *vexAB* and *vexCD* efflux systems are regulated by bile acids and that these pathways have overlapping roles in the resistance to bile (Bina et al., 2006). Surprisingly though, they demonstrated that *vexB*, *vexD* and *vexB-vexD* deletion strains efficiently colonized the infant mouse, suggesting the dispensability of these systems *in vivo* or the involvement of other as of yet uncharacterized efflux systems.

Food-processing and storage expose the gram-positive bacterium *Listeria monocytogenes* to growth challenges including cold temperatures, acidic pH and high osmolarity (Vazquez-Boland et al., 2001). The surviving bacteria contaminate food and subsequently can infect a human host, where temperatures are higher, pH is lower, osmolarity shifts during transit through the gastrointestinal tract, and bile is present (Gahan and Hill, 1999). Similar to *S. Typhimurium* described above, pre-exposure of *Listeria* to sublethal concentrations of bile prepares cells for subsequent growth in otherwise lethal amounts of bile. Begley et al. mimicked experiments performed by Flahaut et al. in *E. faecalis* (Flahaut et al., 1996) and showed this adaptation phenomenon in low levels of bile to occur in as little as five seconds (Begley et al., 2002). Although bile sensing yields coordinate changes to cellular physiology through as of yet unknown
mechanisms, it has been suggested that adaptation to other stresses also confers cross-protection to bile. Indeed, exposing exponential phase *L. monocytogenes* to acidic pH, high temperature, 5% NaCl or 0.01% SDS increased bile tolerance (Begley et al., 2002).

*Listeria* expresses bile salt hydrolases (BSHs) thought to be important for bile resistance. These enzymes catalyze hydrolysis of the amide bond between the C-24 position of the steroid moiety and the amino acid side chain of bile acids. It has been proposed that deconjugation of bile salts by BSHs represents a detoxification mechanism that promotes *Listeria* persistence in the gastrointestinal tract (Begley et al., 2005a). A *bsh* gene deletion in *L. monocytogenes* created by Dussurget et al. decreased the MICs for both bile and bile salts and displayed reduced bacterial faecal carriage after oral infection of guinea pigs (Dussurget et al., 2002). In addition, the *bsh* gene is activated by PrfA, the major virulence gene regulator of *L. monocytogenes*. Interestingly, when wild type cells carried the *bsh* gene on a plasmid, researchers observed an increase in intestinal multiplication.

Begley et al. corroborated these results using a rifampin marked strain in a murine model of infection, showing that *bsh* mutants could not be found in the intestine three days post oral inoculation (Begley et al., 2005b). They also showed involvement of the general stress responsive sigma factor σ^B in *bsh* gene regulation. This indicates that anaerobic conditions of the intestine trigger the sigma B regulon to prepare *Listeria* for environmental stressors including bile. Genomic sequences revealed *bsh* to be absent from nonpathogenic *Listeria*, further implicating BSH activity as a specific mediator of bile resistance for successful colonization of the host.
Another virulence determinant in *L. monocytogenes* is the recently described bile tolerance locus BilE, which contains two distinct ATP-dependent bile acid permease signature sequences common to multidrug efflux pumps (Sleator et al., 2005). Sleator et al. revealed that a *bilE* mutant retained approximately 1.5 times the amount of radiolabelled bile as the wild type, rendering it bile sensitive. Intriguingly, they showed that bile susceptibility is more pronounced in a *bilE* deletion compared to a *bsh* mutant, suggesting that active extrusion of bile salts is more effective than detoxification of internalized bile. The authors also present data showing that *bilE* transcription is coordinately controlled by sigma B and *prfA*, much like BSH enzymes mentioned above.

In addition to altering the pathogenic course of invasive bacteria, bile is known to affect disease outcome for a variety of viral types. Human immunodeficiency virus type 1 (HIV-1) transmission and pathogenic progression requires infection or propagation of viral replication in CD4⁺ T lymphocytes. This mechanism has been suggested to rely upon an important interaction between the gp120 envelope glycoprotein of HIV-1 with DC-specific ICAM-3 grabbing nonintegrin (DC-SIGN) expressed on dendritic cells (DCs) and a subset of B cells (Geijtenbeek et al., 2000; Hu et al., 2000; Pope et al., 1995b). DC-SIGN is a transmembrane C-type lectin that recognizes the major *Mycobacterium tuberculosis* surface lipoglycans, mannose-capped lipoarabinomannan (ManLAM) and higher-order phosphatidylinositol mannosides (PIMs) (Azad et al., 2008; Torrelles et al., 2006). Naarding et al. demonstrated that Lewis X (Le⁺) found in human milk could inhibit DC-SIGN dependent transfer of HIV-1 to CD4⁺ T lymphocytes by binding to DC-SIGN and blocking the interaction between virus and receptor (Naarding et al., 2005). They further showed that bile salt-stimulated lipase (BSSL), a
LeX-carrying glycoprotein secreted by the pancreas and activated by bile salts in the intestine, was the primary compound in human milk binding to DC-SIGN and inhibiting HIV-1 transfer (Naarding et al., 2006). BSSL variations caused alterations in LeX expression that limited viral activity, suggesting a potential role for bile derivatives against HIV-1 transmission. In another study investigating the effects of bile on HIV-1 activity, Al-Jabri et al. showed that tyrosine-conjugated tri- and dihydroxy bile salt derivatives exhibited high surfactant activity, low cellular toxicity to human tissues, and anti-HIV-1 virucidal activity (Al-Jabri et al., 2000).

The presence of bile acids seems to be required for the growth of porcine enteric calicivirus (PEC), a small, nonenveloped virus responsible for a high proportion of nonbacterial gastroenteritis outbreaks in the United States each year (Chang et al., 2004). The PEC strain Cowden can be propagated by serial passage in a continuous cell line (LLC-PK), but only with addition of intestinal content (IC) fluid filtrate obtained from uninfected gnotobiotic pigs. Chang et al. elucidated bile acids cholic acid, glycocholic acid, chenodeoxycholic and glycochenodeoxycholic acid in IC as the essential active factors mediating growth of PEC in cultured cells. The presence of bile during PEC replication triggered the protein kinase A cell-signaling pathway and induced increased cAMP levels in LLC-PK cells, leading to a down-regulation of innate immunity associated with decreased IFN-mediated signal transducer and activator of transcription 1 phosphorylation. Interestingly, addition of cAMP/protein kinase A pathway inhibitors to infected LLC-PK cells suppressed bile acid-mediated PEC replication. The dependence of PEC growth on the presence of bile suggests that bile salts may actively suppress
innate immune function in susceptible target cells so that enteric viruses such as PEC may utilize this mechanism to establish gut infections (Chang et al., 2004).

1.3 Chronic Carriage, Gallstones, and Carcinoma

During systemic typhoid infections, S. Typhi can reach the gallbladder and establish an acute, active infection (cholecystitis) or persist in this organ long after overt symptoms subside (Vaishnavi et al., 2005; Sinnott and Teall, 1987). Historically, clinically administered antibiotics have demonstrated variable efficacies against dissolution of the persistent bacterial infection. In the early 1940s, regimens of iodophthalein, sulphonamides, phenothiazine, penicillin, streptomycin, and chloromycetin individually or in combination were discredited as appropriate treatment options (Paterson, 1949). A study by Levine et al. in 1982 demonstrated that serovar Typhi could be recovered in stool samples for over 1 year from at least 2 to 5% of those suffering acute typhoid illnesses (Levine et al., 1982), yet bacterial recovery in feces is sporadic and as such a poor diagnostic tool. Shedding of S. Typhi into the environment by chronic carriers accelerates the fecal-oral route of person-to-person transmission in endemic areas, suggesting the need for improved food and water sanitation, awareness of personal hygiene in public health, and the development of novel therapeutic strategies.

An estimated 25% of asymptomatic carriers have no history of typhoid fever (Parry et al., 2002), making the chronic carrier state difficult to confirm and an understudied aspect of global human health. Vi agglutination from blood and bone marrow samples has long been used as a method of identifying typhoid carriers (Bhatnagar, 1948), yet not all clinical isolates express Vi-antigen and patients recovering
from acute typhoid infections have low titres of serum IgG antibodies to Vi antigens (Losonsky et al., 1987). Multiplex PCR is a recent advancement in technology that selectively amplifies Vi antigen (viaB), flagellar antigens (fliC-d and fliC-a), and O-antigen synthesis (tyv and prt) to detect S. Typhi in human samples (Hirose et al., 2002).

Despite difficulties in diagnosis, we understand that the asymptomatic carrier state is frequently associated with gallbladder abnormalities, particularly gallstones (Lai et al., 1992). Cholelithiasis afflicts approximately 10 to 15% of the adult population world-wide and more than 25 million individuals in the United States (Shoheiber et al., 1997). Gallstone formation (cholelithogenesis) is attributed to a combination of environmental and genetic causes including obesity, female gender, multiple pregnancies, nutrition, old age, and Crohn’s disease, and can occur in patients for many years without symptoms (Shoheiber et al., 1997; Afdhal, 1999). Under appropriate physicochemical conditions, cholesterol monohydrate crystals supersaturate from bile and nucleate in the gelled mucin glycoprotein scaffolding (biliary sludge) of the gallbladder wall to form gallstones (Maurer et al., 2009). The primary constituent of gallbladder stones is cholesterol, whereas calcium bilirubinate predominates in bile duct stones (Hofmann, 2005). Biliary epithelial cells express mucin genes and secrete immunoglobulin proteins and a variety of cytokines during innate and adaptive immunity of the gastrointestinal tract (Maurer et al., 2009; Reynoso-Paz et al., 1999; Andrianifahanana et al., 2006), yet gallbladder biology and the associated immune response in typhoid carriage, particularly in those patients with gallstone biofilms, remain unknown.

In patients carrying both S. Typhi and cholesterol gallstones in the gallbladder, clinically administered antibiotics are typically ineffective against dissolution of the
persistent bacterial infection (Lai et al., 1992). Removal of the gallbladder (cholecystectomy) remains the most effective treatment option for these individuals, a treatment option first proposed by Dehler in 1907 (Paterson, 1949). In 1994, the United States alone spent an estimated 8.0 billion dollars treating gallbladder diseases, suggesting a strong need for alternative treatment options in areas of the world where typhoid fever is endemic and surgical intervention is cost-prohibitive. Chronic typhoid carriers with gallstones also experience an 8.47-fold-higher risk for developing hepatobiliary diseases, including carcinoma of the gallbladder (Dutta et al., 2000; Kumar et al., 2006; Surette and Bassler, 1999). In 1994, Caygill et al. demonstrated that gallbladder cancers were associated with long-term typhoid carriage, but not acute infections (Caygill et al., 1994).

The association between chronic bacterial gallbladder infections, gallstones, and carcinoma of this organ has also been a subject of recent investigation in Helicobacter. For example, Fox et al. sequenced PCR-amplified 16S rRNA fragments of DNA isolated from human bile and gallbladder tissue samples and showed that H. bilis was found in the gallbladders of five out of eight Chilean patients with chronic cholecystitis and gallbladder cancer (Fox et al., 1998; Fox et al., 1995). Several reports have evaluated the correlation between gallstones and the presence of H. pylori in the gallbladder. Its existence in this organ is inherently intriguing because H. pylori are susceptible to bile in vitro (Tiwari et al., 2006). Apostolov et al. used immunohistochemistry with H. pylori-specific CagA and VacA antigens and observed a high prevalence of this strain in gallbladder epithelial cells of Ukrainian patients needing cholecystectomy (Ananieva et al., 2002; Apostolov et al., 2005).
On the other hand, Bohr et al. draw little analogy between the presence of *Helicobacter* and cholecystitis (Bohr et al., 2007). Immunohistochemical analysis of gallbladder tissues from German patients with gallstones demonstrated a low prevalence of Helicobacteraceae. In addition, Maurer et al. used C57L/J mice, a strain in which gallstone formation can be induced by a lithogenic diet, to conclude that *H. pylori* infection plays no role in murine cholesterol gallstone formation (Maurer et al., 2006). The limited amount of contradictory literature suggests that the cause and effect relationship between the presence of *Helicobacter* and induction of various hepatobiliary diseases, as influenced by bile during infection, needs further investigation.

1.4 Bacterial Biofilm Formation

Biofilm formation was made clinically relevant in 1978 by Dr. R.J. Gibbons (Jefferson, 2004) and is currently thought to characterize 65% of chronic infections treated by physicians (Stoodley, 2002). Bacterial biofilm infections develop on live and dead tissues and medical devices, are slow to produce overt symptoms, demonstrate heightened resistance to cellular and humoral immune host responses and commonly administered antibiotic regimens and household sanitizers, and persist for long periods of time with intermittent recurrence of symptoms until the community is physically removed (for a review see reference (Costerton et al., 1999)).

Over the past two decades, bacterial biofilms have been increasingly implicated as burdens to food and public safety worldwide, and are broadly defined as heterogeneous communities of microorganisms that adhere to each other and to inert or live substrates (Costerton et al., 1995; Davey and O'toole, 2000; Watnick and Kolter, 2000; Stewart and
Franklin, 2008; Monds and O'Toole, 2009). Biofilm formation occurs in sequential, highly regulated stages that begin with adherence of free-swimming, planktonic bacteria to a surface. Subsequent biofilm maturation is characterized by the production of a self-initiated extracellular matrix (ECM) composed of nucleic acid, proteins, or exopolysaccharides (EPS) that encase the community of microorganisms and provide structure and protection. Planktonic cells from this sessile, matrix-bound population are continuously shed, which can result in reattachment and fortification of the biofilm, or systemic infection and release of the organism into the environment (Fig. 1.4).

Wild-type and mutant bacteria strains have been investigated to determine important ligands mediating biofilm development using static microtiter plates, dynamic flow cell apparati, tissue cultured cells, or even human gallstones (Costerton et al., 1999; Stewart and Costerton, 2001; Prouty and Gunn, 2003; Ma et al., 2009). This includes diverse species of commensal and pathogenic microorganisms including *Streptococcus mutans* (Idone et al., 2003; Wen and Burne, 2002; Svensater et al., 2001), *Staphylococcus aureus* (Caiazza and O'Toole, 2003; Vaudaux et al., 1995; Gross et al., 2001), non-typeable *Haemophilus influenzae* (NTHI) (Jurcisek et al., 2007), *Escherichia coli* (Pratt and Kolter, 1998; Danese et al., 2000), *Vibrio cholerae* (Hung et al., 2006; Lejeune, 2003; Yildiz and Schoolnik, 1999), and *Pseudomonas aeruginosa* (Ma et al., 2009; O'Toole and Kolter, 1998b; Davies et al., 1993; Whiteley et al., 2001). Interestingly, bacterial gene expression profiles differ between planktonic and biofilm phenotypes (Stoodley et al., 2002; Jefferson, 2004), and these changes are likely regulated by external stimuli including nutrient availability, oxygen tension, pH, the presence or
absence of antimicrobials, and the composition of binding substrates, suggesting that biofilm development is a highly-ordered, multifactorial process.

Bacterial biofilms resist the deleterious effects of antibiotics whereas planktonic cells dispersed from the biofilm microenvironment quickly regain susceptibility to those antibiotics, suggesting that biofilm-mediated resistance is not acquired through mutations or horizontal transfer of genes (Stewart and Costerton, 2001; Anwar et al., 1989; Williams et al., 1997). For example, a *Klebsiella pneumoniae* strain lacking β-lactamase production was killed in aqueous solution by ampicillin at concentrations as low as 2 µg/ml but, when grown as a biofilm, was unaffected by 5,000 µg/ml ampicillin (Anderl et al., 2000). It was also demonstrated that established mechanisms of resistance to antimicrobials and bile salts including efflux pumps and the multiple antibiotic resistance (*mar*) locus of *E. coli* did not provide protection to bacteria in biofilms (Williams et al., 1997; Maira-Litran et al., 2000), suggesting that biofilm-mediated mechanisms of resistance may differ from those conventionally employed by planktonic cells.

Biofilm formation by *P. aeruginosa* has been hypothesized to mediate colonization and antibiotic resistance in the context of cystic fibrosis, a lung disease that features persistent airway neutrophil accumulation. Interestingly, human neutrophils were shown in vitro to enhance *P. aeruginosa* biofilm development and even settle on the mature biofilm to become phagocytically engorged, degranulated, immobilized, and rounded during compromised host defense (Walker et al., 2005; Jesaitis et al., 2003). Similarly, *S. epidermidis* biofilm formation mediates a protective response in vitro against complement deposition and neutrophil-dependent killing (Kristian et al., 2008).
Mathematical approaches and empirical observations are constantly challenging and redefining the developmental model of microbial biofilm formation (Monds and O'Toole, 2009; van Loosdrecht et al., 2002; Picioreanu et al., 2007; Saravanan and Sreekrishnan, 2006). Recent work in *P. aeruginosa* has investigated stage-specific factors including twitching motility, cell-to-cell signaling, stationary phase sigma factor expression, and polysaccharide synthesis locus (PSL) activity that contribute to biofilm development in flow cell systems using statistical analysis and direct visualization (Heydorn et al., 2002; Ma et al., 2009). Other polysaccharides important for *P. aeruginosa* ECM biosynthesis include alginate, and Pel, a seven gene operon regulated by mediators of quorum sensing (Ryder et al., 2007; Ramsey and Wozniak, 2005; Sakuragi and Kolter, 2007). The term quorum sensing was introduced in reference to the cell-density-linked, coordinated gene expression observed in *Vibrio fischeri*, and is hypothesized to play a role in biofilm formation for some organisms under certain conditions (reviewed in (von Bodman et al., 2008; Kjelleberg and Molin, 2002)).

The molecular genetic mechanisms mediating adherence and microcolony formation during early biofilm growth are beginning to be elucidated for many microorganisms. Flagella, for example, promote surface binding during the initial stages of biofilm development (Pratt and Kolter, 1998; O'Toole and Kolter, 1998b) as well as adhesion during biofilm-mediated colonization of tissue cultured cells and mucus by *P. aeruginosa* (Rudner et al., 1992; Lillehoj et al., 2002), *Campylobacter jejuni* (McSweegan and Walker, 1986), *E. coli* (Giron et al., 2002), and *S. enterica* spp. (Dibb-Fuller et al., 1999; La Ragione et al., 2003; Budiarti et al., 1991; Allen-Vercoe and Woodward, 1999). Expression of the serovar Typhimurium flagella has been shown to
inhibit biofilm formation on polystyrene wells (Teplitski et al., 2006) and promote biofilm development on human gallstones when bile is added to the growth media (Prouty et al., 2002a; Prouty and Gunn, 2003), but the stage at which this appendage positively or negatively impacts Salmonella biofilms remains undefined.

Interestingly, flagellar-mediated phenotypes including swimming motility and chemotaxis differentially contributed to colonization and biofilm formation depending on environmental cues (O'Toole and Kolter, 1998b; Pratt and Kolter, 1998; Prouty and Gunn, 2003; Huber et al., 2002; Mey et al., 2005). Bile increases motility and is a chemical attractant at physiological concentrations for V. cholerae (Butler and Camilli, 2005), acts as a repellent for Helicobacter pylori (Worku et al., 2004), and enhances tumbling frequency while downregulating motility in S. enterica (Prouty et al., 2004b). Swimming contributes positively to biofilm formation of P. aeruginosa (O'Toole and Kolter, 1998b) and E. coli (Pratt and Kolter, 1998), but a motA mutant of serovar Typhimurium did not affect mature biofilm formation on gallstones in vitro (Prouty and Gunn, 2003). In the motA595::Tn10 strain, flagellar motor function is defective while biosynthesis remains unaffected (Kutsukake et al., 1990). Mutations in the same gene of E. coli and Burkholderia cepacia affected early biofilm formation, but each recovered from the transient defect to form mature biofilms (Huber et al., 2002). Bacterial chemotaxis (tumbling motility) is controlled by clockwise flagella rotation and has been shown to be dispensable for E. coli biofilms in a PVC microtitre dish assay (Pratt and Kolter, 1998), but important for V. cholerae biofilms on polystyrene wells (Mey et al., 2005).
Fimbriae have been shown to mediate adherence during biofilm initiation and cell-cell interactions during biofilm growth for a variety of microorganisms (Davey and O'toole, 2000; O'Toole and Kolter, 1998b). The serovar Typhimurium genome encodes 13 putative fimbrial operons, some of which are not expressed in vitro (Nuccio et al., 2007). Type 1 fimbriae have been shown to be important for biofilm formation on HEp-2 tissue culture cells, the murine intestinal epithelium, and chicken intestinal epithelium (Boddicker et al., 2002; Ledeboer and Jones, 2005), but not on human gallstones incubated with bile (Prouty et al., 2002a). Production of filamentous type IV pili was recently described in the airway pathogen NTHI and demonstrated to enhance adherence to human respiratory epithelial cells, colonization of the chinchilla respiratory tract as well as biofilm formation in vitro and in vivo (Bakaletz et al., 2005; Jurcisek et al., 2007). Expression of type IV pili also contributes to biofilm development in \textit{P. aeruginosa} and \textit{V. cholerae} (Deziel et al., 2001; Moorthy and Watnick, 2004).

ECM material within bacterial biofilms is thought to be heterogeneous and to provide both rigidity to the biofilm and protection to bacteria embedded within established biofilms. It has previously been shown that \textit{Salmonella} biofilm-associated ECM factors are not uniformly required for biofilm formation on all surfaces (Prouty and Gunn, 2003). Cellulose and colonic acids have been shown to play a role in biofilm formation on biotic as well as abiotic surfaces, as mutations in either \textit{wcaM} or \textit{yhjN} have been shown to disrupt serovar Typhimurium biofilm formation in flow through chambers on HEp-2 cells (Ledeboer and Jones, 2005). However, in the presence of bile, cellulose and colanic acid are not a factor in biofilm formation on human gallstones (Prouty and
Gunn, 2003). In *E. coli*, cellulose mediated biofilm formation occurs independently of known regulators csgD and adrA (Da Re and Ghigo, 2006).

Atomic force microscopy of serovar Typhimurium biofilms grown on agar plates or mica slides demonstrated that curli and cellulose were important for ECM production and suggested that expression of surface appendages may be co-regulated during biofilm formation (Jonas et al., 2007). AgfD (CsgD) is an important regulator of *Salmonella* biofilm formation (Brombacher et al., 2003; Romling et al., 1998) and has been demonstrated to control biosynthesis of the recently identified O-antigen (O-ag) capsule (Gibson et al., 2006). This exopolysaccharide is membrane linked by 2-hydroxymyristic acid (like a capsule), similar in structure to the LPS O-ag of serovar Enteritidis, conserved amongst salmonellae, and encoded by two divergent operons (*yihU*-yshA and *yihV*-yihW) (Gibson et al., 2006). Despite having similar repeating sugar units, the O-ag capsule and the LPS O-ag differ in size, charge, substitution patterns, and immunoreactivity (White et al., 2003). O-ag capsule expression was found to be necessary for serovar Enteritidis biofilm formation leading to enhanced plant colonization (Barak et al., 2007). Interestingly, *Salmonella* biofilm formation has also been observed on growing bean sprouts and parsley sprigs (Warriner et al., 2003; Lapidot et al., 2006).

In addition to polysaccharides, biofilm-associated proteins (BAPs) contribute to ECM production in *S. aureus* and *E. faecalis* biofilms (Latasa et al., 2006). Salmonellae express a large cell surface protein encoded by *bapA* that was shown to be important for serovar Enteritidis biofilm formation but not for serovar Typhimurium (Latasa et al., 2005; Jonas et al., 2007). Two protein domain superfamilies, glutamate-alanine-leucine (EAL) and GGDEF, have been demonstrated to control *Salmonella* and *Vibrio* biofilm
formation by regulating the levels of the novel second messenger cyclic di-guanylic acid (c-di-GMP) (Tischler and Camilli, 2004; Simm et al., 2005; Simm et al., 2004; Garcia et al., 2004). The role of these and other proteins in ECM production during biofilm formation needs further investigation.

1.5 Hypothesis and Goals

The asymptomatic, chronic carrier state of *Salmonella enterica* serovar Typhi occurs in the human gallbladder, is frequently associated with the presence of cholesterol gallstones, and does not subside with antibiotic treatment (Levine et al., 1982; Lai et al., 1992), yet the progression from infection to persistence remains unknown. The gallbladder is also the storage organ for bile, suggesting novel mechanisms utilized by the bacterium to mediate colonization in a hostile environment. Therefore, we hypothesize that biofilm formation on cholesterol gallstone surfaces enhances persistence in this hostile environment and, thus, person-to-person transmission of typhoid fever in impoverished nations.

We have previously demonstrated that bile promotes biofilm formation in an in vitro human gallstone test tube assay, and that the ECM of these biofilms can be preserved and visualized by SEM (Fig. 1.5) (Prouty et al., 2002a). Interestingly, the support matrix seemed to provide signals that determined *Salmonella* biofilm components, and those differed for gallstones and glass surfaces (Prouty and Gunn, 2003). This dissertation attempts to define *Salmonella* constituents mediating adherence (early) and ECM production (late) events during biofilm development specifically on cholesterol surfaces, and evaluate the contribution of gallstone biofilms during
asymptomatic typhoid carriage in humans and a new murine model. A better understanding of the regulatory mechanisms that mediate *Salmonella* biofilm formation and maintenance promises to elucidate important therapeutic targets for eliminating chronic carriage in the gallbladder in patients with bile-induced typhoid gallstone biofilms.
Figure 1.1: Karl Eberth’s typhoid bacilli, hand drawn with the camera lucida and presented in The British Medical Journal on March 25, 1882 (Coats, 1882). Intestinal tissue from an infected individual was stained with methyl-violet and observed at 1,500 diameters. Pale circles represent red corpuscles while the darker ones are nuclei of lymphatic cells. Typhoid bacilli were detected as aggregates or isolated rods.
Figure 1.2: Typhoid endemic nations and global incidence of antimicrobial resistant serovar Typhi strains adapted from Bhan et al. (Bhan et al., 2005). Clinical isolates are increasingly tolerant to nalidixic acid (NAR) in southeastern Asia (1990-2004).
Figure 1.3: Bacterial intestinal pathogens discussed in this chapter that are affected by bile. Listed are microbes known to cause acute or chronic disease in the gallbladder or the intestinal tract. Interactions between invading microorganism and bile, which is produced in the liver and found in the intestine and gallbladder, affect various genes and virulence properties (listed) that enhance colonization and persistence.
Figure 1.4: The sequential, highly regulated stages of biofilm development. Planktonic, free-swimming bacteria adhere to biotic or abiotic surfaces as mediated by environmental conditions and swim along the substrate to facilitate monolayer formation. The growing community is enlarged and fortified by replication and recruitment of new cells. Subsequent biofilm formation is characterized by the production of a self-initiated extracellular matrix (ECM) composed of nucleic acid, proteins, or exopolysaccharides (EPS) that encase the microorganisms and provide structure and protection. Planktonic cells from this sessile, matrix-bound population are continuously shed and can reattach or spread to distal sites.
Figure 1.5: *S. enterica* bile-induced gallstone biofilms visualized by confocal and scanning electron microscopy. Human gallstones were incubated with salmonellae and 3% bile for 12 or 18 days, washed in LB, fixed in glutaraldehyde, and observed. (A) CLSM micrograph of *S. enterica* serovar Typhi biofilm constitutively expressing green fluorescent protein. (B) Three-dimensional assembly of Z-scan CLSM images demonstrating serovar Typhi biofilm thickness at approximately 31 μm. (C) SEM micrograph showing the multilayered, heterogeneous surface of a cholesterol gallstone incubated in LB broth without bacteria. (D) Serovar Typhimurium biofilm visualized by SEM. The sample was air-dried overnight prior to fixation to harden extracellular matrix constituents. (E and F) SEM micrographs of serovar Typhimurium mature biofilms. The web-like strands or flocculent material were preserved by immediate fixation and are indicative of exopolysaccharides in the biofilm ECM.
The *Salmonella enterica* serovar Typhimurium flagellum mediates attachment to and biofilm development on cholesterol-coated surfaces

2.1 Abstract

The asymptomatic, chronic carrier state of *Salmonella enterica* serovar Typhi occurs in the bile-rich gallbladder and is frequently associated with the presence of cholesterol gallstones. We have previously demonstrated that salmonellae form biofilms on human gallstones and cholesterol-coated surfaces in vitro and thus hypothesize that bile-induced biofilm formation on cholesterol gallstones promotes gallbladder colonization and maintenance of the carrier state. Random transposon mutants of *S. enterica* serovar Typhimurium were screened for impaired adherence and biofilm formation on cholesterol-coated Eppendorf tubes in the Tube Biofilm Assay (TBA), but not on glass and plastic surfaces. We identified 49 mutants with this phenotype. The results indicate that genes involved in flagella biosynthesis and structure primarily mediated attachment to cholesterol. Subsequent analysis in the TBA suggests that the presence of the flagellar filament and functional chemotaxis enhance binding/biofilm formation in the presence of bile, while flagellar motility and expression of type 1 fimbriae are unimportant. Purified *Salmonella* flagellar proteins used in a modified TBA
ELISA showed that FliC was the critical subunit mediating binding to cholesterol. These studies provide a better understanding of early events during biofilm development, specifically how salmonellae bind to cholesterol, and suggest a target for therapies that may alleviate biofilm formation on cholesterol gallstones and the chronic carrier state.

2.2 Introduction

The serovars of *Salmonella enterica* are diverse, infect a broad array of hosts, and cause significant morbidity and mortality in impoverished and industrialized nations worldwide. *Salmonella enterica* serovar Typhi is the etiologic agent of typhoid fever, a severe illness characterized by sustained bacteraemia and a delayed onset of symptoms that afflicts approximately 20 million people each year (Butler et al., 1978; Crump et al., 2004). Serovar Typhi can establish a chronic infection of the human gallbladder, suggesting that this bacterium utilizes novel mechanisms to mediate enhanced colonization and persistence in a bile-rich environment.

There is a strong correlation between gallbladder abnormalities, particularly gallstones, and development of the asymptomatic *Salmonella* carrier state (Lai et al., 1992). Antibiotic regimens are typically ineffective in carriers with gallstones (Lai et al., 1992), and these patients have an 8.47-fold-higher risk for developing hepatobiliary carcinomas (Dutta et al., 2000; Kumar et al., 2006). Elimination of chronic infections usually requires gallbladder removal (Lai et al., 1992), but surgical intervention is cost-prohibitive in developing countries where serovar Typhi is prevalent. Thus, understanding the progression of infection to the carrier state and developing alternative treatment options are of critical importance to human health.
The formation of biofilms on gallstones has been hypothesized to be a facilitator for enhanced colonization of and persistence in the gallbladder. Over the past two decades, bacterial biofilms have been increasingly implicated as burdens to food and public safety worldwide, and are broadly defined as heterogeneous communities of microorganisms that adhere to each other and to inert or live surfaces (Costerton et al., 1995; Davey and O'toole, 2000; Watnick and Kolter, 2000; Stewart and Franklin, 2008; Monds and O'Toole, 2009). This sessile environment provides selective advantages in natural, medical, and industrial ecosystems to a diverse species of commensal and pathogenic bacteria including *Streptococcus mutans* (Idone et al., 2003; Wen and Burne, 2002; Svensater et al., 2001), *Staphylococcus aureus* (Caiazza and O'Toole, 2003; Vaudaux et al., 1995; Gross et al., 2001), *Escherichia coli* (Pratt and Kolter, 1998; Danese et al., 2000), *Vibrio cholerae* (Hung et al., 2006; Lejeune, 2003; Yildiz and Schoolnik, 1999), and *Pseudomonas aeruginosa* (Ma et al., 2009; O'Toole and Kolter, 1998b; Davies et al., 1993; Whiteley et al., 2001). Bacterial biofilms are increasingly associated with many chronic infections in humans, and demonstrate heightened resistance to commonly administered antibiotics and engulfment by professional phagocytes of the host innate immune system (Lewis, 2001; Lewis, 2007; Mah et al., 2003). Bacterial gene expression profiles differ between planktonic and biofilm phenotypes (Stoodley et al., 2002; Jefferson, 2004), and these changes are likely regulated by external stimuli including nutrient availability, the presence or absence of antimicrobials, and the composition of binding substrates.

Biofilm formation occurs in sequential, highly ordered stages that begin with attachment of free-swimming, planktonic bacteria to a surface. Subsequent biofilm
maturation is characterized by the production of a self-initiated extracellular matrix (ECM) composed of nucleic acid, proteins, or exopolysaccharides (EPS) that encase the community of microorganisms. Planktonic cells from this sessile, matrix-bound population are continuously shed, which can result in reattachment and fortification of the biofilm, or systemic infection and release of the organism into the environment. Shedding of serovar Typhi by asymptomatic carriers can contaminate food and water to account for much of the person-to-person transmission in underdeveloped countries.

Our laboratory has previously reported that bile is required for mature biofilm formation with characteristic EPS production of *S. enterica* serovars Typhimurium, Enteritidis, and Typhi on human gallstones and cholesterol-coated Eppendorf tubes (Prouty et al., 2002a; Crawford et al., 2008). It was also demonstrated that *Salmonella* biofilms formed on different surfaces had unique phenotypes and required expression of specific EPS (Prouty and Gunn, 2003; Crawford et al., 2008), yet the factors mediating *Salmonella* binding to gallstones/cholesterol-coated surfaces during the initiation of biofilm formation remain unknown. Here, we show that the presence of serovar Typhimurium flagella promotes binding specifically to cholesterol in the early stages of biofilm development and that the FliC subunit is a critical component. Bound salmonellae expressing intact flagella provided a scaffold for other cells to bind during later stages of biofilm growth. Elucidating key mechanisms that mediate adherence to cholesterol during *Salmonella* bile-induced biofilm formation on gallstone surfaces promises to reveal novel drug targets for alleviating biofilm formation in chronic cases.
2.3 Materials and Methods

2.3.1 Bacterial strains, growth conditions, and molecular biology techniques

The *Salmonella* strains used in this study are listed in Table 2.1. LB broth and agar were used for bacterial growth, creation of mutants, biofilm assays, and flagella purification. For biofilm formation experiments, strains were grown on a rotating drum in the presence or absence of 3% crude ox bile extract (Sigma, St. Louis, MO) to mid- to late exponential phase (optical density at 600 nm [OD$_{600}$] of 0.6 to 0.8). When necessary, antibiotics were added at appropriate concentrations: ampicillin, 50 μg/ml; kanamycin, 25 μg/ml; chloramphenicol, 25μg/ml; and tetracycline, 15 μg/ml. Molecular cloning and PCR were completed according to established protocols. Plasmids were purified using QIAprep spin mini prep kits (Qiagen, Valencia, CA) and transformed by electroporation as previously described (Schmid and Roth, 1983).

2.3.2 Transposon mutagenesis and screening

Cholesterol-binding deficient, tetracycline (Tet) resistant serovar Typhimurium strains were created by random transposon mutagenesis using established methodology (Schmid and Roth, 1983). Briefly, a pool of Tn$^{10}$d-Tet transposons was introduced into wild-type serovar Typhimurium by generalized, P22 HT int-$10^5$-mediated phage transduction. Strains containing Tn$^{10}$d transposon insertions were selected on LB agar-Tet plates and 40,000 individual colonies were pooled together in LB broth-Tet. The serovar Typhimurium transposon mutant pool was grown to log phase in LB broth-Tet with 3% crude ox bile extract and added in 100 μl amounts to siliconized Eppendorf tubes (Fisher Scientific, Pittsburgh, PA) coated with 1 mg each of chromatography-grade
cholesterol (Sigma, St. Louis, MO). Following incubation for 24 hours at room
temperature on a Nutator shaker (Labnet International, Edison, NJ), 10 μl of planktonic,
non-adherent culture was removed and added to 90 μl of fresh LB broth-Tet with 3%
crude ox bile in a new cholesterol-coated, siliconized Eppendorf tube. This panning for
cholesterol binding deficient bacteria was repeated every 24 hours for 10 days. Serial
dilutions of the final planktonic culture were plated on LB agar-Tet, and 500 individual
colonies were screened for loss/defect of biofilm formation on cholesterol-coated
surfaces (0-25% of wild-type values) and preservation of biofilm formation on glass and
plastic coverslips (75-100% of wild-type values). Colonies were reconfirmed for a non-
biofilm forming (on cholesterol) phenotype by back-transduction using P22 HT int-105.

2.3.3 Cholesterol, glass, and plastic surface biofilm assays

Salmonella strains were tested for their ability to form biofilms in the tube biofilm
assay (TBA) and on cholesterol-coated, glass, and plastic coverslips as described
previously (Crawford et al., 2008). In brief, log phase Salmonella strains grown with or
without 3% crude ox bile were added to cholesterol-coated Eppendorf tubes or plastic 24-
well plates (Fisher Scientific, Pittsburgh, PA) containing Chrommerge-cleaned glass,
plastic, and cholesterol-coated coverslips. These cultures were incubated on a Nutator
shaker at room temperature for 6 days. Every 24 hours, the medium was removed, the
tubes were washed three times with LB, and fresh medium (LB with or without 3% bile)
was added. Bound bacterial samples were fixed at 60°C for 1 hour and a solution of
0.1% crystal violet (gentian violet in isopropanol-methanol-1 x PBS [1:1:18]) was then
added to stain cells for 5 minutes at room temperature. The dye was extracted using 33%
acetic acid and then quantified with optical density readings at 570 nm to determine the amount of dye retained by each biofilm.

2.3.4 DNA sequencing and bioinformatics

Sequencing of transposon mutant genomic DNA from serovar Typhimurium cholesterol binding deficient colonies with primer JG1787 (5’-CCTTTTTCGATGGTA-3’) was performed using an Applied Biosystems 3730 DNA Capillary Analyzer and BigDye cycle fluorescent terminator chemistry in the Plant-Microbe Genomics Facility at The Ohio State University. Transposon insertion sites of recovered sequences were determined using Blastx at the NCBI (Johnson et al., 2008).

2.3.5 Mutant and complement construction

A serovar Typhimurium strain containing mutations in both a putative fimbrial protein (FimW) and a transcriptional regulator (FimZ) was created by P22 HT int-105-mediated, generalized transduction between fimW and fimZ single mutant strains (Schmid and Roth, 1983). The correct construct was verified by a high frequency of co-transduction of the antibiotic markers from a potential ΔfimWΔfimZ double mutant strain into a wild-type serovar Typhimurium background. For complementation, the fimW gene from serovar Typhimurium was amplified with primers JG1843 (5’-GCAAGCTTTTTTTATGCTGCCTGCTATTAA-3’) and JG1844 (5’-CCCTCTAGATTACTTACTGAGTAAGAATGAAG-3’), and cloned into the XbaI and HindIII sites of the arabinose inducible pBAD18 (ColEl ori, P_{BAD} L(+) Ap^+) vector.
The resulting construct was introduced into the \textit{fimW} single mutant strain of serovar Typhimurium by electrotransformation.

2.3.6 Adherence assay of live and dead bacteria

Wild-type and flagellar transcriptional activator (FlhC) mutant strains of serovar Typhimurium were grown overnight at 37°C in LB broth with or without 3% crude ox bile extract, diluted 1:100, and allowed to reach an optical density at 600 nm of 0.6. Bacteria in these cultures were killed by incubation for 40 minutes in 10% formalin or heat fixation for 20 minutes at 65°C. Triplicates of live and dead salmonellae were added in 100 \( \mu \)l aliquots to 24-well polystyrene tissue culture plates (Becton Dickinson Labware, Franklin Lakes, NJ) coated with 1 mg of cholesterol per well, and plates were centrifuged at 165 relative centrifugal force (rcf) for 5 minutes to initiate contact between flagellin and cholesterol. A 3 hour adherence incubation at room temperature was followed by 5 washes in 1 x PBS, staining of wells with a solution of 0.1% crystal violet (gentian violet in isopropanol-methanol-1 x PBS [1:1:18]) for 5 minutes, and 5 more washes with 1 x PBS. The dye retained by bound cells was extracted using 33% acetic acid and quantified by optical density measurements at 570 nm.

2.3.7 Live and dead scaffold assay

Late log phase cultures of serovar Typhimurium wild-type and flagellar transcriptional activator (FlhC) mutant strains were formalin fixed or heat killed as described above and added in 100 \( \mu \)l aliquots to siliconized Eppendorf tubes coated with 1 mg each of chromatography grade cholesterol. Following incubation at room
temperature on a Nutator shaker for 24 hours, cultures were removed and tubes were washed three times in LB broth to remove non-adherent bacteria. Live cultures of late log phase wild-type or ΔflhC serovar Typhimurium strains were added to these tubes and examined for biofilm formation using the TBA as indicated earlier.

2.3.8 Purification of serovar Typhimurium flagellin

Serovar Typhimurium wild-type, phase 1 (FliC; H:i) mutant, and phase 2 (FljB; H:i,2) mutant, and type C flagellin (FliC) mutant strains were grown to late log phase in LB at 37°C. These cultures (500 ml) were centrifuged at 8,000 g for 15 minutes. Cell pellets were washed once, resuspended in 15 ml 1 x PBS, and sheared mechanically for 3 minutes at 30,000 rpm using a Power Gen 125 tissue homogenizer (Fisher Scientific, Pittsburgh, PA). Flagellar filaments were separated from cellular debris by spinning 10 minute at 8,000 g. The flagellin containing supernatants were collected and centrifuged at 100,000 g for 1 hour. Filaments were gently resuspended overnight in 1 x PBS by slow shaking at 4°C and centrifuged at 100,000 g. This 24 hour wash cycle was repeated twice and the final pellets of purified serovar Typhimurium flagellin were resuspended in 1 x PBS and stored at -20°C. Protein concentrations of isolated flagellin were measured with a bicinchoninic acid (BCA) assay kit (Pierce Biotechnology Inc., Rockford, IL), and purity was confirmed by sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE) and staining with GelCode Blue Reagent (Pierce Biotechnology Inc., Rockford, IL).
2.3.9 Western blotting

Monoclonal antibodies against *Salmonella* species flagellum (Maine Biotechnology Services, Portland, ME) and FliC subunit protein (BioLegend, San Diego, CA) were used to probe purified flagellin from serovar Typhimurium wild-type, FljB mutant, and FliC mutant strains in Western blot analysis. For each sample, 10 μl containing 1 μg purified flagellin was mixed with an equal volume of SDS-PAGE loading buffer and boiled for 15 minutes. Preparations were separated by 10% SDS-PAGE and transferred to Hybond-ECL nitrocellulose (Amersham Biosciences, Pittsburgh, PA) using a Trans-Blot semi-dry transfer apparatus (Bio-Rad, Hercules, CA). Membranes were blocked overnight in 5% bovine serum albumin (BSA, Sigma, St. Louis, MO) and incubated with anti-flagella (diluted 1:200 in PBS) or anti-FliC (5 μg diluted in 5 ml PBS) antibody for 4 hours. Goat anti-mouse-horseradish peroxidase (HRP) conjugate (Bio-Rad, Hercules, CA) diluted 1:5000 in PBS (2 hour incubation) and enhanced chemiluminescence (ECL) reagents (GE Healthcare, Pittsburgh, PA) were used to detect bound antibodies. Bands were visualized post exposure and development on HyBlot CL autoradiography film (Denville Scientific Inc., Metuchen, NJ). All washes were performed in 1 x PBS.

2.3.10 Subunit binding ELISA

Purified flagellin from serovar Typhimurium wild-type, FljB mutant, and FliC mutant strains was assayed for cholesterol binding ability using a modified enzyme-linked immunosorbent assay (ELISA) approach. Chromatography-grade cholesterol was dissolved in anhydrous ether (J. T. Baker, Phillipsburg, NJ) at a concentration of 25
mg/ml and delivered in 100 μl aliquots to polystyrene wells of a 96-well Microtest tissue culture plate (Becton Dickinson Labware, Franklin Lakes, NJ). Six replicates of 1 μg each of a purified flagellin sample were then added to the cholesterol-coated wells. Following a 3 hour binding-incubation at room temperature, plates were washed three times in 1 x PBS and blocked overnight with 3% BSA. Wells were emptied, washed, and incubated with anti-flagella (diluted 1:20 in 0.3% BSA) or anti-FliC (diluted 1:100 in 0.3% BSA) antibody for 2 hours, each in triplicate. Another wash step was followed by addition of goat anti-rabbit HRP peroxidase conjugate (Bio-Rad, Hercules, CA) diluted 1:5,000 in 0.3% BSA for 1 hour. Measurements of flagellin binding to cholesterol were obtained using a Bio-Rad HRP substrate kit according to the manufacturer’s specifications. Reaction products were transferred to an uncoated 96-well plate for optical density readings at 415 nm.

2.3.11. Statistical analysis

Two-tailed Student’s t tests were used to determine associations of significance between or within groups where indicated. Observed differences were considered statistically significant for p values < 0.05.

2.4 Results

2.4.1 Identification of serovar Typhimurium mutants deficient for binding to cholesterol but not glass or plastic

The molecular genetic mechanisms mediating initiation of biofilm formation vary with growth media and substrate, and are beginning to be elucidated for microorganisms
such as *E. coli* (Prigent-Combaret et al., 2000; Danese et al., 2000; Pratt and Kolter, 1998), *S. aureus* (Caiazza and O'Toole, 2003; Vaudaux et al., 1995; Gross et al., 2001), *Enterococcus faecalis* (Hufnagel et al., 2004; Toledo-Arana et al., 2001), and *Pseudomonas* spp. (O'Toole and Kolter, 1998b; Ma et al., 2009; Sauer et al., 2002; O'Toole and Kolter, 1998a). As described previously, *S. enterica* serovars Typhimurium, Enteritidis, and Typhi form mature biofilms on cholesterol-coated Eppendorf tubes (Tube Biofilm Assay, TBA) when grown in 3% crude ox bile extract. To identify novel genes regulating binding to cholesterol, a pool of Tn10 d-Tet transposons was introduced into wild-type serovar Typhimurium 14028s by generalized, P22 HT int-105-mediated phage transduction. A total of 40,000 colonies were pooled together to generate a mutant library of random Tn10 d transposon insertions. The mutant pool was grown to log phase in LB broth-Tet with 3% crude ox bile extract and added in 100 μl amounts to siliconized Eppendorf tubes coated with 1 mg each of cholesterol. Following incubation for 24 hours, 10 μl of planktonic, non-adherent culture was removed and added to 90 μl of fresh LB broth-Tet with 3% bile in new cholesterol-coated tubes. The panning for cholesterol binding deficient bacteria was repeated every 24 hours for 10 days and the final supernatant was diluted and plated.

To examine whether these mutations affected biofilm formation on other surfaces, 500 individual colonies were assayed in the presence of bile for binding to cholesterol-coated tubes or to glass or plastic coverslips. We identified 49 strains deficient in biofilm formation on cholesterol-coated tubes but not on glass or plastic surfaces, demonstrating specificity of binding mediated by these mutations. To rule out second-site mutations,
mutants were transduced back into the parent strain and their biofilm forming phenotypes were confirmed.

2.4.2 Sequencing of Tn10d transposon insertion sites

Genomic DNA sequencing from the transposon 5’ end and sequence analyses revealed that the Tn10d insertions mapped to several genes corresponding to serovar Typhimurium LT2 loci \textit{fimW}, \textit{ompC}, \textit{flhA}, \textit{fliF}, \textit{fliA}, \textit{fliJ}, \textit{fliL}, and \textit{sseI} (Table 2.2). Eighteen Tn10d insertion sites were within the \textit{fimW} open reading frame, one at base pair (bp) position 98 (JSG3058) and seventeen others at 125 (JSG3055) within the open reading frame (orf). Colonies recovered with transposon insertions in \textit{fimW} were more numerous than for any other gene recovered in the cholesterol binding screen. FimW negatively regulates production of type 1 fimbriae by interacting with FimY (Saini et al., 2009) and possibly with FimZ (Tinker et al., 2001), two proteins that independently activate the P_{fimA} promoter which controls the expression of the \textit{fim} structural genes (Saini et al., 2009). Serovar Typhimurium chromosomal \textit{fimW} mutants expressed four- to eight-fold more type 1 fimbriae compared to the parent strain (Tinker et al., 2001). Therefore, serovar Typhimurium strains carrying Tn10d insertions in \textit{fimW} would exhibit hyper-fimbriate phenotypes, suggesting that over production of type 1 fimbriae inhibited adherence to cholesterol.

Nine identical Tn10d transposon insertions within \textit{flhA} were located at bp position 1917 (JSG3059). The cytoplasmic membrane protein FlhA mediates type III export of axial components of the \textit{Salmonella} flagellum and the scaffolding proteins for its assembly (Kihara et al., 2001; Saijo-Hamano et al., 2005). All three Tn10d insertions
within the *fliF* open reading frame were located downstream of start at bp position 872 (JSG3060). Structural subunits of the transmembrane protein FliF comprise the membrane-supramembrane ring (MS-ring) and proximal rod of the flagellar basal body of serovar Typhimurium, and have been shown to interact with the chemotactic motor/switch protein FliG (Ueno et al., 1992; Thomas et al., 2001; Thomas et al., 2006). Two Tn10d insertion sites within *fliA* (JSG3061) were located at bp position 532 relative to the start codon. The conserved alternative sigma factor $\sigma^{28}$ encoded by the class 2 regulatory gene *fliA* directs transcription from class 3 promoters of flagellar filament late assembly genes in *E. coli* (Liu and Matsumura, 1995; Chilcott and Hughes, 2000) and serovar Typhimurium (Ohnishi et al., 1990; Chilcott and Hughes, 2000; Frye et al., 2006). The Tn10d insertion in JSG3062 was located in the *fliJ* open reading frame at bp position 142 relative. FliJ is a soluble, cytoplasmic chaperone of the type III flagellar protein apparatus that associates with the transmembrane domain of FlhA and is implicated in export of filament (FliC) and rod/hook substrates (FlgD) (Minamino et al., 2000; Fraser et al., 2003; McMurry et al., 2004). One Tn10d transposon insertion site was located downstream of the *fliL* start site at bp position 252 (JSG3063). The function of the membrane-associated component of the flagellar basal body encoded by *fliL* is relatively unknown (Schoenhals and Macnab, 1999). Recent evidence suggests that FliL prevents rod fracture caused by torsional stress during swarming motility (Attmannspacher et al., 2008).

Genomic DNA sequences revealed insertions in serovar Typhimurium outside of flagellar genes that also contribute to cholesterol binding. Of the fourteen Tn10d insertions within *ompC*, five were located downstream of the start codon at bp position
252 (JSG3064) and nine others were found at position 346 (JSG3073). The outer membrane protein OmpC is a porin influenced differentially by medium osmolarity to allow passage of nutrients and antibiotics between the extra- and intra-cellular milieu, and has been well characterized in *E. coli*, *V. cholerae*, and *Salmonella* (Puente et al., 1991; Toro et al., 1990; Pagel et al., 2007; Yang et al., 2008; Alba and Gross, 2004). One Tn10d insertion site was within *sseI* at bp position 552 relative to the start codon. SseI is an effector protein translocated via the *Salmonella* pathogenicity island 2 (SPI-2) type III secretion system (TTSS) encoded by the lambdoid phage Gifsy-2 (Miao et al., 2003; Ehrbar and Hardt, 2005). Although SseI colocalizes with the polymerizing actin cytoskeleton, no virulence defects were detected during infection of mice with an *sseI* mutant strain of serovar Typhimurium (Miao et al., 2003; Ehrbar and Hardt, 2005; Ho and Slauch, 2001a). The roles of OmpC and SseI in mediating binding of serovar Typhimurium to cholesterol will not be investigated further here, but will be considered in the discussion.

In sum, the majority (34/49, or 70%) of the Tn10d insertions disrupted genes involved in flagellar and type 1 fimbrial biosynthesis and structural components, suggesting that the presence of the serovar Typhimurium flagella mediates binding to cholesterol to initiate biofilm formation whereas over production of fimbriae is unimportant. A schematic of the tranposon insertion sites within fimbrial- and flagellar-related genes found through random mutagenesis is depicted in Fig. 2.1.
2.4.3 Fimbriae production and FimW-mediated over expression of type 1 fimbriae in serovar Typhimurium play negative roles in biofilm formation on cholesterol

Fimbriae have been shown to mediate adherence during biofilm initiation and cell-cell interactions during biofilm growth for a variety of microorganisms (Davey and O'toole, 2000; O'Toole and Kolter, 1998b). The serovar Typhimurium genome encodes 13 putative fimbrial operons, some of which are not expressed in vitro (Nuccio et al., 2007). Type 1 fimbriae have been shown to be important for biofilm formation on HEp-2 tissue culture cells, the murine intestinal epithelium, and chicken intestinal epithelium (Boddicker et al., 2002; Ledeboer and Jones, 2005), but not on human gallstones incubated with bile (Prouty et al., 2002a). A serovar Typhimurium SR11 strain containing mutations in four fimbrial operons (fim, agf, lpf, and pef) was added to the TBA to examine biofilm formation on cholesterol-coated surfaces. In both the presence and absence of bile, the amount of biofilm formed by the quadruple mutant SR11 strain equaled that of wild-type serovar Typhimurium 14028s (Fig. 2.2), suggesting that fimbriae encoded by the fim, agf, lpf, and pef operons do not contribute to biofilm formation in this static assay. To test whether serovar Typhimurium cholesterol binding deficient mutants affecting type 1 fimbriae could form mature biofilms, strains carrying Tn10d transposon insertions in fimW were added to cholesterol-coated Eppendorf tubes in the TBA as described previously (Crawford et al., 2008). As shown in Fig. 2.2, a fimW mutation renders serovar Typhimurium deficient for biofilm formation in the presence and absence of 3% bile, whereas complementation of fimW with pBAD18 restores the amount of biofilm growth to wild-type levels in both media conditions. These results suggest that type 1 fimbriae play a negative role during Salmonella binding to
cholesterol, and that this interference prevents subsequent cholesterol biofilm maturation by the bacterium.

2.4.4 Flagella-mediated binding to cholesterol is necessary for biofilm development in serovar Typhimurium

The highly ordered transcriptional hierarchy controlling expression of flagella is comprised of three classes of genes and is regulated by many global signals (Frye et al., 2006; Karlinsey et al., 2000). Therefore, it is no surprise that flagella contribute differentially to biofilm formation depending on environmental conditions such as binding substrate material, nutrient limitation, temperature, media flow rate, and others factors for enterobacteriacae V. cholerae (Watnick and Kolter, 2000; Watnick et al., 2001; Moorthy and Watnick, 2004; Lauriano et al., 2004) and E. coli (Pratt and Kolter, 1998; Prigent-Combaret et al., 2000; McClaine and Ford, 2002; Landini and Zehnder, 2002), as well as P. aeruginosa (Deziel et al., 2001; O'Toole and Kolter, 1998b; Barken et al., 2008; Merritt et al., 2007). Expression of the serovar Typhimurium flagella has been shown to inhibit biofilm formation on polystyrene wells (Teplitski et al., 2006) and promote biofilm development on human gallstones when bile is added to the growth media (Prouty et al., 2002a; Prouty and Gunn, 2003), but the stage at which this appendage positively or negatively impacts Salmonella biofilms remains undefined. To examine whether production of the flagellar filament is necessary for biofilm formation on cholesterol, a mutation at the apex of transcriptional regulation (flhC) was created in serovar Typhimurium. This mutant strain does not form a mature biofilm on cholesterol,
providing direct evidence for the importance of flagella during biofilm development (Fig. 2.2).

2.4.5 Serovar Typhimurium flagellar motility inhibits biofilm formation on cholesterol surfaces whereas functional chemotaxis appears important

As demonstrated above, serovar Typhimurium mutations in flagella structural and biosynthesis genes affected binding to and biofilm formation on cholesterol. To determine if the physical presence of the flagellar filament or flagella-mediated motility was required for biofilm formation, mutants that maintained flagella but could not engage in swimming or chemotaxis were examined. In the TBA, a serovar Typhimurium motA mutation did not reduce biofilm levels on cholesterol surfaces in the presence or absence of bile compared to the parent strain, suggesting that motility is not critical to serovar Typhimurium biofilm development on cholesterol-coated surfaces (Fig. 2.2).

Chemotactic behavior responds to chemical attractants or deterrents with appropriate movement (Adler, 1975) and is controlled in serovar Typhimurium by the five gene Meche operon (Stock and Stock, 1987). To determine the affects of chemotaxis during biofilm formation on cholesterol, a cheZ serovar Typhimurium mutant strain characterized by an increased tumbling phenotype was created and added to the TBA (Parkinson, 1993). Loss of this critical phosphatase significantly reduced the amount of biofilm formed by the mutant when grown in bile, but did not alter biofilm formation in LB alone (Fig. 2). Bile-mediated tumbling may be an important mechanism that steers planktonic salmonellae away from a detergent-like signal toward an appropriate surface to initiate biofilm development, and protection.
2.4.6 The number of surface-expressed serovar Typhimurium flagella is not regulated by bile

The presence of bile was shown to moderately down regulate serovar Typhimurium flagellar gene expression in β-galactosidase assays using MudJ fusions to flhC, flgC, and fliC (Prouty et al., 2004b). Interestingly, bile is required for mature biofilm formation on cholesterol-coated Eppendorf tubes, and flagella biosynthesis mediates, at least in part, attachment to this surface for a biofilm to develop. To determine if the bile-mediated downregulation of flagellar genes resulted in a loss of flagella, or whether bile altered the expression of flagella at a post-transcriptional level, wild-type and flhC mutant strains of serovar Typhimurium were grown to late exponential phase with or without 3% bile and examined by TEM. The wild-type strain averaged nearly 6 flagella per individual bacteria regardless of growth conditions, whereas no detection of flagella was observed for the flhC mutant (Fig. 2.3). Therefore, while exposure to bile may transcriptionally downregulate flagellar genes, there is no effect of bile on the number of flagella.

2.4.7 Formalin fixed and heat killed salmonellae expressing an intact flagella bind to cholesterol

To test whether dead salmonellae expressing intact flagella could bind to cholesterol in the absence of active metabolism and secreted effectors, wild-type and ΔflhC strains of serovar Typhimurium were fixed in 10% formalin for 40 minutes or heat killed at 65°C for 20 minutes and added to cholesterol-coated wells of a 24-well tissue culture plate. Live and dead wild-type cells bound to cholesterol following a 3 hour
incubation period, and this association was only modestly enhanced by bile (Table 2.3). Strains lacking FlhC production did not bind to cholesterol in any of the tested conditions, further suggesting that the serovar Typhimurium flagellar filament mediates binding to cholesterol in the early stages of biofilm formation.

2.4.8 Dead salmonellae bound to cholesterol provide a scaffold for biofilm formation of live cells

Mathematical approaches and empirical observations are constantly challenging and redefining the developmental model of microbial biofilm formation (Monds and O'Toole, 2009; van Loosdrecht et al., 2002; Picioreanu et al., 2007; Saravanan and Sreekrishnan, 2006). Recent work in *P. aeruginosa* has investigated stage-specific factors including twitching motility, cell-to-cell signaling, stationary phase sigma factor expression, and polysaccharide synthesis locus (PSL) activity that contribute to biofilm development in flow cell systems using statistical analysis and direct visualization (Heydorn et al., 2002; Ma et al., 2009). To determine if flagella-mediated binding could provide a scaffold for serovar Typhimurium biofilm maturation, formalin fixed salmonellae were incubated in cholesterol-coated Eppendorf tubes for 24 hours and washed vigorously with 1 x PBS. A late logarithmic culture of wild-type serovar Typhimurium grown in 3% bile was added atop the bound cells and the standard 6 day TBA was performed. Formalin fixed salmonellae, while shown above to bind to cholesterol at 3 hours, did not progress to a mature biofilm in the TBA (Table 2.4). Interestingly, killed, bound cells were able to support biofilm formation of live wild-type
serovar Typhimurium, and the amount of these biofilms were higher than for salmonellae grown in the TBA without this scaffold (Table 2.4).

To test whether the presence of flagella contributed to later events during biofilm development, a live serovar Typhimurium \( flhC \) mutant culture was added to the scaffold of bound, dead wild-type cells and a TBA was performed. Loss of flagella in an \( flhC \) mutant, shown to alleviate biofilm formation on cholesterol-coated surfaces (Fig. 2.2A and B), did not affect biofilm formation on a substrate of dead cells, suggesting that the presence of serovar Typhimurium flagellar filament, while necessary for binding to cholesterol, does not contribute to subsequent biofilm development (Table 2.4). Furthermore, the amount of biofilm formed by an \( flhC \) mutant on dead cells significantly exceeded that of the wild-type strain in the same condition, suggesting that flagella may be inhibitory to biofilm growth (Table 2.4).

Bacterial biofilms are characterized by the production of a self-initiated extracellular matrix (ECM), and we have previously demonstrated that the O-antigen (O-ag) capsule is a bile-induced exopolysaccharide necessary for \( Salmonella \) biofilm formation on cholesterol surfaces (Crawford et al., 2008). Interestingly, a live serovar Typhimurium \( yihO \) mutant strain deficient for O-ag capsule production did not form a mature biofilm on a bound, dead scaffold of wild-type cells (Table 2.4). These results suggest that biofilm development will not progress without ECM production, and that the O-ag capsule is a critical component.
2.4.9 The serovar Typhimurium flagellar subunit FliC is critical for binding to cholesterol

The flagellar filament of *S. enterica* is approximately 10 μm long and is comprised of two antigenically distinct flagellin proteins, FliC (H:i) and FljB (H:i,2) (de Vries et al., 1998; Chilcott and Hughes, 2000). During the well characterized process of phase variation, these subunits are alternatively expressed by a mechanism of posttranscriptional control (Silverman et al., 1979; Bonifield and Hughes, 2003; Aldridge et al., 2006). To determine which subunit protein was mediating binding of the serovar Typhimurium flagellar filament to cholesterol, antibody to *Salmonella* whole flagella and FliC was used in a quantitative binding ELISA as described above. Briefly, flagella were isolated and purified from serovar Typhimurium wild-type, ΔfliC, ΔfljB, and ΔfliCfljB strains using a protocol of mechanical shearing adapted from Andersen-Nissen et al. (Andersen-Nissen et al., 2007). Wild-type and phase-locked mutant flagellin preparations (1 μg each) were separated by 10% SDS-PAGE gel electrophoresis. Amino acid sequences of FliC and FljB predicted 51.6 and 52.5 kDa proteins, respectively (Uchiya and Nikai, 2008), and this approximate 1 kb difference was detected by Coomassie staining (Fig. 2.4A) and Western blotting with monoclonal anti-flagellin antibodies (Fig. 2.4B) that recognized either a peptide common to FliC and FljB, or specifically FliC. The identity of each band was confirmed by comparing wild-type with mutant lanes (Fig. 2.4A and B). Purified proteins from all samples were added to cholesterol-coated wells of a 96-well tissue culture plate and analyzed by a modified ELISA. Flagella from wild-type and ΔfljB strains bound to cholesterol in equal amounts, and these levels were significantly higher than those observed for a fliC mutant,
suggesting that FliC is the critical serovar Typhimurium flagellar subunit mediating binding to cholesterol (Fig. 2.5).

2.5 Discussion

Approximately 5% of patients suffering from typhoid fever carry *Salmonella* in the gallbladder (Levine et al., 1982), and evidence indicates a high correlation between those individuals who become carriers and those who have gallstones (Dutta et al., 2000; Lai et al., 1992). Because antibiotic treatments are ineffective at removing chronic infections in such cases (Lai et al., 1992), we hypothesize that the asymptomatic existence of serovar Typhi is frequently if not always a result of biofilm formation on cholesterol gallstone surfaces. In this work we show that the flagellar filament, and specifically the FliC subunit, of serovar Typhimurium is necessary for and specific toward cholesterol binding during the initiation of biofilm formation.

Bacteria express a diverse arsenal of factors during biofilm development that are uniquely required depending on environmental stimuli such as growth media and substrate hydrophobicity and charge. It was previously shown that *S. enterica* serovars Typhi, Typhimurium, and Enteritidis formed bile-induced biofilms on human cholesterol gallstones and cholesterol-coated Eppendorf tubes in vitro (Prouty et al., 2002a; Crawford et al., 2008). To determine the critical genes of early biofilm events, specifically those involved in attachment, a randomly mutagenized serovar Typhimurium strain was screened in the TBA for loss of adherence. Genomic DNA from mutants that did not bind to cholesterol but retained biofilm formation on glass and plastic surfaces were sequenced to locate the Tn10d insertion site. Disruptions in genes affecting type 1
fimbriae and flagella structure and biogenesis were most frequently recovered, and \textit{fimW} was prevalent.

\textit{Tn}10\textit{d} insertions were also recovered in genes encoding an outer membrane protein (\textit{ompC}) and a translocated effector of the SPI-2 TTSS (\textit{sseI}). The OmpC porin is regulated by osmolarity, permits passive diffusion of nutrients and antibiotics between the extra- and intra-cellular environments, and mediates intestinal colonization of serovar Typhimurium in chickens and adherence of serovar Typhimurium to macrophages (Puente et al., 1991; Methner et al., 2004; Negm and Pistole, 1999). Bacteria within biofilms are thought to encounter higher osmolarity than in the liquid phase (Prigent-Combaret et al., 1999), and preliminary experiments performed in our laboratory indicate that loss of OmpC causes a decrease in serovar Typhimurium biofilm formation in conditions of high osmolarity (3\% bile, LB + 0.3 M NaCl) but leads to increased biofilm growth compared to the wild-type strain in low media osmolarity (LB + 0.05 M NaCl) (data not shown). These results suggest that OmpC is necessary for binding and biofilm formation in the presence of bile, possibly by mediating electrostatic interactions between salmonellae and the cholesterol surface, promoting overall biofilm health as a nutrient channel, or regulating cell-cell interactions as is the case in \textit{E. coli} biofilms.

\textit{SseI} is a secreted effector protein belonging to the GDSL family of lipases that colocalizes with the polymerizing actin cytoskeleton (Miao et al., 2003). SPI-1 has been implicated in directing cholesterol distribution during mammalian cell entry (Garner et al., 2002), and GDSL proteins associated with SPI-2 have been shown to hydrolyse phospholipids and esterify cholesterol intracellularly (Nawabi et al., 2008; Buckley et al., 1984). The lambdoid phage Gifsy-2 encodes \textit{SseI} (Miao et al., 2003; Ehrbar and Hardt,
2005), and the receptor for Gifsy-2 is OmpC (Ho and Slauch, 2001b). Although the Gifsy bacteriophages and SPI-2 secreted effectors are generally virulence regulators of serovar Typhimurium in mice, the association between SseI and OmpC, and the known interaction of SseI with cholesterol, suggests a role for adherence to/modification of cholesterol during biofilm formation.

Fimbriae are attributed, in part, to serovar Typhimurium biofilm formation on HEp-2 tissue culture cells and the intestinal epithelium of mice and chickens (Boddicker et al., 2002; Ledeboer and Jones, 2005; Baumler et al., 1996). In studies by Prouty et al., a serovar Typhimurium SR11 strain containing mutations in four fimbrial operons was observed to adhere and form biofilms on cholesterol gallstones in a manner similar to wild-type serovar Typhimurium (Prouty et al., 2002a). Similarly, the amount of biofilm formed by the quadruple mutant SR11 strain equaled that of wild-type serovar Typhimurium in both the presence and absence of bile in the TBA, suggesting that fimbriae encoded by the \textit{fim}, \textit{agf}, \textit{lpf}, and \textit{pef} operons do not drastically contribute to biofilm formation on cholesterol-coated surfaces.

FimW has recently been shown to downregulate production of type 1 fimbriae by inhibiting FimY, a protein that enhances expression of the \textit{fim} structural genes by activating the \textit{fimA} promoter (Saini et al., 2009), and chromosomal \textit{fimW} mutants expressed four- to eight-fold more type 1 fimbriae compared to the parent strain (Tinker et al., 2001). Therefore, frequent recovery of serovar Typhimurium colonies carrying \textit{Tn10d} insertions in \textit{fimW} during a screen for adherence mutants suggests that over production of type 1 fimbriae is inhibitory to cholesterol binding. To test whether a serovar Typhimurium mutant deficient for attachment to cholesterol could form mature
biofilms, a strain carrying a Tn10d transposon insertion in fimW was added to cholesterol-coated Eppendorf tubes in the TBA and shown to significantly effect biofilm formation. Complementation of fimW with pBAD18 restored the amount of biofilm growth to wild-type levels. These results suggest that over expression of type 1 fimbriae play a negative role during *Salmonella* binding to cholesterol and subsequent biofilm formation by the bacterium.

Flagella promote surface binding during biofilm formation (Pratt and Kolter, 1998; O'Toole and Kolter, 1998b) as well as adhesion during colonization of tissue cultured cells and mucus by *P. aeruginosa* (Rudner et al., 1992; Lillehoj et al., 2002), *Campylobacter jejuni* (McSweegan and Walker, 1986), *E. coli* (Giron et al., 2002), and *S. enterica* spp. (Dibb-Fuller et al., 1999; La Ragione et al., 2003; Budiarti et al., 1991; Allen-Vercoe and Woodward, 1999). Flagella biogenesis is controlled by the FlhDC master operon (Frye et al., 2006) and has been shown to regulate biofilm formation of *Yersinia pseudotuberculosis* (Wang et al., 2007). To examine whether the physical presence of the flagellar filament is necessary for biofilm formation on cholesterol, a serovar Typhimurium *flhC* mutant was examined in the TBA and shown not to form a mature biofilm on cholesterol. These results provide direct evidence for the importance of flagella during biofilm development.

Flagellar-mediated motility and chemotaxis differentially contribute to colonization and biofilm formation depending on environmental cues (O'Toole and Kolter, 1998b; Pratt and Kolter, 1998; Prouty and Gunn, 2003; Huber et al., 2002; Mey et al., 2005). Bile, for example, increases motility and is a chemical attractant at physiological concentrations for *V. cholerae* (Butler and Camilli, 2005), acts as a
repellent for *Helicobacter pylori* (Worku et al., 2004), and enhances tumbling frequency while downregulating motility in *S. enterica* (Prouty et al., 2004b). Serovar Typhimurium mutant strains lacking motor function (*motA*) and control of chemotactic behavior (*cheZ*) were analyzed for biofilm formation in the TBA. Loss of the CheZ phosphatase significantly decreased biofilm formation in LB broth with bile, whereas neither *motA* or *cheZ* affected biofilm development in LB broth. These results indicate that flagellar motility is dispensable to biofilm formation on cholesterol surfaces, but regulation of tumbling in the presence of bile is an important factor.

Bile has also been shown to affect transcription of serovar Typhimurium flagella structural genes *flhC*, *flgC*, and *fliC* (Prouty et al., 2004b). We report here that wild-type salmonellae grown with or without bile and visualized by TEM express equal amounts of flagella, suggesting that prolonged exposure to bile does not alter the ultimate production of flagella. Thus, reductions in the levels of *flhC*, *flgC*, and *fliC* transcription in bile, while slightly below those observed in the absence of bile, are still above the threshold necessary for normal flagella production in this condition and allow flagella-mediated binding to cholesterol.

Flagella are necessary for the initial stages of biofilm development on a variety of surfaces (Pratt and Kolter, 1998; O'Toole and Kolter, 1998b) and colonization of tissue cultured cells and mucus by *P. aeruginosa* (Rudner et al., 1992; Lillehoj et al., 2002), *Campylobacter jejuni* (McSweegan and Walker, 1986), *E. coli* (Giron et al., 2002), and *S. enterica* spp. (Dibb-Fuller et al., 1999; La Ragione et al., 2003; Budiarti et al., 1991; Allen-Vercoe and Woodward, 1999). To determine whether dead cultures of serovar Typhimurium could bind to cholesterol in the absence of secreted factors, formalin fixed
or heat killed wild-type and \textit{flhC} mutant strains were added to cholesterol-coated wells of 24 well plates and incubated for 3 hours. The results of these experiments indicate that surface expression of intact flagella is sufficient and necessary to mediate binding to cholesterol. Interestingly, bound and inactivated cells provided a scaffold for biofilm formation of live, wild-type cells in the TBA, and the amount of these biofilms exceeded those of salmonellae grown in the TBA without this scaffold. To elucidate factors important for microcolony growth and recruitment, serovar Typhimurium flagella (\textit{FlhC}) or O-antigen capsule (\textit{YihO}) mutants were added to the layer of adherent, dead cells and examined for biofilm development. The \textit{flhC} mutant showed an increase in biofilm formation on a substrate of bound bacteria, suggesting that the flagellar filament, while critical for initial binding to and biofilm formation on cholesterol, does not mediate subsequent biofilm growth following attachment. The live serovar Typhimurium \textit{yihO} mutant did not form a mature biofilm when tested under the same conditions, indicating that O-antigen capsule production is a mandatory component of developing bile-induced biofilms.

The flagellar filament of \textit{S. enterica} is comprised of two antigenically distinct subunit proteins, FliC (H:i) and FljB (H:1,2), and is a pathogen-associated molecular pattern detected by mammalian cells via surface-expressed toll-like receptor 5 and cytosolic Nod-like receptors (Rochon and Romling, 2006; Simon and Samuel, 2007). Secretion of interleukin-1 beta by mouse cell lines is activated comparably by the FliC and FljB flagellins (Simon and Samuel, 2008), but memory CD4+-T-cells preferentially recognize FliC during adaptive immunity and \textit{Salmonella} committed to expressing only FljB is attenuated in vivo (Bergman et al., 2005; Ikeda et al., 2001). To determine which
flagellar subunit mediated adherence to cholesterol, ELISA was performed using anti-flagella or anti-FliC antibodies against purified flagella proteins from serovar Typhimurium wild-type, *fliC*, *fljB*, and *fliC fljB* mutant strains bound to cholesterol-coated wells. The FliC subunit was shown to be a critical factor mediating attachment to cholesterol. Interestingly, *S. Typhi* is monophasic, harboring only the *fliC* gene (Baker et al., 2007), and FliC expression was anatomically restricted to certain tissues during systemic serovar Typhimurium mouse infections (Cummings et al., 2006).

In this work, we have shown that expression of the serovar Typhimurium flagellar filament mediates bile-induced adherence to cholesterol surfaces primarily with the FliC subunit to initiate biofilm formation, but is inhibitory to subsequent biofilm development. It could be hypothesized that FliC production in the human gallbladder may be an evolutionary adapted mechanism facilitating binding to cholesterol gallstones or the gallbladder epithelium and biofilm formation during the asymptomatic, chronic carrier state. This new understanding of how bile regulates early events during *Salmonella* biofilm formation on cholesterol-coated surfaces via flagella provides a therapeutic target with the potential for alleviating asymptomatic gallbladder carriage of *S. Typhi* and thus the human-to-human transmission that makes this pathogen a global concern.
<table>
<thead>
<tr>
<th>Strain</th>
<th>Characteristic(s)</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. Typhimurium</td>
<td></td>
<td></td>
</tr>
<tr>
<td>JSG210</td>
<td>ATCC 14208s (CDC6516-60); wild-type</td>
<td>ATCC</td>
</tr>
<tr>
<td>JSG526</td>
<td>flhC98::Tn10 (TH2934)</td>
<td>Gift of Hughes</td>
</tr>
<tr>
<td>JSG1174</td>
<td>Δ(fim-aph-11::Tn10)-391 lpfC::Kan agfB::Cam pefC::Tet</td>
<td>Gift of Baumler</td>
</tr>
<tr>
<td>JSG1178</td>
<td>Hin108::Tn10d-Cam</td>
<td>Gift of Cookson</td>
</tr>
<tr>
<td>JSG1179</td>
<td>fliC::Tn10</td>
<td>Gift of Cookson</td>
</tr>
<tr>
<td>JSG1190</td>
<td>Hin108::Tn10d-Cam fliC::Tn10</td>
<td>Gift of Cookson</td>
</tr>
<tr>
<td>JSG1547</td>
<td>motA595::Tn10</td>
<td>Gift of T. Lino</td>
</tr>
<tr>
<td>JSG2897</td>
<td>yihO unmarked deletion</td>
<td>Crawford</td>
</tr>
<tr>
<td>JSG2953</td>
<td>fimZ::Kan</td>
<td>Gift of S. Clegg</td>
</tr>
<tr>
<td>JSG2954</td>
<td>JSG2953 complemented with fimZ</td>
<td>Gift of S. Clegg</td>
</tr>
<tr>
<td>JSG3024</td>
<td>cheZ::Tn10</td>
<td>Gift of B. Ahmer</td>
</tr>
<tr>
<td>JSG3055</td>
<td>fimW::Tn10d-Tet; Site 1, 17 insertions</td>
<td>This study</td>
</tr>
<tr>
<td>JSG3056</td>
<td>JSG3055 complemented with fimW</td>
<td>This study</td>
</tr>
<tr>
<td>JSG3057</td>
<td>fimW::Tn10d-Tet fimZ::Kan</td>
<td>This study</td>
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<tr>
<td>JSG3058</td>
<td>fimW::Tn10d-Tet; Site 2, 1 insertion</td>
<td>This study</td>
</tr>
<tr>
<td>JSG3059</td>
<td>flhA::Tn10d-Tet; 9 insertions</td>
<td>This study</td>
</tr>
<tr>
<td>JSG3060</td>
<td>fliF::Tn10d-Tet; 3 insertions</td>
<td>This study</td>
</tr>
<tr>
<td>JSG3061</td>
<td>fliA::Tn10d-Tet; 2 insertions</td>
<td>This study</td>
</tr>
<tr>
<td>JSG3062</td>
<td>fliJ::Tn10d-Tet; 1 insertion</td>
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</tr>
<tr>
<td>JSG3063</td>
<td>fliL::Tn10d-Tet; 1 insertion</td>
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</tr>
<tr>
<td>JSG3064</td>
<td>ompC::Tn10d-Tet; 5 insertions</td>
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</tr>
<tr>
<td>JSG3065</td>
<td>sseI::Tn10d-Tet; 1 insertion</td>
<td>This study</td>
</tr>
<tr>
<td>JSG3073</td>
<td>ompC::Tn10d-Tet; 9 insertions</td>
<td>This study</td>
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</table>

Table 2.1: Bacterial strains and relevant characteristics
<table>
<thead>
<tr>
<th>Gene</th>
<th>No. Colonies</th>
<th>No. Insertions</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>fimW</td>
<td>18</td>
<td>2</td>
<td>Inhibitor of type 1 fimbriae regulator FimZ</td>
</tr>
<tr>
<td>ompC</td>
<td>14</td>
<td>2</td>
<td>Outer membrane protein; passive diffusion of ions, hydrophilic solutes</td>
</tr>
<tr>
<td>flhA</td>
<td>9</td>
<td>1</td>
<td>Flagellar export apparatus, biosynthesis membrane protein</td>
</tr>
<tr>
<td>flIF</td>
<td>3</td>
<td>1</td>
<td>Flagellar cytoplasmic anchor MS-ring protein</td>
</tr>
<tr>
<td>flIA</td>
<td>2</td>
<td>1</td>
<td>Flagellar biosynthesis sigma factor</td>
</tr>
<tr>
<td>flIJ</td>
<td>1</td>
<td>1</td>
<td>Rod/hook and filament biosynthesis chaperone</td>
</tr>
<tr>
<td>flIL</td>
<td>1</td>
<td>1</td>
<td>Basal body protein; stability of MotAB complexes of MS-ring</td>
</tr>
<tr>
<td>ssel</td>
<td>1</td>
<td>1</td>
<td>Secreted effector; colocalizes with host polymerizing actin cytoskeleton</td>
</tr>
</tbody>
</table>

Table 2.2: Transposon insertion sites, frequencies, and functions
Strain and growth condition | Live | Dead
--- | --- | ---
Serovar Typhimurium, LB | + | +
Serovar Typhimurium, LB + 3% Bile | + | +
Serovar Typhimurium *flhC* mutant, LB | - | -
Serovar Typhimurium *flhC* mutant, LB + 3% Bile | - | -

Table 2.3: Binding quantification assay. Three hour binding quantification assay on cholesterol-coated wells of live or dead serovar Typhimurium wild-type and *flhC* mutant strains. Crystal violet stained material was extracted with acetic acid, and optical density at 570 nm for quantification. +, binding amounts equivalent to wild-type in appropriate condition; -, severe defect in or complete loss of adherence (0 to 10% of wild-type values). Shown is a representative experiment performed in triplicate (n = 3).
<table>
<thead>
<tr>
<th>Organism grown in 3% bile</th>
<th>Biofilm development on *</th>
<th>Cholesterol</th>
<th>Dead Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serovar Typhimurium</td>
<td></td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>Serovar Typhimurium <em>flhC</em> mutant</td>
<td></td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td>Serovar Typhimurium <em>yihO</em> mutant</td>
<td></td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 2.4: Summary of biofilm formation on cholesterol or formalin fixed bacteria. *Biofilms grown on cholesterol-coated Eppendorf tubes or a substrate of inactivated, bound cells in the TBA. Dye was extracted with acetic acid, and optical density at 570 nm for quantification. +, robust, mature biofilm formation at wild type levels; ++, increased biofilm present (1.5 – 2.0-fold higher than wild-type values); -, severe defect in or complete loss of biofilm formation (0 to 10% of wild-type values). These data correspond to 3 independent experiments done in triplicate.
Figure 2.1: Schematic of Tn10d transposon insertion sites in serovar Typhimurium cholesterol-binding mutants affecting type 1 fimbriae and flagella structure and biogenesis. Arrows indicate the size and orientation of neighboring genes and triangles depict the location of each transposon insertion.
Figure 2.2: Biofilm formation of serovar Typhimurium strains grown with and without 3% crude ox bile extract on cholesterol-coated Eppendorf tubes. Crystal violet-stained TBA biofilms were extracted with acetic acid, and absorbance was measured at 570 nm. Data, presented as means ± SD, are a representative experiment done in triplicate (n = 3). *, statistically significant reduction in biofilm formation (P < 0.005) based on a two-tailed Student t test. OD570, optical density at 570 nm.
Figure 2.3: Expression of flagella on the surface of serovar Typhimurium strains grown in the presence or absence of bile visualized by negative staining under transmission electron microscopy. (A) Serovar Typhimurium flhC mutant. (B) Serovar Typhimurium flhC mutant + 3% bile. (C) Serovar Typhimurium wild-type. (D) Serovar Typhimurium wild-type + 3% bile. Shown are micrographs representative of 3 independent experiments.
Figure 2.4: Identification of flagellin proteins from wild-type and mutant serovar Typhimurium strains. Flagella were isolated from late log phase bacterial cultures using mechanical shearing, purified, separated by 10% SDS-PAGE gel electrophoresis, and analyzed by GelCode Blue staining (A) and Western blotting (B). Detection with anti-flagella (B, top panel) and anti-FliC monoclonal antibodies (B, lower panel) are shown. Lanes: 1, wild-type serovar Typhimurium; 2, \textit{fliC} mutant strain; 3, \textit{fljB} mutant strain; 4, \textit{fliC fljB} double mutant strain. FliC; 51.6 kD, FljB; 52.5 kD. The stain and blot is representative of at least 3 independent experiments.
Figure 2.5: Expression of purified flagella proteins from serovar Typhimurium wild-type, \textit{fliC}, \textit{fljB}, and \textit{fliC fljB} mutant strains adhered to cholesterol-coated wells. ELISA was performed using anti-flagella or anti-FliC monoclonal antibodies and binding to cholesterol was quantified by measuring bound HRP substrate at optical density 415 nm. Means + SD are reported for an independent experiment done in triplicate (n = 3). Statistical significance for reduction in cholesterol binding based on a two-tailed Student \textit{t} test, *; \(P < 0.05\), **; \(P < 0.005\).
Chapter 3

Identification of a bile-induced exopolysaccharide required for Salmonella Biofilm formation on gallstone surfaces

3.1 Abstract

Salmonella enterica serovar Typhi can establish a chronic, asymptomatic infection of the human gallbladder, suggesting that this bacterium utilizes novel mechanisms to mediate enhanced colonization and persistence in a bile-rich environment. Gallstones are one of the most important risk factors for developing carriage, and we have previously demonstrated that salmonellae form biofilms on human gallstones in vitro. Thus, we hypothesize that bile-induced biofilms on gallstone surfaces promote gallbladder colonization and maintenance of the carrier state. A colanic acid/cellulose S. enterica serovar Typhimurium double mutant formed a mature biofilm on gallstones in a test tube assay and in a new, gallstone-independent assay using cholesterol-coated Eppendorf tubes. These data suggest the presence of an unidentified exopolysaccharide necessary for mature biofilm development and demonstrate specific binding affinity between salmonellae and cholesterol. Our experiments indicate that the Salmonella O-antigen capsule (yihU-yshA and yihV-yihW) is a crucial determinant in gallstone and cholesterol biofilms but that expression of this exopolysaccharide is not necessary for
binding to glass or plastic. Real-time PCR revealed that growth in bile resulted in upregulation of the O-antigen capsule-encoding operon in an agfD-independent manner. Thus, the O-antigen capsule genes are bile induced, and the capsule produced by the enzymes of this operon is specifically required for biofilm formation on cholesterol gallstones. These studies provide new therapeutic targets for preventing asymptomatic serovar Typhi gallbladder carriage.

3.2 Introduction

Salmonellae remain pathogens of worldwide concern, particularly in nonindustrialized societies with inefficient food and water sanitation systems. The diverse Salmonella enterica serovars infect a broad range of hosts and are increasingly resistant to commonly administered antibiotics (Boyle et al., 2007). Gastroenteritis and enteric (typhoid) fever are the two most prevalent clinical manifestations, both of which cause significant morbidity and mortality.

Salmonella enterica serovar Typhi is the etiologic agent of typhoid fever in humans, a systemic illness afflicting approximately 20 million people globally each year (Crump et al., 2004). Bacteria reaching the gallbladder can induce an active local infection (cholecystitis) or exist asymptptomatically in a chronic carrier state. Nearly 5% of patients suffering from typhoid fever become Salmonella carriers (Levine et al., 1982). This condition is frequently associated with abnormalities of the gallbladder, including gallstones, and Salmonella carriage is the largest risk factor for cancer of this organ (Dutta et al., 2000; Kumar et al., 2006; Lai et al., 1992).
During colonization of the intestine and gallbladder, *Salmonella* spp. interact with bile, a complex, lipid-rich, and protein poor digestive secretion produced by the liver at nearly 1 liter per day (Boyle et al., 2007). Bile consists of many components, including bile acids, cholesterol, phospholipids, and bilirubin, and serves as a potent antimicrobial agent in the gastrointestinal tract (Hofmann, 1999). Salmonellae, however, resist its surface-acting, amphipathic, detergent-like properties (van Velkinburgh and Gunn, 1999), indicating that bile resistance is an important phenotype associated with *Salmonella* pathogenesis during both acute and chronic infections. The presence of bile is also known to cause pleiotropic responses that affect production of virulence factors (SPI-I-mediated type III secretion, motility, adhesion, antibiotic and bile resistance, efflux pump expression, and biofilm formation) in *S. enterica* (Prouty and Gunn, 2000; Prouty et al., 2004b) and in enteric commensals and pathogens, including *Vibrio cholerae* (Bina and Mekalanos, 2001; Chatterjee et al., 2007; Hung and Mekalanos, 2005; Hung et al., 2006)(Provenzano et al., 2000; Schuhmacher and Klose, 1999; Wibbenmeyer et al., 2002), *Campylobacter jejuni* (Lin et al., 2005b), *Escherichia coli* (Torres et al., 2007), and *Listeria monocytogenes* (Sleator et al., 2005).

The main site of human serovar Typhi carriage is the bile-rich gallbladder, suggesting unique or enhanced bile resistance mechanisms utilized by the bacterium that lead to survival in this environment. The formation of biofilms on gallstones has been hypothesized to be a facilitator for enhanced colonization of and persistence in this organ. A biofilm is defined as a community of microorganisms that adhere to each other and to a biotic or abiotic surface, resulting in stability and protection from antibiotics and other environmental factors mediated in part by a self-initiated exopolysaccharide (EPS).
matrix. Bacterial biofilms have been implicated as the causes of many chronic infections in humans and are associated with medical and industrial ecosystems (Costerton et al., 1995). Planktonic cells from this sessile, matrix-bound population are continuously shed, which can result in systemic infection or release of the organism into the environment. Bacteria shed by asymptomatic carriers contaminate food and water and account for much of the person-to-person transmission of serovar Typhi in underdeveloped countries.

As mentioned above, it has been demonstrated that patients with gallbladder abnormalities, such as gallstones, are at a much higher risk for serovar Typhi carriage, yet the progression of infection to the carrier state is undefined. Antibiotic regimens can be ineffective in carriers with gallstones (Lai et al., 1992), and the cost of surgery to remove gallbladders is prohibitive in developing countries where serovar Typhi is prevalent. Interestingly, bile has been shown to promote biofilm formation of *S. enterica* serovar Typhimurium and serovar Typhi in an in vitro human gallstone test tube assay (Prouty et al., 2002a). To date, the EPS matrix of *Salmonella* bile-induced biofilms on gallstones remains poorly characterized. A better understanding of the key EPS constituents of *Salmonella* bile-induced biofilms promises to better characterize the asymptomatic carrier state and elucidate important therapeutic targets for alleviating biofilm formation in chronic cases. Here, we define a bile-induced EPS, the O-antigen (O-ag) capsule, required for biofilm formation specifically on gallstone and cholesterol-coated surfaces.
3.3 Materials and Methods

3.3.1 Bacterial strains and growth conditions

The *Salmonella* strains and plasmids used in this study are listed in Table 3.1. Luria-Bertani (LB) broth and agar were used for bacterial growth, creation of mutants, biofilm assays, and expression studies. When necessary, antibiotics were added at appropriate concentrations: ampicillin, 50 µg/ml; kanamycin, 25 µg/ml; and tetracycline, 15 µg/ml.

3.3.2 Molecular biology and genetic techniques

DNA purification and PCR were completed according to established procedures (using a QIAprep spin mini prep kit) (1989). Plasmids were transformed by electroporation as previously described (Schmid and Roth, 1983). Quantitative real-time PCR was performed using RNA extracted from log-phase (optical density of 0.6 at 600 nm) *Salmonella* cultures with an RNeasy kit (Qiagen, Valencia, CA). Samples grown in 3% crude ox bile extract (Sigma, St. Louis, MO) were quickly washed three times in 1x phosphate-buffered saline (PBS) before extraction. RNA quality and quantity were measured with the Experion automated electrophoresis system (Bio-Rad, Hercules, CA), and 1 microgram was reverse transcribed to cDNA with Superscript II RNase H− reverse transcriptase (Invitrogen, Carlsbad, CA). Five nanograms of converted cDNA was added to Sybr green PCR master mix for quantitative real-time PCR with a Bio-Rad iCycler apparatus. Table 3.2 shows the primers used for expression of the target gene *yihU* and the housekeeping gene *dnaN*. Relative copy numbers were calculated according to a published protocol (Gavrilin et al., 2006).
3.3.3 Construction of Δ\textit{bcsE ΔwcaA}, \textit{yihO}, and \textit{yihP} mutants

The serovar Typhimurium cellulose/colanic acid double mutant (\textit{ΔbcsE ΔwcaA}) was created by P22 HT105 \textit{int-102}-mediated, generalized transduction between strains carrying single mutations in \textit{bcsE} or \textit{wcaA} (Schmid and Roth, 1983). The lambda Red-mediated mutagenesis method of gene disruption was used to create a deletion of \textit{yihO} in serovar Typhimurium and of \textit{yihP} in serovar Typhi (Datsenko and Wanner, 2000). Primer pairs JG1281/JG1282 and JG1643/JG1644 (Table 3.2) were designed to amplify \textit{yihO’-kan-’yihO} and \textit{yihP’-kan-’yihP}, respectively, using pKD4 as a template. The kanamycin cassettes and surrounding regions of homology were exchanged into the chromosome of serovar Typhimurium or serovar Typhi, generating strains carrying \textit{ΔyihO::kan} in serovar Typhimurium and \textit{ΔyihP::kan} in serovar Typhi. Correct placement of these constructs in the \textit{Salmonella} genome was confirmed by PCR, using primers JG1283 and JG1284 for serovar Typhimurium and primers JG1647 and JG1648 for serovar Typhi. The kanamycin cassettes were then resolved by introducing pCP20, and the resulting strains were named JSG2897 (carrying \textit{ΔyihO}) and JSG2950 (carrying \textit{ΔyihP}) (Table 3.1). For complementation, the \textit{yihO} gene from serovar Typhimurium was amplified and cloned into the HindIII and EcoRI sites of vector pUC18 (GenScript Corporation, Piscataway, NJ) and expressed from the \textit{lac} promoter. The resulting construct was introduced into JSG2897 by electrotransformation.
3.3.4 Gallstone tube assay

Uniform gallstone samples composed primarily of cholesterol and retrieved from a single patient were used in this study. *Salmonella* strains were grown overnight in LB broth with or without 3% crude ox bile extract. Each overnight culture was diluted 1:10, added to LB broth in test tubes containing human gallstones (with or without bile), and incubated in a rotating drum at 37°C with aeration. To attempt to mimic normal gallbladder physiology, the medium and bacteria were removed from the tubes every 24 hours, the gallstones were washed three times in LB by light vortexing, and fresh medium (with or without bile) was returned. The optimal growth period was 12 days, as described previously (Prouty et al., 2002a).

3.3.5 Tube biofilm assay (TBA)

Chromatography-grade cholesterol (product number C8667; Sigma, St. Louis, MO) was dissolved in anhydrous ether (J. T. Baker, Phillipsburg, NJ) at a concentration of 10 mg/ml and delivered in 100 µl aliquots to siliconized Eppendorf tubes (Fisher Scientific, Pittsburgh, PA). The ether was allowed to evaporate, consistently leaving tubes with a 1 mg coating of cholesterol to which 100 µl of bacterial culture could be added. *Salmonella* strains were grown overnight in LB broth with or without 3% crude ox bile extract, diluted 1:100 the following day, and subsequently grown to an optical density at 600 nm of 0.6. These cultures (100 µl) were incubated in cholesterol-coated Eppendorf tubes at room temperature on a Nutator shaker (Labnet International, Edison, NJ). Every 24 hours, the medium was removed, the tubes were washed three times with
LB, and fresh medium (LB with or without 3% bile) was added. Each strain was examined in triplicate, and the experiment proceeded for a period of 6 days.

3.3.6 Plastic/glass binding assay

Round, Chromerge-cleaned glass coverslips were placed in plastic 24-well plates (Fisher Scientific, Pittsburgh, PA). Liquid cultures of *S. enterica* diluted 1:100 following overnight growth were allowed to reach an optical density at 600 nm of 0.6 in the presence or absence of 3% crude ox bile extract and then added to each coverslip-containing well in 2 ml volumes and incubated at room temperature on a Nutator shaker. The assay continued for 6 days, with removal of medium, washing, and addition of fresh medium occurring every 24 hours. Each strain was tested with triplicate wells. Biofilms on the plastic 24-well plates and on the glass coverslips were examined as described below.

3.3.7 Crystal violet quantification of biofilms

Following bacterial growth on cholesterol-coated tubes in the TBA or on glass/plastic in binding assays, samples were washed three times in LB and incubated at 60°C for 1 hour to fix the cells. A solution of 0.1% crystal violet (gentian violet in isopropanol-methanol-1x PBS [1:1:18]) was then added to stain cells for 5 minutes at room temperature. Specimens were washed thoroughly with 1x PBS until the liquid ran clear. The dye was extracted using 33% acetic acid and then quantified with optical density readings at 570 nm to determine the amount of dye retained by the biofilm cells.
3.3.8 Immunofluorescence microscopy of tube biofilms

After completion of the TBA as described above, cells were visualized using immunofluorescence microscopy for the production of O-ag capsule EPS in the presence or absence of 3% crude ox bile extract. Three vigorous washes with 1x PBS were followed by 4 hours of incubation with 5% bovine serum albumin (Sigma, St. Louis, MO). Cells were washed and then exposed to capsule-specific primary antibody (diluted 1:2,000 in PBS) at room temperature for 2 hours. This polyclonal antibody was generated in rabbits and showed no cross-reactivity with commercially purified lipopolysaccharide (LPS) from *S. enterica* serovar Enteritidis (Gibson et al., 2006). The tube contents were washed three additional times and incubated with a 1:5,000 dilution of fluorescein-conjugated, goat affinity-purified antibody to rabbit immunoglobulin G (ICN/Cappel, Aurora, OH) in the dark for 2 hours. Biofilm samples were washed, scraped gently from the tubes by using a microspatula, diluted in 1x PBS, transferred to glass slides, and visualized on a BX51 Olympus fluorescence microscope.

3.3.9 Tube biofilm ELISA

Cells from the TBA were measured for production of O-ag capsule EPS by using a modified enzyme-linked immunosorbent assay (ELISA) approach. After 6 days in the TBA, the tubes were washed three times with 1x PBS and incubated with 5% bovine serum albumin for 4 hours. After three more washes with 1x PBS, capsule-specific antibody (diluted 1:2,000 in PBS) was added to the tubes at room temperature for 2 hours. Another set of three washes was followed by a 2 hour incubation period with a goat anti-rabbit immunoglobulin G (H+L) horseradish peroxidase (HRP) conjugate
diluted 1:5,000 in PBS (Bio-Rad, Hercules, CA). Measurements of O-ag capsule expression in biofilms were obtained using a Bio-Rad HRP substrate kit according to the manufacturer’s specifications. Reactions were performed with the TBA Eppendorf tubes, and the reaction products were transferred to 96-well plates for optical density readings at 415 nm.

3.3.10 Scanning electron microscopy (SEM)

Following sample growth for 12 days, gallstones were washed twice with LB. Specimens were either fixed immediately in 2% glutaraldehyde or air dried overnight in a Laminar flow hood before being fixed. All samples were dehydrated postfixation using a Cressington critical point drier at The Ohio State University Campus Microscopy and Imaging Facility. The samples were sputter coated with a Pelco Model 3 and viewed using a FEI Nova NanoSEM.

3.3.11 Murine infections with serovar Typhimurium wild-type and yihO mutant strains

Female BALB/c mice (Harlan Laboratories, Indianapolis, IN) weighing 16 to 18 grams (6 to 8 weeks) were inoculated orally with approximately 1x10^6 CFU wild-type (n = 7) or O-ag capsule mutant (ΔyihO) (n = 7) strains of serovar Typhimurium. Dilutions of the stationary phase culture inoculum were plated on LB agar to determine the actual number of administered bacteria. Animals were euthanized upon attainment of early removal criteria at days 7, 10, or 14 post infection.
3.3.12 Statistics

To determine associations of significance between groups, two-tailed Student’s $t$ tests were performed. Observed differences were considered statistically significant for $p$ values $< 0.05$.

3.4 Results

3.4.1 A serovar Typhimurium colanic acid/cellulose double mutant forms mature gallstone biofilms

The extracellular matrix produced by bacterial biofilms is frequently composed of polysaccharides and has been well characterized for organisms such as *E. coli*, *V. cholerae*, and *Pseudomonas aeruginosa* (Danese et al., 2000; Hung et al., 2006; Read and Costerton, 1987). As described previously, the EPS of bile-induced *Salmonella* biofilms on gallstones can be preserved and visualized by SEM (Prouty et al., 2002a). Individual mutants unable to produce the known *Salmonella* EPS components cellulose and colanic acid and the ViAg capsule (serovar Typhi) were previously shown to maintain the ability to form a biofilm on gallstones (Prouty and Gunn, 2003). To extend these results, a Δ*bcsE ΔwcaA* double mutation was created in serovar Typhimurium. Mutant and wild-type strains were incubated in the presence of 3% crude ox bile extract and a human gallstone under established assay conditions. Gallstones were fixed immediately in 2% glutaraldehyde and examined by SEM (Fig. 3.1). Biofilm formation and the presence of EPS (flocculent and stringy material) were equally robust for wild-type and mutant strains, suggesting involvement of an as-yet-unidentified EPS.
3.4.2 *S. enterica* binds to and forms biofilms on cholesterol-coated Eppendorf tubes

Though regional differences in diet and other environmental and genetic factors can alter gallstone development, human gallstones are primarily composed of cholesterol or calcium bilirubin. To eliminate dependence on human gallstone samples and to allow gallstone constituents to be analyzed individually for their abilities to interact with salmonellae, siliconized Eppendorf tubes were coated with 1 mg of cholesterol to mimic gallstone surfaces.

As shown in Fig. 3.2A, Eppendorf tubes required a coating of cholesterol to support *Salmonella* biofilm formation, suggesting that salmonellae bind to cholesterol. The presence of bile in this TBA significantly enhanced biofilm formation on cholesterol as it did for human gallstones, further promoting the hypothesis that bile in the gallbladder helps to establish biofilm formation on cholesterol gallstones (Fig. 3.2A and B). It was also observed that bacteria preincubated with bile (grown overnight in 3% bile) formed more-robust biofilms and formed them earlier than bacteria encountering bile for the first time upon addition to cholesterol-coated tubes (data not shown). Additionally, tubes coated with bilirubin supported significantly less biofilm than those coated with cholesterol, though bile still induced bilirubin-coated tube biofilm formation (Fig. 3.3). Therefore, bile is an important environmental signal for salmonellae that enhances biofilm formation on cholesterol-coated surfaces.
3.4.3 The *Salmonella* O-ag capsule is necessary for binding to cholesterol versus binding to glass or plastic

In addition to cellulose and colanic acid, *E. coli* expresses a variety of chemically and serologically distinct capsules (Whitfield and Roberts, 1999). One such EPS is the O-ag capsule, the composition of which is similar to that of the LPS O polysaccharides (Roberts, 1996). Recently, Gibson and colleagues identified an O-ag capsule associated with the serovar Enteritidis extracellular matrix that was shown to be important for environmental persistence (Gibson et al., 2006). Assembly and translocation of the conserved O-ag capsule involve the enzymes produced by two operons, *yihU-yshA* and *yihVW*. Mutations in several genes of these operons, including *yihO*, have been shown to eliminate expression of this capsule.

To determine the role of the O-ag capsule in biofilm formation on cholesterol-coated surfaces, deletions of *yihO* in serovar Typhimurium and of *yihP* in serovar Typhi were created by lambda Red-mediated recombination and were examined in the TBA. The data show that the O-ag is a necessary component of *Salmonella* biofilms on cholesterol-coated Eppendorf tubes in serovar Typhimurium, serovar Typhi, and serovar Enteritidis (Fig. 3.2A and B). Real-time PCR experiments confirmed that the serovar Typhimurium *yihO* deletion did not have a polar effect on downstream genes (data not shown). Thus, we performed complementation experiments with plasmid-borne *yihO*. These studies resulted in amounts of bile-induced biofilm formation on cholesterol of the *yihO* mutant similar to those for wild-type serovar Typhimurium (Fig. 3.2B).

The O-ag capsule was originally identified by White et al. (White et al., 2003) in association with the AgfD-regulated extracellular matrix. AgfD (CsgD) is an important
regulator of *Salmonella* biofilm formation (Brombacher et al., 2003; Romling et al., 1998) and has been demonstrated to control O-ag capsule biosynthesis (Gibson et al., 2006). To determine the role of AgfD in bile-mediated biofilm formation on gallstone/cholesterol-coated surfaces, a serovar Typhimurium *agfD* mutant was added to the TBA. As shown in Fig. 3.2B, loss of AgfD had no effect on biofilm formation in these assays.

To examine whether the O-ag capsule was important for biofilm formation on other surfaces, wild-type and *yihO* and *yihP* deletion strains were assayed in the presence of bile for their abilities to bind to glass or plastic coverslips. As shown in Table 3.3, wild-type salmonellae formed mature biofilms on glass, plastic, or cholesterol-coated surfaces. However, absence of the O-ag capsule eliminated biofilm formation on cholesterol-coated tubes but not on glass or plastic coverslips. Furthermore, a Δ*bcsE* Δ*wcaA* double mutant formed poor biofilms on glass and plastic surfaces but formed a biofilm similar to that of the wild-type strain on cholesterol-coated tubes (Table 3.3). These data suggest specificity of binding to cholesterol mediated directly or indirectly by the O-ag capsule.

3.4.4 The extracellular-matrix-associated O-ag capsule is a critical component of *Salmonella* gallstone biofilms

To evaluate the role of the O-ag capsule in bile-induced *Salmonella* gallstone biofilms, O-ag mutants were examined by electron microscopy for biofilm formation on gallstone surfaces. As shown in Fig. 3.4, wild-type serovar Typhimurium, serovar Typhi, and serovar Enteritidis formed mature biofilms on human gallstones after 12 days of
incubation with 3% bile. The extracellular-matrix material was preserved in a more natural state by air drying samples overnight before fixation in 2% glutaraldehyde. This extra step accounted for the visual differences in Salmonella gallstone biofilm ECM composition observed in Fig. 3.1 (samples subjected to immediate fixation) and Fig. 3.4 (samples air dried before fixation). Unlike the wild-type strains, none of the three O-ag capsule mutant strains formed detectable biofilms on gallstones (Fig. 3.4B, D, and F). These results implicate the extracellular-matrix-associated O-ag capsule as a crucial component of bile-mediated biofilm formation on gallstones.

3.4.5 Bile increases Salmonella O-ag capsule production in biofilms

The presence of bile has been shown to alter protein production, antibiotic and bile resistance, virulence, and EPS synthesis for enteric bacterial groups, including Shigella flexneri (Olive et al., 2007; Pope et al., 1995a), Helicobacter pylori (Tiwari et al., 2006), Campylobacter spp. (Lin et al., 2005b; Stintzi et al., 2005), and V. cholerae ((Bina and Mekalanos, 2001; Hung et al., 2006); for a review, see reference (Begley et al., 2005a)). To examine the effect of bile on capsule expression in biofilms grown on cholesterol-coated surfaces, antibody to Salmonella O-ag was used in a quantitative TBA ELISA. Growth in 3% bile significantly enhanced O-ag capsule induction for wildtype serovar Typhimurium, serovar Typhi, and serovar Enteritidis as well as the serovar Typhimurium colanic acid/cellulose double mutant (Fig. 3.5A). This was not seen with strains deficient for O-ag production.

Because some of the differences observed in Fig. 3.5A may be due to the reduction in biofilm in the absence of bile, fluorescence microscopy of Salmonella
biofilm material removed from the TBA was also used to measure O-ag capsule expression (Fig. 3.5B). As with the modified ELISA, the images show that production of the O-ag capsule increased in biofilms for all wild-type strains and the ΔbcsE ΔwcaA mutant when bacteria were grown in bile. Quantification of signal intensity indicated that bile mediated a 166 to 278% fluorescence increase in the *Salmonella* strains examined. The *yihO* and *yihP* mutants of serovar Typhimurium, serovar Typhi, and serovar Enteritidis showed little O-ag expression in the presence or absence of bile (Fig. 3.5B). In addition, there was no significant difference in thickness of biofilm material between samples from tubes with and without bile (data not shown). Furthermore, a fluorescence increase was also observed on a single-cell basis for bacteria grown in the presence of 3% bile versus those grown without bile (data not shown). These results further implicate the importance of the O-ag capsule in *Salmonella* biofilm ECM and demonstrate that its production is bile induced.

3.4.6 Transcription of O-ag capsule genes is upregulated by bile in an AgfD-independent manner

Bile has been shown to influence the expression of microbial virulence factors at the level of gene transcription. For example, eukaryotic cell invasion by serovar Typhimurium is repressed by bile, as evidenced by transcriptional downregulation of genes within *Salmonella* pathogenicity island I (Prouty and Gunn, 2000). To examine the effect of bile on transcription of O-ag genes, real-time PCR was performed using wild-type serovar Enteritidis, serovar Typhimurium, and serovar Typhi and an *agfD* mutant of serovar Typhimurium, all grown in the presence or absence of bile (Fig. 3.6).
Transcription of \textit{yihU}, a putative oxidoreductase located in the O-ag operon, was enhanced 18-fold for serovar Enteritidis, 24-fold for serovar Typhimurium, 11-fold for serovar Typhi, and 23-fold for serovar Typhimurium \textit{\Delta agfD} when strains were grown in the presence of bile, suggesting that bile is a signal resulting in increased transcription of the O-ag cluster independently of AgfD.

3.4.7 Serovar Typhimurium O-ag capsule mutants are attenuated for virulence during oral murine infections

Serovar Typhimurium is a facultative intracellular pathogen that mediates a systemic, typhoid-fever like illness and rapid death in mouse strains lacking the macrophage phagosome-specific protein Nramp1, and certain genetic manipulations in \textit{Salmonella} prevent pathogenesis in this model (reviewed in (Skamene et al., 1998)). To determine whether the loss of O-ag capsule expression attenuates serovar Typhimurium virulence, female BALB/c mice were orally inoculated with wild-type and \textit{yihO} mutant strains (\(10^6\) bacteria) at 2 logs above the dose necessary to kill 50\% (LD\(_{50}\)). Infections with wild-type salmonellae resulted in death at 7 to 10 days P.I., whereas mice given a \textit{yihO} mutant experienced overt symptoms and subsequent recovery, and were euthanized 2 weeks P.I. These results are preliminary, (performed twice, total \(n = 7\)), and suggest that expression of the O-ag capsule is necessary for full virulence during oral infection of susceptible murine animals.
3.5 Discussion

Bacterial biofilms are increasingly associated with serious illnesses in humans and animals. We hypothesize that the *Salmonella* chronic carriage state is frequently if not always a result of biofilm formation on gallstones. In limited studies, a high correlation was shown to exist between those individuals who became carriers and those who have gallstones (Dutta et al., 2000; Lai et al., 1992), and the protection afforded the bacteria within the biofilm would help explain their resistance to killing by bile and antibiotics. In this work, we describe a bile-induced EPS necessary for and specific toward biofilm formation on gallstones and cholesterol-coated surfaces.

ECM material within bacterial biofilms is thought to be heterogeneous and to provide both rigidity to the biofilm and protection to bacteria embedded within these biofilms. It was previously shown that the serovar Typhi Vi antigen did not contribute to the establishment of gallstone biofilms (Prouty and Gunn, 2003). Furthermore, serovar Typhimurium and serovar Enteritidis do not produce this EPS but form biofilms on gallstones that are indistinguishable from those of serovar Typhi. To further examine the role of EPSs in *Salmonella* gallstone biofilms, a double mutation in the known *Salmonella* ECM constituents colanic acid and cellulose was created. When assayed by SEM for biofilm formation on human gallstones, robust biofilms and ECM material were still clearly present. These data suggested the presence of an undetermined EPS in the *Salmonella* gallstone microenvironment.

Previous work performed by Gibson et al. (Gibson et al., 2006) identified a conserved serovar Enteritidis EPS termed the O-ag capsule. This substance was similar in structure to the LPS O-ag of serovar Enteritidis and was encoded by two divergent
operons \((yihU\text{-}yshA \text{ and } yihV\text{-}yihW)\) (Gibson et al., 2006). Despite having similar repeating sugar units, the O-ag capsule and the LPS O-ag differ in size, charge, substitution patterns, and immunoreactivity (White et al., 2003). In addition, this capsule is linked to the membrane via a lipid anchor. O-ag capsule expression was found to be necessary for serovar Enteritidis biofilm formation leading to enhanced plant colonization (Barak et al., 2007). To examine the role of the O-ag capsule in \emph{Salmonella} gallstone biofilms, a \emph{yihO} deletion was constructed in serovar Typhimurium and \emph{yihP} was deleted in serovar Typhi. Lack of O-ag capsule production proved to be critical, as these strains failed to form a biofilm on gallstone surfaces. Similar results were observed for an O-ag serovar Enteritidis mutant, though they were not as exaggerated as those observed for serovar Typhimurium or serovar Typhi.

Human gallstones in North America are primarily cholesterol but also contain calcium bilirubin and other components. Interaction of salmonellae with cholesterol has been described to occur in host cells, as cholesterol is recruited to the \emph{Salmonella}-containing vacuole and esterified by the SPI-2 effector SseJ (Catron et al., 2002; Nawabi et al., 2008). Because of such known cellular associations with cholesterol and because cholesterol is the primary constituent of the human gallstones used in our assays, we examined \emph{Salmonella} biofilm formation on Eppendorf tubes coated with cholesterol (TBA). The results of these experiments mimicked those of the experiments performed with gallstones in test tubes for all wild-type and mutant strains examined, suggesting that \emph{S. enterica} binds to cholesterol. Biofilms on gallstones were previously shown to often occur in patches (Prouty et al., 2002a), which, given this new knowledge, may reflect the local distribution of cholesterol on the gallstone surface. Furthermore, bile
was shown to enhance biofilm formation on cholesterol as it did for human gallstones. This model has advantages over that of human gallstones as it is more efficient, is amenable to large-scale mutagenesis screens, avoids retrieval of patient materials, and will allow other gallstone constituents to be analyzed for *Salmonella* binding in the future.

It has previously been shown that *Salmonella* biofilm-associated factors are not uniformly required for biofilm formation on all surfaces (Prouty and Gunn, 2003). Interestingly, serovar Typhimurium, serovar Typhi, and serovar Enteritidis O-ag capsule mutants were all shown to be deficient in biofilm formation on cholesterol-coated surfaces and human gallstones but produced robust biofilms on glass and plastic coverslips when grown in the presence of bile. Cellulose and colanic acid mutants had the opposite phenotype, as they did not form biofilms on glass or plastic coverslips but formed mature biofilms on human gallstones and in the TBA. Interestingly, cellulose and colonic acids have been shown to play a role in biofilm formation on biotic as well as abiotic surfaces, as mutations in either *wcaM* or *yhjN* have been shown to disrupt serovar Typhimurium biofilm formation in flow through chambers on HEp-2 cells (Ledeboer and Jones, 2005). However, in the presence of cholesterol and bile, cellulose and colanic acid are not a factor in biofilm formation.

A modified ELISA and immunofluorescence microscopy were used to determine if bile enhanced O-ag capsule expression in *Salmonella* biofilms on cholesterol. Both experiments showed that bile increased not only biofilm formation but also production of the O-ag capsule in all wild-type strains as well as the serovar Typhimurium Δ*bcsE* Δ*wcaA* double mutant. Real-time PCR analysis indicated that bile-mediated O-ag
capsule induction occurred at the level of transcription of the \textit{yih} genes. Gibson and colleagues showed that AgfD, an important regulatory factor of biofilm formation (Brombacher et al., 2003; Romling et al., 1998), controls expression of the \textit{yih} operons (Gibson et al., 2006). However, loss of AgfD had no effect on biofilm formation in the TBA. Furthermore, real-time PCR revealed that bile upregulated transcription of the \textit{yih} operon in an \textit{agfD}-independent manner, suggesting that another bile-responsive transcription factor is involved in \textit{yih} gene expression.

To date, our understanding of serovar Typhi pathogenesis and associated pathology during typhoid fever has been inferred from infections of inbred mouse strains lacking the Nramp1 protein with serovar Typhimurium (Jones and Falkow, 1996). Mutations in a variety of virulence related genes render \textit{Salmonella} incapable of killing otherwise susceptible mice, including constitutive expression of the PhoP regulon, and an observed effect can depend on the route of infection (Galan and Curtiss, 1991; Miller and Mekalanos, 1990). Here, the effect of O-ag capsule expression on pathogenesis in this model was examined. Our results, though preliminary, indicate that production of the O-ag capsule is a necessary component of full virulence, as mice inoculated orally with $10^6$ CFU of a \textit{yihO} serovar Typhimurium mutant cleared all infections. To account for any bile-mediated virulence effects on the O-ag capsule that may have occurred during oral inoculation, these experiments will be repeated intraperitoneally to bypass the gastrointestinal tract.

We have identified a critical EPS in the extracellular matrix of \textit{Salmonella} bile-induced biofilms on gallstones and a new method of examining \textit{Salmonella}-gallstone interactions (the TBA). It could be hypothesized that bile induction of the O-ag capsule
has evolved to aid chronic carriage of *S. enterica* specifically in the gallbladder environment. This new understanding of how bile mediates *Salmonella* gallstone biofilm formation via O-ag capsule expression provides a therapeutic target for potentially alleviating carriage in the gallbladder and subsequently much of the human-to-human transmission that makes this pathogen a relevant global concern.
<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Characteristics</th>
<th>Source or reference</th>
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<td>ATCC 14208s (CDC6516-60), wild type</td>
<td>ATTC</td>
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<td>JSG210 ΔyihiO</td>
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Table 3.1: Bacterial strains, plasmids and relevant characteristics
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Table 3.2: Oligonucleotide primers
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<tr>
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<tr>
<td>Serovar Typhimurium <em>yihO</em> mutant</td>
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<td>Serovar Typhimurium <em>bcsE wcaA</em> mutant</td>
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</tr>
<tr>
<td>Serovar Typhi</td>
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<tr>
<td>Serovar Typhi <em>O-ag capsule yihP</em> mutant</td>
<td>-</td>
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</table>

Table 3.3: Summary of biofilm formation on cholesterol, glass, and plastic surfaces from a representative experiment performed in triplicate (n = 3). *Biofilms grown on cholesterol-coated, glass, and plastic surfaces were stained with crystal violet. Dye was extracted with acetic acid and optical density was measured at 570 nm for quantification. ++, robust mature biofilm formation compared to that of the wild-type; +, reduced biofilm present (50-80% of wild-type values); -, severe defect in or complete loss in biofilm formation (0-10% of wild-type values).
Figure 3.1: The loss of colanic acid and cellulose does not affect *Salmonella* gallstone biofilm formation. (A) SEM micrograph showing the multi-layered, heterogeneous surface of a cholesterol gallstone incubated in LB broth without bacteria. (B) *S.* Typhimurium wild-type (14028s) strain exposed to a human gallstone for 12 days in the presence of 3% bile forms a mature biofilm. (C) *S.* Typhimurium *bcsE* *wcaA* strain exposed to a human gallstone for 12 days in the presence of 3% bile also forms a mature biofilm. The web-like strands or flocculent material are indicative of exopolysaccharide in the biofilm ECM. Micrographs are representative of 3 independent experiments.
Figure 3.2: Eppendorf tubes coated with 1 mg cholesterol support *Salmonella* biofilm formation in the tube biofilm assay (TBA). The TBA spanned 6 days with washings every 24 hours to mimic emptying of the gallbladder. (A) *Salmonella* biofilms from the TBA were stained with crystal violet and photographed using the BioChemi System (UVP BioImaging). (B) Crystal violet stained TBA biofilms were extracted with acetic acid and absorbance was measured for at 570 nm. All experiments were performed in triplicate and means ± SD are given (n = 3). * denotes statistical significance between bile and no bile groups (P < 0.005) based on two-tailed Student t-Test.
Figure 3.3: Comparison of *Salmonella* biofilm formation in the tube biofilm assay (TBA) on cholesterol- or bilirubin-coated tubes. The TBA spanned 6 days with washings every 24 hours to mimic emptying of the gallbladder. Crystal violet stained biofilms were extracted with acetic acid and absorbance was measured for at 570 nm. Data are presented as means + SD from a representative experiment done in triplicate (n = 3). * denotes statistically significant difference in biofilm formation on cholesterol vs. bilirubin (P < 0.005) based on two-tailed Student t-Test.
Figure 3.4: Mutations in the O-ag operon affect *Salmonella* gallstone biofilm formation. Human gallstones were incubated with *Salmonella* and 3% bile for 12 days, washed in LB, air-dried overnight, fixed in glutaraldehyde, and visualized by SEM. (A) *S.* Typhimurium wild-type (B) *S.* Typhimurium *yihO* mutant (C) *S.* Typhi wild-type (D) *S.* Typhi *yihP* mutant (E) *S.* Enteritidis wild-type (F) *S.* Enteritidis *yihO* mutant. Experiments were performed at least 3 times and presented are representative micrographs.
Figure 3.5: The effect of bile on *Salmonella* O-ag capsule expression. (A) Modified ELISA in the TBA. Salmonellae were incubated with or without bile in cholesterol-coated Eppendorf tubes for six days with washings every 24 hours. Samples in the tubes were then blocked with BSA, exposed to O-ag capsule antisera and the appropriate HRP-conjugated secondary antibody, and developed. (B) Immunofluorescent microscopy of TBA biofilms gently removed from the tubes. Primary O-ag capsule antibody was used with a fluorescein-conjugated secondary for visualization with an Olympus fluorescent microscope. All experiments were performed in triplicate and means ± SD are given (n = 3). * denotes statistical significance of P < 0.005 between bile and no bile groups based on two-tailed Student t-Test.
Figure 3.6: Real-time PCR measuring O-ag capsule transcription for *S. enterica* serovars Enteritidis, Typhimurium and Typhi and *S. Typhimurium* Δ*agfD* grown in the presence or absence of bile. Primers target *yihU*, a putative oxido-reductase located in the O-ag capsule encoding operon. Means + SD are given for a representative experiment done in triplicate (n = 3). * denotes statistical significance of P < 0.005 between bile and no bile groups based on two-tailed Student t-Test.
Chapter 4

Gallstones play a significant role in *Salmonella* spp. gallbladder colonization and carriage

4.1 Abstract

Approximately 5% of patients suffering from typhoid fever carry *S. enterica* serovar Typhi in the bile-rich gallbladder long after overt symptoms subside, suggesting that this bacterium utilizes novel mechanisms to mediate enhanced colonization of and persistence in this hostile environment. The asymptomatic carrier state is frequently associated with the presence of gallstones and is a significant risk factor for carcinoma of this organ. We have previously shown that salmonellae form bile-mediated biofilms on human gallstones and cholesterol-coated surfaces in vitro and thus hypothesize that biofilm formation on cholesterol gallbladder stones facilitates typhoid carriage. Naturally resistant 129X1/SvJ mice fed a lithogenic diet (normal chow supplemented with 1% cholesterol and 0.5% cholic acid) developed cholesterol gallstones and, as a result, demonstrated enhanced colonization of gallbladder tissue and bile as well as fecal shedding during persistent serovar Typhimurium infection. This was specific to the gallbladder as there were no significant differences in bacteria recovered from the spleen over 21 days post infection. Furthermore, cholesterol gallstones were shown to associate with significant amounts of salmonellae that increased with time post infection.
Individuals seeking cholecystectomy due to gallstones were analyzed for asymptomatic serovar Typhi carriage at the Hospital Infantil in Mexico City to test our hypothesis in humans from an area endemic to typhoid fever. Results indicate that 5% of patients with cholelithiasis carried serovar Typhi and that bacterial biofilms could be visualized on gallstones from 3/4 of these carriers. Biofilm formation was not observed on patients positive for other bacteria or whose gallbladders failed to possess culturable aerobic bacteria, suggesting that gallstone biofilms are a typhoid specific phenomenon. These findings offer direct evidence that gallstone biofilms occur in humans and mice carrying salmonellae without symptoms, which facilitates gallbladder colonization and shedding. In addition, this work brings forward a new model for evaluating therapeutic targets against typhoid fever transmission.

4.2 Introduction

Global public health continues to be burdened with significant morbidity and mortality caused by salmonellae. The greater than 2,400 serovars of *Salmonella enterica* are ubiquitous in nature, distinguished by somatic differences in surface antigen expression, and as such have evolved to infect a broad array of host organisms and resist commonly administered antibiotics (Boyle et al., 2007; Rabsch et al., 2001). *S. enterica* serovar Typhi has adapted to induce disease exclusively in humans and is the etiologic agent of typhoid fever, a debilitating illness characterized by interstitial inflammation, a gradual onset of symptoms, and, ultimately, bacterial dissemination to systemic sites (Tsolis et al., 2008). The Centers for Disease Control and Prevention estimate that at least 22 million people world-wide are stricken with typhoid fever each year, yet many
more go unreported due in part to poor diagnostic testing world-wide (Butler et al., 1978; Crump et al., 2004; Parry et al., 2002).

The hallmarks of salmonellosis caused by non-typhoidal *S. enterica* serovars Enteritidis or Typhimurium differ from those of enteric fever and include intestinal neutrophilia, localized inflammatory diarrhea, the loss of electrolytes, serum protein and fluid, as well as sepsis-induced death in immunocompromised individuals (Tsolis et al., 2008; Dharmana et al., 2002; Fleisher, 1991; Santos et al., 2002). Interestingly, much of what is known about the systemic pathogenesis of *S. Typhi* during typhoid fever has been extrapolated from infections of inbred mouse strains lacking *Nramp1* (BALB/c and C57BL/6) with non-typhoidal serovar Typhimurium. In this model, translocation of the intestinal epithelium at microfold (M) cells is followed by invasion of phagocytic cells of the underlying lymphoid tissue, a primary bacteraemia in the blood, and uncontrolled replication within macrophages of the liver, spleen, and bone marrow leading to death at 7 to 10 days post infection (Jepson and Clark, 2001; Jones and Falkow, 1996; Galan and Curtiss, 1991; Richter-Dahlfors et al., 1997; Salcedo et al., 2001).

In humans, serovar Typhi reaching the gallbladder can establish an acute, active infection (cholecystitis) or persist in this organ long after symptoms subside, suggesting novel mechanisms utilized by the bacterium to mediate colonization in a bile-rich environment (Vaishnavi et al., 2005; Sinnott and Teall, 1987). Bile is an important digestive secretion manufactured in the liver that serves as a potent emulsifying and antimicrobial agent in the gastrointestinal tract, yet salmonellae have adapted to resist its surface-acting, amphipathic, detergent-like properties (van Velkinburgh and Gunn, 1999).
Development of chronic typhoid carriage is frequently associated with the presence of gallbladder abnormalities, especially gallstones, yet the progression from infection to the carrier state remains undefined (Lai et al., 1992). Gallstone formation (cholelithogenesis) is attributed to a combination of environmental and genetic causes typically linked to cholesterol supersaturation from bile, and can occur in patients for many years without symptoms (Maurer et al., 2009; Shoheiber et al., 1997). The primary constituent of gallbladder stones is cholesterol, whereas calcium bilirubinate predominates in bile duct stones (Hofmann, 2005). In patients carrying both S. Typhi and cholesterol gallstones in the gallbladder, clinically administered antibiotics are typically ineffective against dissolution of the persistent bacterial infection (Lai et al., 1992) and the risk factor for developing hepatobiliary carcinomas is high (Dutta et al., 2000; Kumar et al., 2006). Similarly, survival of *Helicobacter pylori* in the hostile, acidic stomach often leads to persistence of this microorganism and an increased risk of adenocarcinoma (Blaser et al., 1995). To date, removal of the gallbladder (cholecystectomy) remains the most effective treatment option for chronic typhoid carriers with gallstones. In 1994, the United States alone spent an estimated 8.0 billion dollars treating gallbladder diseases, suggesting a strong need for alternative treatment options in areas of the world where typhoid fever is endemic and surgical intervention is cost-prohibitive.

This description led us to hypothesize that asymptomatic S. Typhi carriage in the presence of bile was facilitated by the formation of biofilms on cholesterol gallstones. Over the past two decades, bacterial biofilms associated with medical and industrial ecosystems have been implicated as the cause of many antibiotic resistant, persistent infections in humans (Stewart and Costerton, 2001; Costerton et al., 1999). Biofilm
formation occurs in sequential, highly regulated stages that include adherence of free-swimming bacteria to a surface, development of a protective, self-initiated extracellular (ECM) matrix, and continual release of planktonic cells from this sessile community into the environment (Costerton et al., 1995). Shedding of S. Typhi from asymptomatic, chronic carriers is thought to account for much of the person-to-person transmission of typhoid fever in impoverished societies.

We have demonstrated that bile-dependent mature biofilm formation with characteristic ECM production of S. enterica serovars Typhimurium, Enteritidis, and Typhi occurs on human gallstones and on cholesterol-coated Eppendorf tubes in vitro (Prouty et al., 2002a; Crawford et al., 2008). However, S. Typhi biofilm formation on cholesterol gallstones has never been investigated as a mechanism mediating the chronic carrier state of typhoid fever in vivo. To address this issue, we present clinical evidence that serovar Typhi forms biofilms on gallstones in asymptomatic, human carriers. In addition, we have developed a murine model of Salmonella persistence in mice with lithogenic diet-induced cholesterol gallstones. Interestingly, mice with gallstones harbor significantly more serovar Typhimurium in the gallbladder tissue, bile, and fecal matter than control mice, and salmonellae can be recovered from homogenized gallstones in increasing amounts over time post infection. These findings offer direct evidence that gallstone biofilms occur in individuals carrying S. Typhi, and point to a new therapeutic target against the spread of typhoid fever.
4.3 Materials and Methods

4.3.1 Bacterial strains and growth conditions

The *S. enterica* strains used in this study are listed in Table 4.1. Cultures were grown in Luria-Bertani (LB) broth with or without 3% crude ox bile extract (Sigma, St. Louis, MO) overnight on a rotating drum at 37°C and diluted to mid- to late exponential phase (optical density at 600 nm [OD\textsubscript{600}] of 0.6 to 0.8) for use in biofilm and mouse colonization assays. When necessary, antibiotics were added at appropriate concentrations: ampicillin, 50 µg/ml; kanamycin, 25 µg/ml; chloramphenicol, 25µg/ml; and tetracycline, 15 µg/ml.

4.3.2 Serovar Typhimurium infections in mice

Female 129X1/SvJ mice (Jackson Laboratories, Bar Harbor, ME) weighing 16 to 18 grams (6 to 8 weeks) were fed a normal rodent diet (Harlan-Teklad cat. 2014) or a lithogenic, gallstone-inducing diet consisting of mouse chow (Harlan-Teklad cat. 7912) supplemented with 1% cholesterol (Sigma, St. Louis, MO) and 0.5% cholic acid (Sigma, St. Louis, MO) for 8 weeks. To make the lithogenic diet, equal parts (weight: volume) powdered ingredients and UltraPure distilled water (Invitrogen, Carlsbad, CA) were mixed in zip-top bags until homogenous, and pellets weighing between 5 to 7 grams were shaped by hand to resemble normal chow and air dried overnight in a Laminar flow hood. Mice were inoculated (n = 42; 21 per diet group) intraperitoneally with approximately 1x10\textsuperscript{4} CFU or left as uninfected controls (n = 18; 9 per diet group), and euthanized at days 3, 7, and 21 post infection. Dilutions of the stationary phase culture inoculum were plated on LB agar to determine the actual number of delivered bacteria. For analysis of
organ burdens (CFU), spleens and gallbladders were aseptically removed at the stated time points, homogenized, serially diluted in 1 x PBS, and plated on LB agar. To enumerate CFU, gallstones (present only in the lithogenic diet groups) and gallbladder tissues were separated, homogenized in a 50 ml conical tube containing 1 ml of 1 x PBS, and gallbladder bile was diluted and plated. To monitor shedding of serovar Typhimurium, infected mice from both diet groups were isolated overnight in individual cages prior to sacrifice at day 21. Approximately 5 to 7 fresh pellets were collected from each mouse, weighed in 50 ml conical tubes, homogenized in 1 x PBS, diluted, and plated on SS-agar (BD, Franklin Lakes, NJ) for CFU determination.

4.3.3 Sample collection from human donors

Patients seeking surgical removal of their gallbladder (cholecystectomy) at the Hospital Infantil in Mexico City were tested for asymptomatic typhoid carriage during a 2 year study. To diagnose the presence of serovar Typhi, gallbladder tissues, gallstone sections, and stool samples were homogenized and plated, and bile was obtained and plated directly. If bacteria were present, serotyping was performed using standard clinical techniques. Genomic DNA from positive samples was purified and selectively amplified by multiplex PCR for Vi antigen (viaB), flagellar antigens (fliC-d and fliC-a), and O-antigen synthesis (tyv and prt) as previously described (Hirose et al., 2002) to confirm identity of serovar Typhi. Gallstones and gallbladder tissues from positive patients and negative controls were sent to The Ohio State University (OSU) and analyzed for gallstone biofilms by scanning electron microscopy (SEM) or tissue inflammation and cellular infiltration by histopathology.
4.3.4 Scanning electron microscopy

Gallstones recovered from patients at the Hospital Infantil in Mexico City positive for asymptomatic carriage of serovar Typhi were rinsed with sterile PBS, fixed in 2% glutaraldehyde, air dried in a Laminar flow hood, and sent by overnight courier to OSU. All specimens were dehydrated postfixation using a Cressington critical point drier at the Campus Microscopy and Imaging Facility (OSU CMIF), sputter coated with a Pelco Model 3, and viewed using a FEI Nova NanoSEM at 5.00 kV under varying magnifications.

4.3.5 Histopathology

Gallbladder tissues from human patients seeking cholecystectomy in Mexico City as well as from infected and uninfected mice belonging to lithogenic and normal diet groups (all time points) were collected for histological examination. Human clinical samples were rinsed in sterile saline, fixed in 10% buffered formalin (Sigma, St. Louis, MO) for 18 hours, washed with purified 1 x PBS, placed in 30% sucrose for 24 hours, frozen at -70°C, and shipped to OSU. Mice gallbladders were removed immediately post sacrifice, rinsed aseptically in 1 x PBS, and fixed in 10% neutral buffered formalin (Fisher Scientific, Pittsburgh, PA) for 72 hours. All tissues were embedded in paraffin blocks, mounted on slides in 4 μm thick sections, and stained with hematoxylin/eosin (H&E) by the Comprehensive Cancer Center Histology Core Facility (OSU) for subsequent pathologic examination by the Comparative Pathology & Mouse Phenotyping Shared Resource (OSU).
4.3.6 MIC assays of bile resistance

Laboratory strains and clinical isolates of serovar Typhi were challenged with crude ox bile extract at concentrations of 0.02% to 60%. Standard MIC testing of susceptibility to bile was performed as previously described (Steinberg et al., 1997). Briefly, strains were grown in Mueller-Hinton broth overnight, diluted, and $10^4$ cells were added to individual round-bottomed wells of polypropylene microtiter plates (Costar Corp., Cambridge, MA). Bacteria were tested for growth (as visualized by a pellet accumulation) against serial dilutions of bile following incubation for 24 hours at 37°C.

4.3.7 Biofilm formation assays on cholesterol

Salmonellae formed biofilms on cholesterol-coated surfaces as described previously (Crawford et al., 2008). Briefly, log phase serovar Typhimurium grown with or without 3% crude ox bile was added to cholesterol-coated Eppendorf tubes in the tube biofilm assay (TBA) or plastic 24-well plates (Fisher Scientific, Pittsburgh, PA) containing Chromerge-cleaned coverslips coated with cholesterol. Chromatography-grade cholesterol (product number C8667; Sigma, St. Louis, MO) was dissolved in anhydrous ether (J. T. Baker, Phillipsburg, NJ) at a concentration of 10 mg/ml or 25 mg/ml and delivered in 100 μl aliquots to siliconized Eppendorf tubes (Fisher Scientific, Pittsburgh, PA) or coverslips, respectively. The ether was allowed to evaporate, consistently leaving a homogenous coating of cholesterol. Bacteria were incubated in tubes or plates on a Nutator shaker at room temperature for 6 days and washed every 24 hours with LB followed by addition of fresh LB media (+/- bile). Bound samples were
fixed at 60°C for 1 hour and stained with 0.1% crystal violet (gentian violet in isopropanol-methanol-1 x PBS [1:1:18]) for 5 minutes at room temperature. The dye was extracted using 33% acetic acid, added to 96-well plates, and measured at optical density 570 nm to quantify biofilm formation.

4.3.8 Antibiotic challenge of biofilm and planktonic cells

Serovar Typhimurium biofilms were grown on cholesterol-coated Eppendorf tubes (TBA). Biofilm and planktonic cells were removed at day 6, diluted, and plated on LB agar for enumeration. TBA assays were performed again, and equivalent amounts of biofilm and planktonic cells obtained from appropriate dilutions of the planktonic supernatant were incubated with 25 μg/ml ciprofloxacin, 25 μg/ml chloramphenicol, or 30% bile for 2, 4, or 6 hours. Samples were diluted and plated at these time points on LB agar to determine bacterial counts.

4.3.9 Neutrophil isolation and effect on established biofilms v. planktonic cells

Human neutrophils were obtained from heparinized venous blood of a healthy adult volunteer (in compliance with IRB protocols at OSU) by density gradient centrifugation as previously described (Pizza et al., 2001). Briefly, blood samples were diluted 1:1 in normal saline and centrifuged at 800 x g for 40 minutes over a Ficoll-Hypaque (Amersham Biosciences, Pittsburgh, PA) density cushion to isolate peripheral blood mononuclear cells (PBMCs). Neutrophils were separated from the red blood cell pellet by sedimentation with 3% dextran, washed in calcium and magnesium free Hanks’ balanced salt solution (HBSS, Invitrogen, Carlsbad, CA), and centrifuged to enrich.
Residual red blood cells were lysed with distilled water, and neutrophils \( (2.82 \times 10^7) \) were suspended in HBSS. The neutrophils were added to serovar Typhimurium biofilms grown on cholesterol-coated coverslips in 24-well plates or planktonic cells removed from each biofilm-containing well, and incubated for 30 or 60 minutes. Biofilms were quantified with crystal violet as described above and the number of planktonic cells was measured by optical density readings at 570 nm.

4.3.10 Statistical analysis

Statistical significance between groups was based on \( p \) values < 0.05 obtained from two-tailed Student’s \( t \) tests.

4.4 Results

4.4.1 *Salmonella* resistant mice form cholesterol gallstones

Serovar Typhimurium is a facultative intracellular pathogen that mediates a systemic, typhoid-fever like illness and rapid death in mouse strains lacking the macrophage-specific protein Nramp1 (for a review, see reference (Skamene et al., 1998)), but persists without symptoms in naturally resistant mice (Nramp1\(^{+/+}\)) (Nauciel et al., 1988). Recent experiments monitoring *Salmonella* infections in wild-type mice reported recovery of salmonellae from the gallbladders of asymptomatic animals at late time points post infection (P.I.) (Monack et al., 2004; Deng et al., 2008). The murine gallbladder has also been shown to carry diet-induced gallstones in certain inbred strains used to study cholesterol gallstone genetics and pathogenesis (Reihner and Stahlberg, 1996; Wittenburg et al., 2003). To determine whether strains resistant to serovar...
Typhimurium infection could harbor gallbladder stones, female 129X1/SvJ mice were fed a gallstone-inducing diet for 8 weeks. Consumption of the lithogenic diet (normal mouse chow supplemented with 1% cholesterol and 0.5% cholic acid) induced cholesterol gallstone formation in 25/30 gallbladders of 129sv mice (Fig. 4.1A), and these materials could be surgically removed for further examination (Fig. 4.1B and C).

4.4.2 Cholesterol gallstones enhance *Salmonella* gallbladder colonization and fecal shedding during persistent murine infections

We hypothesize that *S*. Typhi persistence in the gallbladders of asymptomatic human carriers is facilitated in large part by the formation of biofilms on cholesterol gallstones, and have previously demonstrated that *S*. enterica serovars Typhimurium, Enteritidis, and Typhimurium formed bile-induced biofilms on human gallstones and cholesterol-coated surfaces in vitro (Prouty et al., 2002a; Crawford et al., 2008). To determine whether cholesterol gallbladder stones enhanced gallbladder colonization and carriage of salmonellae in vivo, 129X1/SvJ mice were fed a lithogenic diet or normal mouse chow for 8 weeks and inoculated intraperitoneally with 1.4x10^4 CFU serovar Typhimurium or left as uninfected controls.

Similar spleen burdens between diet groups were observed at days 3, 7, and 21 P.I. (Fig. 4.2A), indicating that hypercholesterolemia did not impair general host immunity during infection, unlike apolipoprotein E-deficient mice that exhibited high susceptibility to *Mycobacterium tuberculosis* infections when fed a high cholesterol diet (Martens et al., 2008). Over 10^6 CFU were recovered from the spleen 3 weeks P.I. (Fig. 2A) whereas no salmonellae were obtained in this organ from uninfected controls.
Biliary epithelial cells express mucin genes and secrete immunoglobulin proteins, and thus participate along with bile in both innate and adaptive immunity of the gastrointestinal tract (Maurer et al., 2009; Reynoso-Paz et al., 1999; Andrianifahanana et al., 2006). To investigate the prevalence of serovar Typhimurium within the gallbladder epithelium during persistent infections, gallbladder tissues were separated from luminal contents, homogenized, and plated to enumerate CFU. The number of salmonellae harbored in the gallbladder epithelium of mice with diet-induced cholesterol gallstones increased over time P.I., and was significantly higher than observed in mice without gallbladder stones at time points 3 (4-fold), 7 (12-fold), and 21 (541-fold) days P.I. (Fig. 4.2B). Gallbladder tissues from uninfected mice in both diet groups yielded no salmonellae.

The presence of gallstones also correlated with higher levels of serovar Typhimurium recovered from gallbladder bile, and these amounts increased as the infection progressed (Fig. 4.3A). Conversely, the amounts of salmonellae detected in gallbladder bile of mice without cholesterol gallstones decreased over time P.I. and were lower than mice with gallstones at time points 3 (5-fold), 7 (24-fold), and 21 (5,300-fold) days (Fig. 4.3A). No bacteria were recovered from uninfected control mice. These results suggest that cholesterol gallstones enhance colonization and persistence of Salmonella in gallbladder tissues and bile during systemic murine infections, possibly by providing a substrate for biofilm-mediated protection and stability against the host immune response.

To determine whether gallstones themselves supported serovar Typhimurium growth, gallbladder stones were removed, homogenized with a microspatula and vigorous
pipetting, diluted, and plated on LB agar. As shown in Fig. 4.3B, the association between salmonellae and cholesterol gallstones was robust, and the amount of salmonellae recovered from gallstone material increased by over 2 logs from days 3 to 21 P.I. Gallstones from uninfected mice were *Salmonella*-free. These results indicate that salmonellae colonize gallbladder stones, likely developing a biofilm as has been demonstrated in vitro (Prouty et al., 2002a; Crawford et al., 2008).

Individuals typically contract serovar Typhi by ingesting food or water contaminated with excretions from typhoid carriers (Parry et al., 2002). It was demonstrated previously that subsets of persistently infected 129sv mice shed high levels of serovar Typhimurium in feces, and that these groups rapidly transmitted the infection to naïve mice (Lawley et al., 2008). To determine whether cholesterol gallbladder stones enhanced shedding of serovar Typhimurium, 129X1/SvJ mice from both diet groups were isolated overnight in individual cages prior to sacrifice at day 21 and approximately 5 to 7 fresh fecal pellets were collected, weighed, and plated on SS agar to detect salmonellae (Fig. 4.4A). Interestingly, mice harboring gallstones shed 3 logs more serovar Typhimurium than infected mice on the control diet, whereas stools from uninfected groups were *Salmonella*-free (Fig. 4.4B). Therefore, the observed association of salmonellae with cholesterol gallstones and its elevated recovery from gallbladder tissue, bile, and feces at late time points post infection suggest that the presence of diet-induced gallstones in 129X1/SvJ mice mediates gallbladder colonization and enhanced shedding following serovar Typhimurium infection, and could be used as a model of asymptomatic typhoid carriage by humans.
4.4.3 Serovar Typhi forms biofilms on gallstones of asymptomatic carriers from Mexico City

In 1982, Levine et al. demonstrated that serovar Typhi could be recovered in stool samples for over 1 year from at least 2 to 5% of those suffering acute typhoid illnesses (Levine et al., 1982). It is now understood that gallbladder stones are frequently present in asymptomatic carriers of serovar Typhi (Lai et al., 1992). To better define the association between gallstones and typhoid carriage in an area endemic to typhoid fever, individuals seeking surgical gallbladder removal due to gallstones (cholecystectomy) at the Hospital Infantil in Mexico City (age range 21-92 yrs; mean = 44.3 yrs; 12 unreported) were analyzed for the asymptomatic presence of serovar Typhi during a study lasting 2 years. Multiplex PCR using genomic DNA purified from gallbladder tissues, bile, gallstone sections, and stool samples revealed that 5/103 patients with cholelithiasis (4.9%) tested positive for typhoid carriage, of which 3 were female (ages 52, 58, and 60) and 2 were male (ages 41 and 46). Colony forming units (CFU) for each typhoid positive biological sample are depicted in Table 4.2. All 5 carriers were positive for serovar Typhi in gallstone sections, 2 of which also harbored salmonellae in gallbladder epithelial tissues. Only 1 carrier tested positive for serovar Typhi in bile, and none were diagnosed by stool determinations. Additionally, the presence of *Escherichia coli* was detected in 13 typhoid negative patients and *Shigella flexneri* was recovered from 3 stool samples.

Gallstones from 4 of the 5 asymptomatic serovar Typhi carriers were sent to OSU and examined by scanning electron microscopy (SEM) to determine whether typhoid biofilms on gallstones contributed to chronic carriage. Interestingly, biofilms were
visualized with an estimated 80-95% surface coverage on 3/4 gallstones from typhoid carriers. A representative sample is highlighted in Fig. 4.5 at various magnifications (panels A-D), while specimens from the other typhoid positive patients are presented in Fig. 4.6A and B. A single gallstone from this group lacked biofilm formation (Fig. 4.6C), an observation likely attributed to a variety of gallstone and patient specific factors including the uniqueness in surface structure, smallness of the sample size, and the possibility that the primary constituent of this stone was not cholesterol, but rather calcium bilirubin as indicated by its black color. We have demonstrated previously that Eppendorf tubes coated with bilirubin supported significantly less biofilm than those coated with cholesterol in vitro, suggesting that serovar Typhi may not form biofilms on gallstones comprised of calcium bilirubinate in vivo (Crawford et al., 2008). To determine if gallstone biofilm formation was mediated specifically by serovar Typhi, gallstones from 6/13 *E. coli* positive patients were also sent to OSU and examined by SEM. Of this group, 4 samples lacked biofilm formation, whereas small patches of bacteria covering an estimated 5-20% of the surface were visualized on 2 others. Additionally, no biofilm formation was observed on gallstones from a patient positive for both *E. coli* and *S. flexneri* (Fig. 4.6D) or from patients free of culturable enterobacteriaciae. Table 4.2 provides a summary of biofilm formation and respective tissue burden for patients in this study. These results suggest that bacterial biofilms on gallstones of individuals presenting with cholelithiasis are mediated by serovar Typhi, providing the first clinical evidence that gallstone biofilms facilitate asymptomatic typhoid carriage.
4.4.4 Histology of infected gallbladder tissues

*Salmonella enterica* serovars Typhimurium and Typhi mediate different clinical manifestations in humans and, not surprisingly, distinct changes to intestinal pathology during infection (Raffatellu et al., 2006). Severe typhoid fever is marked by hypertrophy/ischaemia of intestinal tissue and necrosis or ulceration of the mesenteric lymph including Peyer’s patches (Everest et al., 2001), yet the histopathology of the gallbladder epithelium in response to infection remains unknown. To examine the amount of gallbladder inflammation/damage caused by *Salmonella* infections and/or the presence of gallstones, gallbladder tissues from infected and uninfected mice with or without cholesterol gallstones were collected at days 3, 7, and 21 P.I., fixed in formalin, embedded in paraffin, sectioned and mounted on slides, and stained with H&E. Microscopic evaluation demonstrated that the presence of eosinophils, lymphocytes, and plasma cells was minimal or nonexistent at acute and persistent time points P.I. and did not vary with infection or gallstones, and that epithelial cells appeared normal (Fig. 4.7). Any observed tissue mucosal hyperplasia or intralumenal crystals were not associated with infection, lithogenic diet, or time, but, rather, were artifacts of either instrumental crushing during removal, or hyalinosis, a condition affecting the nasal cavity, trachea, lung, esophagus, stomach, bile, pancreatic duct, and gallbladder of genetically engineered mice (2000).

Human gallbladder tissues from patients seeking cholecystectomy were rinsed in sterile saline, fixed in formalin, washed, and frozen in 30% sucrose in Mexico City, and processed for histological analysis at OSU. Microscopic examination revealed no pathological damage, including loss of epithelial folds, thickening of the mucosa, or
formation of debris, attributed to asymptomatic carriage of serovar Typhi (Fig. 4.8B) and 
E. coli (Fig. 4.8C), or in individuals without culturable bacteria (Fig. 4.8A). Occasional
rush or cautery artifacts due to surgery were observed. Collectively, these results suggest that gallbladder inflammation and associated histopathological alterations to epithelial integrity are minimal during bacterial infection and gallstone formation, and that this response does not change appreciably with time.

4.4.5 Serovar Typhi laboratory strains and clinical isolates demonstrate the same level of bile tolerance, and this resistance is independent of Vi antigen

Bile is known to affect virulence phenotypes including adhesion, antibiotic resistance, motility, and biofilm-related factors in many enterobacteraciae (Prouty and Gunn, 2000; Prouty et al., 2004b; Crawford et al., 2008; Lin et al., 2005b; Sleator et al., 2005; Torres et al., 2007; Chatterjee et al., 2007; Hung et al., 2006; Hung and Mekalanos, 2005; Whitfield and Roberts, 1999). Serovar Typhi strains express a polysaccharide capsule termed the Vi-antigen (Vi-ag) that is encoded by Salmonella pathogenicity island (SPI) 7 (Parkhill et al., 2001). It has been demonstrated that Vi expression in vitro is influenced by culture conditions including osmolarity (Pickard et al., 1994) and that SPI7 can be lost upon laboratory passage (Nair et al., 2004).

Although Vi agglutination can be used to identify typhoid carriers (Bhatnagar, 1948), not all clinical isolates express Vi-ag (Losonsky et al., 1987). We hypothesize that the Vi-ag capsule may play a role in bile resistance and be differentially expressed by systemic and gallbladder serovar Typhi isolates compared with laboratory strains. To compare bile tolerance amongst laboratory and clinical isolates, minimal inhibitory
concentration (MIC) assays were performed by challenging strains overnight with 0.2 to 60% crude ox bile extract. Serovar Typhi blood and gallstone isolates shared a 1% MIC value with laboratory strain Ty2, and this was far lower than observed for serovar Typhimurium 14028s (10%, Table 4.3). Interestingly, serovar Typhi Vi-capsule mutants constructed in the laboratory (tviB and rcsB) or obtained from 2 clinical isolates (unpublished observation, Geoffrey Gonzalez) were more tolerant to bile (6%) than wild-type Ty2 or clinical isolates. These results suggest that laboratory and patient serovar Typhi strains demonstrate equal resistance to bile, and that Vi-capsule expression may prevent tolerance to higher concentrations.

4.4.6 Cholesterol-bound serovar Typhimurium biofilms resist antibiotics but planktonic cells are susceptible

In general, bacterial biofilms resist commonly administered antibiotics while the viability of dispersed planktonic cells in such antimicrobials is drastically reduced (Costerton et al., 1999). Here, serovar Typhimurium biofilms were grown on cholesterol-coated Eppendorf tubes in the TBA and equivalent amounts of biofilm and planktonic cells were challenged with 25 μg/ml ciprofloxacin, 25 μg/ml chloramphenicol, or 30% bile for 2, 4, or 6 hours. Salmonellae embedded in biofilms resisted killing by all three antimicrobial agents, whereas planktonic cells were highly susceptible. Ciprofloxacin, a potent fluoroquinolone of clinical last resort, mediated a dramatic 2.5 log reduction in planktonic salmonellae but did not affect biofilm bacteria (Fig. 4.9). Chloramphenicol treatment reduced planktonic viability by 1.5 logs and a high concentration of bile decreased growth of free-swimmers by nearly 1 log (Fig. 4.9). These results further
suggest a strong need for alternative treatment methods against biofilm-mediated persistent infections, particularly asymptomatic carriage facilitated by serovar Typhi biofilms on cholesterol gallstones.

4.4.7 Serovar Typhimurium biofilm bacteria, but not dispersed planktonic cells, are resistant to neutrophil-mediated killing

Human PMNs have been demonstrated in vitro to enhance biofilm formation by *Pseudomonas aeruginosa* (Walker et al., 2005; Jesaitis et al., 2003) and be unable to kill or disperse *Staphylococcus epidermidis* biofilms (Kristian et al., 2008). To investigate the response of serovar Typhimurium biofilms on cholesterol to an important component of innate immunity, purified human neutrophils were added to biofilm bacteria and dispersed planktonic cells. These results are preliminary (performed once in triplicate), and suggest that free swimming cells are rapidly susceptible to neutrophil-mediated killing (30 and 60 minutes) whereas those in biofilms were resistant (Fig. 4.10). Therefore, typhoid biofilms on gallstones may mediate, in part, resistance to the phagocytic array of toxic oxygen species, defensins, and other degrading enzymes associated with neutrophils, should this host defense cell type be present in the gallbladder during typhoid colonization and persistence.

4.5 Discussion

Bacterial biofilm infections develop on live and dead tissues and medical devices, are slow to produce overt symptoms, resist cellular and humoral immune host responses and antibiotic regimens, and persist for long periods of time with intermittent recurrence
of symptoms until the sessile community is surgically removed (for a review see reference (Costerton et al., 1999)). Biofilm formation is thought to characterize 65% of chronic infections treated by physicians (Schaechter, 1987) and has been observed directly in a variety of clinical manifestations including nontypable *Haemophilus influenzae* (NTHI) mediated otitis media. NTHI has recently been shown to form biofilms on respiratory epithelial cells and the chinchilla respiratory tract in vitro and in vivo (Bakaletz et al., 2005; Jurcisek et al., 2007). The asymptomatic, chronic carrier state of *Salmonella enterica* serovar Typhi occurs in the bile-rich gallbladder, is almost always associated with the presence of cholesterol gallstones, and does not subside with antibiotic treatment (Levine et al., 1982; Lai et al., 1992), yet the progression from infection to persistence remains unknown. We hypothesize that gallbladder typhoid carriage is frequently a result of biofilm formation on cholesterol gallstone surfaces and have demonstrated that salmonellae form bile-induced biofilms on human gallstones and cholesterol-coated surfaces in vitro (Prouty et al., 2002a; Crawford et al., 2008). In this work, we develop a murine model of *Salmonella* persistence in naturally resistant mice harboring cholesterol gallstones and present clinical evidence that biofilms occur on gallstones from asymptomatic, human typhoid carriers.

Our understanding of *S. Typhi* pathogenesis and associated pathology during typhoid fever has been inferred from infections of susceptible inbred mouse strains lacking *Nramp1* (BALB/c and C57BL/6) with non-typhoidal serovar Typhimurium (Jones and Falkow, 1996; Galan and Curtiss, 1991). Naturally resistant Nramp<sup>+/+</sup> mice inoculated with serovar Typhimurium carried salmonellae asymptotically in spleens and gallbladders for long extensions of time (Nauciel et al., 1988; Monack et al., 2004).
Interestingly, gallbladder abnormalities are typically studied using a C57 variant that develops gallstones following 8 week consumption of a cholesterol-rich diet (Reihner and Stahlberg, 1996; Wittenburg et al., 2003). Here, resistant 129X1/SvJ mice fed a lithogenic diet (normal chow supplemented with 1% cholesterol and 0.5% cholic acid) consistently formed gallstones that enhanced colonization and persistence of serovar Typhimurium in gallbladder tissue and bile following intraperitoneal infection. The presence of this gallstone material associated with significant numbers of salmonellae that increased over time, and mediated a 3-log increase in fecal shedding compared with infected controls lacking gallstones. Equivalent numbers of serovar Typhimurium were recovered from spleens between groups with or without gallstones at all time points suggesting that, unlike observations in *Mycobacterium tuberculosis* (Martens et al., 2008), hypercholesterolemia did not impair general host immunity during murine infection with salmonellae.

Taken together, these results indicate that persistently infected 129X1/SvJ mice harboring diet-induced cholesterol gallstones increased gallbladder colonization and thus have the potential to serve as a model for the study of typhoid carriage. Mutants of serovar Typhimurium in factors shown to be important for gallstone biofilm formation in vitro including flagellar filament and O-antigen capsule expression could be tested for effects on gallbladder persistence in vivo. Furthermore, treatments shown to resolve murine cholesterol gallstones such as beta-muricholic acid or fatty acid bile conjugates (Wang and Tazuma, 2002; Leikin-Frenkel et al., 2008) could be added to mice carrying salmonellae to examine how the dissolution of stones impacts gallbladder carriage.
An estimated 25% of asymptomatic carriers have no history of typhoid fever and shedding is known to be sporadic (Parry et al., 2002), making the chronic carrier state difficult to confirm and an understudied aspect of global human health. It has been shown that typhoid carriage accelerates person-to-person transmission, is almost always associated with abnormalities of the gallbladder, including gallstones, and represents the largest risk factor for cancer of this organ (Dutta et al., 2000; Kumar et al., 2006; Lai et al., 1992). To better understand the association between gallstones and the chronic carrier state, individuals seeking cholecystectomy in an area endemic to typhoid fever were analyzed for the asymptomatic presence of serovar Typhi. Approximately 5% of this population was shown to be positive for typhoid in gallbladder tissue, bile, or gallstone sections analyzed by multiplex PCR. Interestingly, 3 of 4 gallstones from serovar Typhi positive patients visualized by SEM showed bacterial biofilms completely covering the surface. The negative sample was black pigmented and had a unique surface composition, suggesting that the main constituent of this gallstone was calcium bilirubinate, a substrate shown in vitro to support significantly less Salmonella biofilm formation than cholesterol (Crawford et al., 2008).

Gallstones from patients positive for E. coli were also analyzed by SEM and shown to be biofilm negative, although 2 of the 6 samples had small patches of bacteria on approximately 5-20% of the surface. Additionally, no biofilm formation was detected on gallstones from an E. coli and S. flexneri double positive individual or gallbladders free of bacteria, suggesting that, of those culturable bacteria, biofilm formation on gallstones is mediated specifically by serovar Typhi.
The human intestine is a dynamic organ that regulates nutrient and ion transport and acts as a critical mucosal immune barrier distinguishing the commensal microflora from enteric pathogens (Lippolis, 2008). Human enterocolitis induces massive neutrophilia whereas typhoid fever is characterized by mononuclear infiltrate, suggesting that the intestine employs separate response arsenals of pathogen recognition receptors (PRRs) and cytokines depending on the pathogen-associated molecular patterns (PAMPs) (for a review, see references (Tukel et al., 2006; Harris et al., 1972)). Interestingly, histopathological examination of H&E stained sections from human and mouse single cell-layered gallbladder epithelium samples did not reveal tissue damage or inflammatory mediators resulting from exposure to gallstones and/or acute or persistent bacterial infections.

Successful host colonization by enteric pathogens requires resistance to the detergent-like activity of bile salts. The mechanisms mediating bile tolerance are beginning to be elucidated, such as the Acr and Emr efflux pumps in *E. coli* (Thanassi et al., 1997), ToxR and ToxT regulated genes, and Vex efflux systems in *Vibrio cholerae* (Provenzano et al., 2000; Schuhmacher and Klose, 1999; Bina et al., 2006; Chatterjee et al., 2007), and the PhoP-PhoQ two-component regulatory system, Tol and MarRAB operons, Acr efflux pump, enterobacterial common antigen, and DNA adenine methylase activity in *S. enterica* serovars Typhimurium and Typhi (van Velkinburgh and Gunn, 1999; Prouty et al., 2002b; Prouty et al., 2004a; Ramos-Morales et al., 2003; Heithoff et al., 2001; Prieto et al., 2004). The SPI7 locus of serovar Typhi encodes expression of the Vi-capsule antigen, is genetically unstable and can be lost upon laboratory passage, and is absent from the serovar Typhimurium chromosome altogether (Nair et al., 2004). The
Vi-capsule has been shown to prevent Toll-like receptor 4 recognition (Wilson et al., 2008), and its loss in serovar Typhi isolates increased TNF-α production or IL-8 secretion production in macrophage or epithelial cell lines, respectively (Hirose et al., 1997; Sharma and Qadri, 2004). Vi agglutination from blood and bone marrow samples has long been used as a method of identifying typhoid carriers (Bhatnagar, 1948), yet not all clinical isolates express Vi-antigen and patients recovering from acute typhoid infections have low titres of serum IgG antibodies to Vi antigens (Losonsky et al., 1987). To compare resistance of laboratory and clinical isolates as well as Vi-antigen mutants, MIC assays were performed with crude ox bile extract at concentrations from 0.2 to 60%. Blood and gallstone isolates tolerated bile at the same levels as laboratory strain Ty2 whereas Vi mutants resisted bile at higher levels similar to serovar Typhimurium, suggesting that Vi-capsule expression may be inhibitory to bile resistance. We have demonstrated previously that Vi polysaccharides are not necessary for biofilm formation on gallstones in vitro (Prouty and Gunn, 2003). During typhoid carriage in the gallbladder, it is possible that Vi expression is lower than in other systemic sites to facilitate bile-induced gallstone biofilms. This hypothesis is purely speculative and needs investigation.

As mentioned above, bacterial biofilms resist commonly administered antibiotics and as a result are frequently implicated in persistent infections (Stewart and Costerton, 2001; Costerton et al., 1999). Bacteria dispersed from the biofilm microenvironment quickly regain susceptibility to those antibiotics, suggesting that biofilm-mediated resistance is not acquired through mutations or horizontal transfer of genes (Stewart and Costerton, 2001; Anwar et al., 1989; Williams et al., 1997).
Interestingly, established mechanisms of resistance including efflux pumps and the multiple antibiotic resistance (*mar*) locus of *E. coli* did not provide protection to bacteria in biofilms (Williams et al., 1997; Maira-Litran et al., 2000). In this study we grew serovar Typhimurium biofilms on cholesterol and challenged an equivalent amount of biofilm-embedded bacteria and planktonic cells with 25 μg/ml ciprofloxacin, 25 μg/ml chloramphenicol, or 30% bile for 2, 4, or 6 hours. The results from these experiments support a protective role for biofilm formation, showing that salmonellae encased in biofilms tolerate high concentrations of antimicrobials whereas planktonic cells are highly susceptible. Thus, there is a strong need for alternative treatment methods against biofilm-mediated persistent infections.

Biliary epithelial cells express mucin genes and secrete immunoglobulin proteins and a variety of cytokines during innate and adaptive immunity of the gastrointestinal tract (Maurer et al., 2009; Reynoso-Paz et al., 1999; Andrianifahanana et al., 2006), yet gallbladder biology and the associated immune response in typhoid carriage, particularly in those patients with gallstones biofilms, remain unknown. Biofilm formation by *P. aeruginosa* has been hypothesized to mediate colonization and antibiotic resistance in the context of cystic fibrosis, a lung disease that features persistent airway neutrophil accumulation. Interestingly, human neutrophils were shown in vitro to enhance *P. aeruginosa* biofilm development and even settle on the mature biofilm to become phagocytically engorged, degranulated, immobilized, and rounded during compromised host defense (Walker et al., 2005; Jesaitis et al., 2003). Similarly, *S. epidermidis* biofilm formation mediates a protective response in vitro against complement deposition and neutrophil-dependent killing (Kristian et al., 2008). Preliminary experiments reported
here suggest that serovar Typhimurium bacteria in cholesterol-bound biofilms resist neutrophil-mediated killing mechanisms but planktonic cells are susceptible.

We have developed a murine model of cholesterol gallstone-mediated typhoid carrier state, and provided the first clinical evidence that typhoid biofilms on gallstones may facilitate bacterial persistence in the hostile gallbladder during chronic carriage. We also demonstrated that biofilm-embedded bacteria are more resistant than dispersed planktonic cells to high concentrations of antimicrobials and innate immune mediators. These new observations and model system provide a better understanding of the typhoid carrier state and an important alternative, therapeutic target for alleviating asymptomatic gallbladder carriage of *S. Typhi* and human-to-human transmission.
<table>
<thead>
<tr>
<th>Strain</th>
<th>Characteristic(s)</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. Typhimurium</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>JSG210</td>
<td>ATCC 14208s (CDC6516-60); wild-type</td>
<td>ATCC</td>
</tr>
<tr>
<td><em>S. Typhi</em></td>
<td></td>
<td></td>
</tr>
<tr>
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<td>Blood isolate, San Antonio, TX</td>
<td>Univ. Hospital, J. Jorgensen</td>
</tr>
<tr>
<td>JSG698</td>
<td>Ty2; wild-type</td>
<td>ATCC</td>
</tr>
<tr>
<td>JSG791</td>
<td>Clinical isolate, Mexico City</td>
<td>U.N.A.M., C. Alpuche-Aranda</td>
</tr>
<tr>
<td>JSG792</td>
<td>Clinical isolate, Mexico City</td>
<td>U.N.A.M., C. Alpuche-Aranda</td>
</tr>
<tr>
<td>JSG1213</td>
<td>Ty2; <em>tviB::Kan</em></td>
<td>Popoff laboratory</td>
</tr>
<tr>
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<td>Ty2; <em>rcsB::Kan</em></td>
<td>Popoff laboratory</td>
</tr>
<tr>
<td>JSG3074</td>
<td>Gallstone isolate, Mexico City</td>
<td>U.N.A.M., C. Alpuche-Aranda</td>
</tr>
<tr>
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<td>Gallstone isolate, Mexico City</td>
<td>U.N.A.M., C. Alpuche-Aranda</td>
</tr>
<tr>
<td>JSG3076</td>
<td>Gallbladder tissue isolate, Mexico City</td>
<td>U.N.A.M., C. Alpuche-Aranda</td>
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</tbody>
</table>

Table 4.1: Bacterial strains and relevant characteristics
Table 4.2: Gallstone biofilm formation\(^a\) and bacterial burden\(^b\) in patient samples.

\(^a\)Gallstones from patients seeking cholecystectomy in Mexico City were visualized by SEM for the presence of bacterial biofilms. ++, robust biofilm formation covering an estimated 80-95% of gallstone surface; +, little biofilm present (covering approximately 5-20% gallstone surface); -, absence of biofilm formation on gallstone surface.

\(^b\) Bacteria recovered from the gallbladder tissues, bile, and gallstone sections in patients with gallstones were plated for CFU and identified using multiplex PCR as described in materials and methods.

<table>
<thead>
<tr>
<th>No.</th>
<th>GB Tissue</th>
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<th>Gallstones</th>
<th>Biofilm</th>
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<tr>
<td>1</td>
<td><em>S. Typhi</em> 1.2x10(^3) CFU/ml</td>
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<td><em>S. Typhi</em> 1.4x10(^3) CFU/ml</td>
<td>++</td>
</tr>
<tr>
<td>2</td>
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<td>No Growth</td>
<td><em>S. Typhi</em> 80 CFU/ml</td>
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<tr>
<td>3</td>
<td><em>S. Typhi</em> 20 CFU/ml</td>
<td><em>S. Typhi</em> 20 CFU/ml</td>
<td><em>S. Typhi</em> 40 CFU/ml</td>
<td>++</td>
</tr>
<tr>
<td>4</td>
<td>No Growth</td>
<td>No Growth</td>
<td><em>S. Typhi</em> 20 CFU/ml</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
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<td><em>E. coli</em> 3x10(^5) CFU/ml</td>
<td><em>E. coli</em> 4.5x10(^5) CFU/ml</td>
<td>+</td>
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<tr>
<td>6</td>
<td><em>E. coli</em> 1x10(^5) CFU/ml</td>
<td><em>E. coli</em> 1x10(^5) CFU/ml</td>
<td><em>E. coli</em> 1x10(^5) CFU/ml</td>
<td>+</td>
</tr>
<tr>
<td>7</td>
<td><em>E. coli</em> 3x10(^4) CFU/ml</td>
<td><em>E. coli</em> 1x10(^5) CFU/ml</td>
<td><em>E. coli</em> 1x10(^5) CFU/ml</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td><em>E. coli</em> 1x10(^5) CFU/ml</td>
<td><em>E. coli</em> 1x10(^5) CFU/ml</td>
<td><em>E. coli</em> 1x10(^5) CFU/ml</td>
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<tr>
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<td><em>E. coli</em> 1x10(^5) CFU/ml</td>
<td><em>E. coli</em> 1x10(^5) CFU/ml</td>
<td><em>E. coli</em> 1x10(^5) CFU/ml</td>
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<tr>
<td>10*</td>
<td><em>E. coli</em> 3.6x10(^5) CFU/ml</td>
<td><em>E. coli</em> 5x10(^5) CFU/ml</td>
<td><em>E. coli</em> 3x10(^5) CFU/ml</td>
<td>-</td>
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<td>Strain</td>
<td>Description</td>
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<td>JSG791</td>
<td>\textit{S}. Typhi clinical isolate (Vi-ag negative)</td>
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<td>\textit{S}. Typhi wild-type Ty2 (ATCC)</td>
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<tr>
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<td>\textit{S}. Typhi gallstone isolate</td>
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</table>

Table 4.3: Bile MICs for clinical isolates and laboratory strains of serovar Typhi from a representative independent experiment done in triplicate (n = 3). \textsuperscript{a}Presented as percentage of crude ox bile extract
Figure 4.1: Effects of an 8 week lithogenic diet (mouse chow supplemented with 1% cholesterol and 0.5% cholic acid) in mice naturally resistant to serovar Typhimurium infections (n = 30, 2 independent experiments). (A) Cholesterol diet-induced gallstones in the gallbladder of Nramp1+/+ 129X1/SvJ mice. (B) Gallbladder stones removed and pictured with a 25-gage needle for scale. (C) Placement of gallstone material in 1.7 ml Eppendorf tubes with 1 x PBS prior to homogenization.
Figure 4.2: CFU enumerations (mg/ml) from organs of 129X1/SvJ mice fed normal chow or lithogenic diet at days 3, 7, and 21 post intraperitoneal infections with $1.4 \times 10^4$ serovar Typhimurium 14028s ($n = 42$, 21 mice for each diet group, 7 per time point; 2 individual experiments; means $\pm$ SD are given). (A) Intake of excessive dietary cholesterol did not enhance general infection or spleen burden at any time point. (B) The presence of gallstones significantly increased the amount of salmonellae recovered from gallbladder tissues at late time points compared with diet control during persistent murine infections. *, statistical significance ($P < 0.01$) based on a two-tailed Student $t$ test.
Figure 4.3: Cholesterol gallstones harbor salmonellae and increase the recovery of serovar Typhimurium from bile of persistently infected 129X1/SvJ mice (presented are means + SD for 2 independent experiments; n = 42; 21 mice per diet group, 7 for each time point). (A) CFU from gallbladder bile increases significantly with time post infection in mice fed a lithogenic diet for 8 weeks compared to infected mice fed normal chow. (B) Salmonellae were recovered from homogenized gallstones, and CFU values increased over time post infection. *, statistical significance ($P < 0.01$) based on a two-tailed Student $t$ test.
Figure 4.4: Serovar Typhimurium is shed in fecal matter from infected 129X1/SvJ mice. (A) Approximately 5 to 7 fresh stool pellets were collected from mice belonging to both diet groups 3 weeks post infection, weighed, homogenized, and plated on SS agar. Salmonellae are indicated by colorless colonies with black centers. (B) Mice harboring cholesterol gallstones shed 3 logs more serovar Typhimurium than those on a normal chow diet. Means ± SD are given for 2 independent experiments using 7 infected animals per diet group. *, statistical significance ($P < 0.01$) based on a two-tailed Student $t$ test.
Figure 4.5: Gallbladder stones from an asymptomatic typhoid carrier in Mexico City support biofilm formation. SEM micrographs show serovar Typhi bacilli embedded in biofilms on the surfaces of gallstones at magnifications of 1,500x (A), 2,000x (B), 2,400x (C), and 16,000x (D).
Figure 4.6: Biofilm formation on human gallstones was observed only in patients seeking cholecystectomy at the Hospital Infantil positive for typhoid carriage. SEM micrographs show bacterial biofilms on gallstones of 2 asymptomatic typhoid carriers in panels A and B. (C) Biofilm formation was not detected on gallstone fragments from a single serovar Typhi carrier. The unique surface texture and pigment were suggestive of black, calcium bilirubinate gallstones. (D) Absence of biofilm formation on gallbladder stones from a patient positive for *E. coli* and *S. flexneri*.
Figure 4.7: Gallbladder tissues stained by hematoxylin and eosin (H&E) from human patients seeking cholecystectomy in Mexico City. After surgical removal, samples were rinsed in sterile saline, fixed in 10% buffered formalin, washed, placed in 30% sucrose, and frozen at -70°C, and shipped to OSU. Tissues were embedded in paraffin blocks, mounted on slides in 4 μm thick sections, and stained with H&E for pathologic examination. (A) Tissue from patient with no culturable bacteria (n = 4). (B) Representative section from individual carrying serovar Typhi (n = 5). (C) Tissue from patient harboring *E. coli* (n = 9).
Figure 4.8: Representative H&E stained gallbladder sections from mice with gallstones at days 3, 7, and 21 post infection from 2 independent experiments. Mice gallbladders (n = 2 per time point) were removed immediately post sacrifice, rinsed aseptically in saline, and fixed in 10% neutral buffered formalin for 72 hours. Samples were paraffin-embedded, sectioned to a thickness of 4 μm, and observed for histopathological changes compared with uninfected controls.
Figure 4.9: Serovar Typhimurium biofilms grown on cholesterol-coated Eppendorf tubes resist antibiotics but planktonic cells are susceptible. Equivalent amounts of *Salmonella* biofilm bacteria and dispersed planktonic cells from a 6 day TBA were challenged with 25 $\mu$g/ml ciprofloxacin, 25 $\mu$g/ml chloramphenicol, or 30% bile for 2, 4, or 6 hours. The viability of planktonic cells decreased by nearly 2.5, 1.5, and 1 logs, after incubation with ciprofloxacin, chloramphenicol, and bile, respectively whereas biofilm cells were unaffected. Shown are mean values from 3 independent experiments.
Figure 4.10: Biofilm embedded serovar Typhimurium resist neutrophil-mediated killing but dispersed planktonic cells are significantly susceptible. Human neutrophils were purified and added to serovar Typhimurium biofilms grown on cholesterol-coated coverslips in 24-well plates or planktonic cells removed from each biofilm-containing well, and incubated for 30 or 60 minutes. Planktonic cells and crystal violet stained biofilms were quantified by measuring optical density at 570 nm. Data are preliminary, and represent a single experiment performed in triplicate.
Chapter 5

General Discussion

The ubiquitous existence of microorganisms in natural reservoirs has shaped the evolutionary dynamics of planet Earth. As a result, microbial pathogens have induced diseases in humans and animals on every continent and in every century throughout history. One such manifestation, typhoid fever, causes greater than 22 million illnesses and 600,000 deaths world-wide each year, with particular concentration in impoverished, endemic societies (Butler et al., 1978; Crump et al., 2004). *S. enterica* serovar Typhi, a gram-negative bacterium exquisitely adapted to induce disease strictly in humans, was first cultured in 1884 and is the etiologic agent of typhoid fever. Person-to-person spread of serovar Typhi occurs via the fecal-oral route and is enhanced by its low infectious dose.

The introduction of multi-drug resistant strains is making typhoid fever management particularly problematic (Bhan et al., 2005). Antibiotics are also influencing the clonal expansion of serovar Typhi. Nalidixic acid and fluoroquinolone resistant variants that are prevalent in southeastern Asia originated in the early 1990s, migrated to Africa, and were shown to be phylogenetically different than the H58 parent (Roumagnac et al., 2006). Despite these recent shifts, the overall population structure of
serovar Typhi remains without large disruptions and is thus indicative of neutral evolution (Roumagnac et al., 2006). Divergence and differentiation resulting from geographic isolation, clonal replacement, selective sweeps, and population bottlenecks is evident in the genomes of monomorphic pathogens including *Mycobacterium tuberculosis* and *Yersinia pestis*, but not serovar Typhi (Roumagnac et al., 2006). All typhoid haplotypes descended from the H45 strain before the Neolithic period and are thought to maintain neutral evolution and genetic buffering by existence in an asymptomatic, chronic carrier state (Roumagnac et al., 2006). Subsequent outbreaks or infections may provide a vehicle for intercontinental transmission and a global distribution of carriers for multiple haplotypes (Roumagnac et al., 2006), making surveillance of the carrier state an important public health initiative.

Serovar Typhi persists asymptotically in the gallbladder of approximately 3-5% of patients suffering from typhoid fever and is shed in feces long after overt symptoms subside (Levine et al., 1982). This percentage is low, but even a single typhoid carrier exacerbates transmission in endemic areas and has a multiplicative effect. The idea of healthy carriers in the epidemiology of typhoid fever was first championed by Robert Koch in 1902 (Mortimer, 1999), long before the implementation of the science of bacteriology and its associated advances to the practice of medicine. Although the most notorious contemporary carrier has come to be Typhoid Mary, Mr. N the milker initiated more outbreaks during the same time period in Folkestone, England (Mortimer, 1999).

Historically, development of chronic typhoid carriage is linked to the presence of gallbladder stones (Lai et al., 1992). Gallstone formation (cholelithogenesis) was first described in 1507, observed in mummified Egyptians, and typically induced by
cholesterol supersaturation from bile (Maurer et al., 2009; Portincasa et al., 2006).

World-wide, the primary constituent of gallbladder stones is cholesterol whereas only 10% are calcified (Portincasa et al., 2006). To date, removal of the gallbladder (cholecystectomy) remains the most effective treatment option for these individuals.

In patients carrying both S. Typhi and cholesterol gallstones in the gallbladder, clinically administered antibiotics are typically ineffective against dissolution of the persistent bacterial infection (Lai et al., 1992). Chronic typhoid carriers with gallstones also experience an 8.47-fold-higher risk for developing hepatobiliary diseases, including carcinoma of the gallbladder (Dutta et al., 2000; Kumar et al., 2006; Surette and Bassler, 1999). In 1994, Caygill et al. demonstrated that gallbladder cancers were associated with long-term typhoid carriage, but not acute infections (Caygill et al., 1994). It is possible that persistent expression of bacterial endotoxin or sustained, low levels of inflammation and epithelial tissue damage due to the formation and presence of gallstones and the acute stage of infection may be carcinogenic.

We hypothesize that biofilm formation on cholesterol gallstones facilitates colonization and persistence of serovar Typhi in the bile-rich gallbladder. Bacterial biofilms are increasingly associated with medical and industrial ecosystems and have been implicated as the cause of many antibiotic resistant, persistent infections in humans (Stewart and Costerton, 2001; Costerton et al., 1999). Biofilm formation is characterized by adherence of bacteria to a surface, growth and recruitment, development of a protective extracellular (ECM) matrix, and release of planktonic cells from this sessile community into the environment (Costerton et al., 1995). Similarly, shedding of S. Typhi from asymptomatic, chronic carriers is thought to account for much of the person-to-
person transmission of typhoid fever in impoverished societies, and we have demonstrated that bile is required for mature biofilm formation with characteristic ECM production of *S. enterica* serovars Typhimurium and Typhi on human gallstones in vitro (Prouty et al., 2002a; Prouty and Gunn, 2003).

The work in this dissertation sought to advance this seminal observation through the following aims: determine the critical genes mediating bile-induced adherence specifically to cholesterol surfaces during the initiation of biofilm formation (early events); identify bile-mediated factors of the extracellular matrix (ECM) of biofilms specifically on gallstones/cholesterol surfaces (late events); evaluate the role of gallstone biofilms in a human population of typhoid carriers, and develop a model of chronic *Salmonella* gallbladder carriage in mice with gallstones.

We show that the flagellar filament of serovar Typhimurium is necessary for and specific toward cholesterol binding during the initiation of biofilm formation, and that the FliC subunit is uniquely required. *Salmonella* flagella subunits FliC (phase 1) and FljB (phase 2) control directional motility and are alternately expressed in a process called phase variation (Bonifield and Hughes, 2003). Recent experiments suggested that a *luxS* serovar Typhimurium mutant strain polarized flagellar phase variation toward the more immunogenic phase 1 flagellin independently of quorum sensing (Karavolos et al., 2008). Quorum sensing is a process of cell-cell communication in bacteria that involves production and detection of signaling molecules (autoinducers (AIs)) and has been hypothesized to play a role in biofilm formation (reviewed in (von Bodman et al., 2008)). Interestingly, a serovar Typhimurium *luxS* mutant unable to produce AI-2 did not affect biofilm formation on cholesterol-coated tubes in the TBA (data not shown).
observation suggests that *Salmonella* biofilm formation does not involve quorum sensing, or, alternatively, that flagella locked in phase 1 (FliC) mediated binding to cholesterol and subsequent biofilm development in the *luxS* mutant. *S. Typhi* is monophasic, harboring only the *fliC* gene (Baker et al., 2007), and FliC expression was anatomically restricted to certain tissues during systemic serovar Typhimurium mouse infections (Cummings et al., 2006). It is possible that control of FliC expression in serovar Typhi during human infection is an evolutionarily adapted mechanism mediating binding to cholesterol gallstones and biofilm formation, thus facilitating typhoid gallbladder colonization and persistence in chronic carriers.

The majority of colonies (18/49) affecting serovar Typhimurium adherence to cholesterol carried transposon insertions in *fimW*, an inhibitor of type 1 fimbriae production. Mutations in *fimW* exhibit a four-to eightfold increase in fimbrial expression (Tinker et al., 2001) compared with parent strains, suggesting that a hyperfimbriate phenotype may inhibit binding to and biofilm formation on cholesterol surfaces. FimW was recently shown to downregulate expression of FimY, a putative regulatory protein that activates transcription of type 1 fimbriae by direct binding to the FimA promoter (Saini et al., 2009). Interestingly, FimY interacts with and enhances expression of FimZ, a molecular switch in serovar Typhimurium that positively regulates production of fimbriae by activating the *fimA* promoter, and negatively impacts SPI-1 mediated invasion by upregulation of *hilE*, a known repressor (Saini et al., 2009). It has also been demonstrated that tetracycline-mediated overexpression of FimZ decreased flagellar antigen production and, in turn, motility (Clegg and Hughes, 2002). However, transmission electron micrographs revealed the presence of intact flagellar filaments in
these strains and it is unknown whether FimZ binds to the FlhDC master operon promoter (Clegg and Hughes, 2002), suggesting that a direct link between FimZ and motility remains unconfirmed.

Fimbriae are attributed, in part, to aiding serovar Typhimurium biofilm formation on HEp-2 tissue culture cells and the intestinal epithelium of mice and chickens (Boddicker et al., 2002; Ledeboer and Jones, 2005; Baumler et al., 1996). In studies by Prouty et al. and unpublished data here, a serovar Typhimurium SR11 strain containing mutations in four fimbrial operons was observed to adhere and form biofilms on cholesterol gallstones and cholesterol-coated Eppendorf tubes in a manner similar to wild-type serovar Typhimurium (Prouty et al., 2002a). These data suggest that fimbriae encoded by the \textit{fim}, \textit{agf}, \textit{lpf}, and \textit{pef} operons do not drastically affect biofilms on cholesterol-coated surfaces. Therefore, regulation of serovar Typhimurium fimbrial expression is a complex process that may be dictated by the extracellular milieu (both time and space) and have overlapping effects on other virulence phenotypes including flagella production, invasion, and biofilm formation. Transcriptional alterations of singular genes in these cascades may not always show predicted phenotypic variations due to functional redundancies, regulation by other genes, or post-translational modifications.

A high number of colonies with transposon insertions in \textit{ompC} were also demonstrated to affect binding of serovar Typhimurium to cholesterol. The OmpC porin is regulated by osmolarity, permits passive diffusion of nutrients and antibiotics between the extra- and intra-cellular environments, and mediates both intestinal colonization of serovar Typhimurium in chickens and adherence of serovar Typhimurium to
macrophages (Puente et al., 1991; Methner et al., 2004; Negm and Pistole, 1999).

Bacteria within biofilms are thought to encounter higher osmolarity than in the liquid phase (Prigent-Combaret et al., 1999), and preliminary experiments performed in our laboratory indicate that loss of OmpC causes a significant decrease in serovar Typhimurium biofilm formation in conditions of high osmolarity (3% bile, LB + 0.3 M NaCl) but leads to increased biofilm growth compared to the wild-type strain in low media osmolarity (LB + 0.05 M NaCl) (data not shown). These results suggest that OmpC is necessary for binding and biofilm formation in the presence of bile, possibly by mediating electrostatic interactions between salmonellae and the cholesterol surface, promoting overall biofilm health as a nutrient channel, or regulating cell-cell interactions as is the case in E. coli biofilms. Interestingly, internal glucose stores did not enhance biofilm formation by exponentially growing cultures of S. enteritidis, whereas OmpC mediated intake of environmental glucose was a critical component (Bonafonte et al., 2000). Similarly, OmpC expression may be needed to allow sensing and internalization of bile salts that positively regulate gene transcription involved in Salmonella biofilm formation on cholesterol surfaces and gallstones.

Our results also suggest that chemotaxis is necessary for biofilm formation on cholesterol in the presence of bile, whereas swimming motility and fimbrial expression are dispensable. Bacterial flagellar filament, motility, and chemotaxis have been implicated in biofilm formation for a variety of enterobacteriaceae including P. aeruginosa (O'Toole and Kolter, 1998b; Watnick et al., 2001) and E. coli (Pratt and Kolter, 1998; Prigent-Combaret et al., 2000). We also demonstrate that swarming, a specialized form of surface movement and colonization unrelated to swimming (Wang et al., 2004), is
important for adherence to cholesterol surfaces and that flagella are not needed during late events of biofilm formation. Therefore, the involvement of flagella during bile-mediated biofilm formation appears to be highly regulated and stage specific, and may occur in the following manner. Initially, planktonic cells growing in bile use chemotactic behavior (enhanced tumbling) rather than traditional swimming motility to locate and approach cholesterol surfaces, and swarming to evaluate substrate fitness. Subsequent adherence to cholesterol is mediated by the flagellar filament subunit FliC in combination with other factors including OmpC, but can be inhibited by overexpression of fimbriae. Late stages of biofilm formation require ECM production independently of flagella, as a flagellar mutant was unaffected for biofilm formation when added to a layer of fixed bacteria. Lack of a flagellum induced exopolysaccharide (EPS) expression and biofilm formation in V. cholerae (Lauriano et al., 2004). Therefore, temporal regulation of flagella may serve as an important signal required for early biofilm events (attachment), but downregulated during late biofilm events (ECM production).

The work presented here also identified a bile-induced EPS, the O-antigen (O-ag) capsule, required for biofilm formation specifically on gallstone and cholesterol-coated surfaces. The O-ag capsule, characterized in the laboratory of William Kay, is conserved amongst salmonellae, similar in structure to the O-ag of LPS, and encoded by two divergent operons (yihU-yshA and yihV-yihW) (Gibson et al., 2006). Despite having similar repeating sugar units, the O-ag capsule and the LPS O-ag differ in size, charge, substitution patterns, and immunoreactivity (White et al., 2003). In addition, this capsule is linked to the membrane via a lipid anchor. O-ag capsule expression was found to be necessary for serovar Enteritidis biofilm formation leading to enhanced plant colonization.
Gibson and colleagues showed that AgfD, an important regulatory factor of biofilm formation (Brombacher et al., 2003; Romling et al., 1998), controls expression of the *yih* operons (Gibson et al., 2006). However, loss of AgfD had no effect on biofilm formation in the TBA. Furthermore, real-time PCR revealed that bile upregulated transcription of the *yih* operon in an *agfD*-independent manner, suggesting that another bile-responsive transcription factor is involved in *yih* gene expression.

Production of the O-ag capsule is encoded by two divergent operons, *yihU-yshA* and *yihVW*, yet little is known about the regulation of these genes during assembly and translocation. Serovar Typhimurium strains carrying *lacZ* reporter fusions to O-ag capsule promoters could be randomly mutagenized using a pool of Tn10d-Tet transposons to characterize bile-mediated transcription of genes in these operons.

Recent work in *P. aeruginosa* has investigated stage-specific factors including twitching motility, cell-to-cell signaling, stationary phase sigma factor expression, and polysaccharide synthesis locus (PSL) activity that contribute to biofilm development in flow cell systems using statistical analysis and direct visualization (Heydorn et al., 2002). To determine the temporal involvement of the O-ag capsule, biofilm formation on cholesterol surfaces could be similarly monitored using a flow cell apparatus (Ma et al., 2009). At various time points, biofilms from GFP-labeled wild-type, *yihO* mutant, and *yihO* overexpressing strains would be examined for O-ag capsule production using specific primary antibody and a fluorescein-conjugated secondary under confocal laser or immunofluorescence microscopy. Alternatively, O-ag expression could be controlled in space and time by an arabinose-inducible promoter in a mutant background during
biofilm formation in the static TBA. Crystal violet staining of biofilms over time could determine when O-ag production is necessary.

Interestingly, much of what is known about the systemic pathogenesis and associated pathology of *S.* Typhi during typhoid fever has been extrapolated from infections of inbred mouse strains lacking *Nramp1* (BALB/c and C57BL/6) with non-typhoidal serovar Typhimurium. *Nramp1* is a macrophage transmembrane protein hypothesized to affect transport of cations (Skamene et al., 1998). In this model, bacterial mediated endocytosis at microfold (M) cells in the intestinal epithelium is followed by interaction with phagocytic cells of the underlying lymphoid tissue, a primary bacteraemia in the blood, and uncontrolled replication within macrophages of the liver, spleen, and bone marrow leading to death at 7 to 10 days post infection (Jepson and Clark, 2001; Jones and Falkow, 1996; Galan and Curtiss, 1991; Richter-Dahlfors et al., 1997; Salcedo et al., 2001).

Recent experiments monitoring *Salmonella* infections in wild-type 129X1/SvJ (*Nramp*+/+) mice reported recovery of salmonellae from the gallbladders of asymptomatic animals at late time points post infection (Monack et al., 2004; Deng et al., 2008). The murine gallbladder has also been shown to carry gallstones following an 8 week lithogenic diet (normal chow supplemented with 1% cholesterol and 0.5% cholic acid) in a C57 inbred variant strain (Reihner and Stahlberg, 1996; Wittenburg et al., 2003). Here, resistant 129X1/SvJ mice fed a lithogenic diet consistently formed gallstones and, as a result, demonstrated enhanced colonization of gallbladder tissue and bile as well as fecal shedding during persistent serovar Typhimurium infection. Equivalent numbers of serovar Typhimurium were recovered from spleens between groups with or without
gallstones at all time points suggesting that, unlike observations in *Mycobacterium tuberculosis* (Martens et al., 2008), hypercholesterolemia did not impair general host immunity during murine infection with salmonellae. Cholesterol gallbladder stones were shown to associate with significant amounts of salmonellae that increased with time post infection. Furthermore, the amount of serovar Typhimurium recovered at 3 weeks post infection from fecal pellets of mice with gallstones was significantly higher than observed for mice on the control diet.

These results indicate that persistently infected 129X1/SvJ mice harboring diet-induced cholesterol gallstones increased gallbladder colonization and fecal shedding, and thus have the potential to serve as a model for the study of typhoid carriage. Mutants of serovar Typhimurium in factors shown to be important for gallstone biofilm formation in vitro including flagellar filament and O-antigen capsule expression could be tested for effects on gallbladder persistence in vivo. However, serovar Typhimurium O-ag capsule mutants were attenuated for virulence during oral infection of BALB/c mice. In these experiments, salmonellae were recovered in the spleen but not the Peyer’s patches or gallbladder. Therefore, O-ag mutants may need to be infected directly into the gallbladders of 129X1/SvJ mice harboring gallstones, and this will be attempted at Ohio State using micro-injection guided by a VisualSonics ultrasound machine. Direct injection of microorganisms into the gallbladder has been demonstrated in cannulated guinea pigs (Lavergne et al., 1977) and, if unsuccessful here, could be attempted using an established model of cholelithiasis in prairie dogs (Giurgiu et al., 1997).

The Vi-capsule antigen of serovar Typhi has been demonstrated to reduce Toll-like receptor (TLR) dependent cytokine production by preventing TLR4 recognition in
tissue cultured models (Wilson et al., 2008). This stealth mechanism has been proposed to facilitate intestinal invasion and systemic infection by masking the proinflammatory effects of LPS (Tsolis et al., 2008). The O-ag capsule is expressed specifically by salmonellae, is larger than LPS, and was shown to be a critical bile-induced component of gallstone biofilms in vitro ECM whereas Vi-capsule was not. The production of O-ag capsule in typhoid gallstone biofilms may similarly prevent TLR-mediated inflammation in the gallbladder as a mechanism of host immune evasion during establishment and maintenance of the asymptomatic carrier state. RNA from gallbladder tissues persistently infected with wild-type and O-ag capsule overexpressing strains could be isolated, converted to cDNA, and analyzed by real-time PCR for the levels of cytokines and chemokines using a mouse immune panel from Applied Biosystems (Ordway et al., 2006).

Treatments shown to resolve murine cholesterol gallstones such as beta-muricholic acid or fatty acid bile conjugates (Wang and Tazuma, 2002; Leikin-Frenkel et al., 2008) could be administered to mice carrying salmonellae to examine how the dissolution of stones impacts gallbladder carriage. In fact, the clinical administration of bile has therapeutic activity. Specifically, bile acids have been shown to be efficacious as topical agents countering sexually transmitted diseases and intravenously for viral hepatitis (Chen et al., 2003; Herold et al., 1999). The viral hepatitides, common causes of liver disease globally, have been shown to encounter bile during pathogenesis, and bile duct damage is a characteristic histopathological consequence of the massive immune response triggered by chronic hepatitis C virus (HCV) infection (Haruna et al., 2001; Lefkowitch et al., 1993). It has been noted that administration of bile acids, such as urso
deoxycholic acid, induced significant improvements in serum transaminase activities in hepatitis B and hepatitis C (Chen et al., 2003). Bile acid therapy may not resolve typhoid biofilms on gallstone surfaces during asymptomatic carriage, but could initiate biofilm dispersal in conjunction with drugs targeting specific ECM constituents, or kill planktonic bacteria to prevent environmental shedding.

Enhancing the bile salt hydrolase properties of probiotics could increase the demand for cholesterol in the liver for de novo bile acid synthesis, thereby removing it from the blood to provide a treatment for patients with hypercholesterolaemia (Begley et al., 2005a). In malnourished individuals who are susceptible to enteric infections, increasing bile acid levels in the intestine via oral administration or by an increase in hormones that result in increased bile production or secretion would bolster their defense arsenal against invading pathogens. As long as the integrity of the normal microbial flora is maintained, compounds such as those discussed above could be manufactured to target known factors contributing to bile resistance. It is likely that future bile research will target temporal and spatial sensing mechanisms by bacteria, as bile-mediated virulence factor expression leading to colonization and persistence must be better understood.

Efflux pump inhibitors (EPIs) also present a new avenue of intervention, and towards this goal, Lin et al. recently showed that oral administration of two different EPIs reduced colonization of *C. jejuni* in chickens at two to four days post inoculation, whereas *cmeABC* mutants failed to do so at any point during the 20 day study (Lin et al., 2003; Lin and Martinez, 2006). The CmeABC efflux pump is required for *Campylobacter* resistance to bile salts, the very same compounds that enhanced both expression of this locus and tolerance to multiple antibiotics including cefotaxime,
novobiocin and fusidic acid in culture media (Lin et al., 2005b). The *Salmonella* AcrAB efflux pump is similarly necessary for bile tolerance (Prouty et al., 2004b), and represents a potential target of EPI therapy.

*S.* Typhi biofilm formation on cholesterol gallstones as a mechanism mediating the chronic carrier state of typhoid fever in humans was investigated here for the first time. Individuals seeking cholecystectomy due to gallstones were analyzed for asymptomatic serovar Typhi carriage at the Hospital Infantil in Mexico City. Results indicate that 5% of patients with cholelithiasis carried serovar Typhi and that bacterial biofilms could be visualized on gallstones from 3/4 of these carriers. Biofilm formation was not observed on patients positive for other bacteria or whose gallbladders failed to possess culturable aerobic bacteria, suggesting that gallstone biofilms are a typhoid specific phenomenon.

It has been demonstrated that salmonellae do not form biofilms on every area of human gallstones in vitro, and that salmonellae form significantly more biofilms on Eppendorf tubes coated with cholesterol than with bilirubin. These results suggest that gallstone composition plays a significant role in biofilm formation during typhoid gallbladder carriage. Interestingly, a study of cholecystectomized patients in Athens, Greece, demonstrated that older patients had multiple small gallstones and little gallbladder inflammation, whereas younger patients presented with solitary gallbladder stones and tissue damage (Domeyer et al., 2008). Because the risk for typhoid carriage is severe in the elderly population and gallbladder inflammation is hypothesized to be low during human cholesterol gallstone formation (Maurer et al., 2009), a collection of small
stones and minimal inflammation may increase the risk of persistent serovar Typhi gallbladder colonization during human infection.

Detection of asymptomatic carriers is made difficult by the irregular nature of shedding from these individuals (Parry et al., 2002). *V. cholerae* is also transmitted via the fecal-oral route, and bile acids have been shown to mediate biofilm of this microorganism by regulating the *vps* genes (*Vibrio* polysaccharide synthesis) and their transcriptional activator VpsR (Hung et al., 2006). A recent epidemiological and ecological survey of *V. cholerae* in the drinking water from an area susceptible to annual cholera outbreaks demonstrated that persistence in aquatic environments required biofilm-induced nonculturability (Alam et al., 2007). *V. cholerae* biofilms were also present in fresh stool samples from infected human volunteers, suggesting that bacterial biofilms enhance environmental fitness and allow stealthy transmission. It would be of great interest to know whether person-to-person passage of serovar Typhi from chronic carriers involves biofilms in feces or contaminated food and water.

Prevention of typhoid fever demands better food and water sanitation methods in impoverished countries that cannot afford them, and improvement from vaccines that generate strong side effects or modest immunogenicity. This issue is further complicated by the many defense mechanisms needed to clear typhoid infections, including mucosal and systemic immune responses. Travel to India, Pakistan, Mexico, Bangladesh, the Philippines, and Haiti accounted for 76% of all travel acquired typhoid fever infections in patients from developed countries (Bhan et al., 2005). Inactivated whole cell vaccines against typhoid have largely been discontinued as a global public health tool due to their high reactogenicity (Guzman et al., 2006), leaving Vi-based subunit vaccines and live
attenuated oral vaccines as the only licensed alternatives. The Vi parenteral vaccine, however, does not provide immunological memory, as immune responses against polysaccharides do not involve T cells (Guzman et al., 2006). Live attenuated oral vaccines were first administered in the 1970s but risk reversion to virulence and increase the amount of genetically manipulated strains shed into the environment (Abd El Ghany et al., 2007; Kotton and Hohmann, 2004). The live typhoid vaccine Ty21a is an attenuated Ty2 derivative with an RpoS-, GalE-, and Vi-negative phenotype (Santander et al., 2007) that required three or four doses to confer protection in controlled field studies, but had variable effectiveness in trials (Wahdan et al., 1982; Levine et al., 1987).

Typhoid fever remains a significant burden to public health and safety worldwide. The hope for serovar Typhi eradication rests with multi-faceted treatment and prevention strategies that seek to improve sanitation and hygiene, diagnostic tools, immunizations, and targeted drug therapies. To that end, this dissertation provides a better understanding of the regulatory mechanisms and bacterial surface components that mediate *Salmonella* biofilm formation on gallstones and cholesterol surfaces and, thus, novel therapeutic targets for potentially alleviating gallbladder carriage and much of the human-to-human transmission linked to the asymptomatic, chronic carrier state.
List of References


44. **Brombacher, E., C. Dorel, A. J. Zehnder, and P. Landini.** 2003. The curli biosynthesis regulator CsgD co-ordinates the expression of both positive and negative determinants for biofilm formation in Escherichia coli. Microbiology **149**:2847-57.


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Notes: CORPORATE NAME: European Listeria Genome Consortium


Notes: GENERAL NOTE: PIP: TJ: ANNALS OF TROPICAL PAEDIATRICS.


