Salmonella Binding to and Biofilm Formation on Cholesterol/Gallstone Surfaces in the Chronic Carrier State

Undergraduate Honors Thesis

Presented in Partial Fulfillment of the Requirements for the Degree Bachelor of Science with Distinction in the School of Allied Medical Professions of The Ohio State University

Kristin E. Reeve
Undergraduate Biomedical Science Major
School of Allied Medical Professions
The Ohio State University
2010

Thesis Committee:

Dr. John S. Gunn, Ph.D., Adviser

Dr. Daniel Wozniak, Ph.D.

Dr. Margaret H. Teaford, Ph.D.

Table of Contents

Abstract

The Gram-negative pathogen *Salmonella enterica* serovar Typhi is the etiologic

agent of human typhoid fever, a systemic illness characterized by high fever, bradycardia,

and muscle pain. A percentage of these infections can result in asymptomatic carriage of

salmonellae in the bile-rich gallbladder, and we have previously demonstrated that

salmonellae can form biofilms on the surface of human cholesterol gallstones as a

mechanism that contributes to the development of the carrier state. To determine which

genes/ligands mediate the ability of *Salmonella* to bind and form biofilms on cholesterol,

mutants of *S. enterica* serovar Typhimurium were created through random transposon

mutagenesis. These mutants were screened for impaired biofilm formation on

cholesterol-coated Eppendorf tubes in the Tube Biofilm Assay (TBA) but normal biofilm

formation on glass and plastic surfaces. Of the 49 mutants with this phenotype, 70% of

the disrupted genes were involved in flagella or fimbriae biosynthesis. Independent

assays demonstrated that the presence of flagella were important for adherence to

cholesterol and biofilm initiation, while over-expression of fimbriae was inhibitory. The

remaining transposon insertions, located in *sseI* and *ompC*, had no effect on cellular

motility, suggesting a mechanism of action independent of flagellar-mediated adherence

to cholesterol. Subsequent analysis of *sseI* and related mutations in the TBA suggested

that SPI-2 genes are important for the formation of biofilms in the presence of bile, but

unimportant for biofilm formation in the absence of bile. Similar analysis of *ompC* in the

TBA demonstrated that the observed loss of biofilm formation was not due to changes in

the osmolarity of the extracellular environment. These studies provide a better

understanding of how salmonellae form biofilms in the presence of bile and suggest a

target for therapies that may alleviate biofilm formation on cholesterol gallstones and the

chronic carrier state.

Chapter 1

Introduction

1.1 Problem Statement

      Salmonella enterica is a diverse gram-negative bacterial species with many

serovars and variations in host specificity. One of the most well-known serovars is

Salmonella enterica serovar Typhi, which causes an acute systemic infection known as

Typhoid fever and can, after overt symptoms wane, persist asymptomatically in the

human gallbladder in a chronic carrier state (Prouty and Gunn, 2003; Prouty et al., 2002).

The bacteria are able to survive in the gallbladder, which is also the storage site for bile, a

detergent-like substance that aids in dispersal of lipids during digestion and acts as a

potent anti-microbial in the gastrointestinal tract (Prouty et al., 2002). Although the

infected individual does not show any signs of illness, they can still infect others through

fecal-oral transmission (Prouty and Gunn, 2003; Prouty et al., 2002).

      It is not known how *Salmonella* colonize and persist in the gallbladder to establish

this asymptomatic, chronic carrier state. This thesis project sought to characterize

Salmonella genes that mediate cholesterol binding and subsequent biofilm formation.

We compared biofilm formation between wild-type salmonellae and mutants to identify

those with impaired biofilm-forming abilities specifically on cholesterol, and these assays

were performed in the presence and absence of bile. Results from a screen for *S.*

Typhimurium mutants deficient in binding to cholesterol-coated surfaces but not glass or

plastic surfaces focused on four groups of genes. Potential applications of our research

include targeted drug therapy, which could be used to reduce or eliminate the number of chronic carriers of *S.* Typhi.

1.2 Review of Literature

      The *Salmonella enterica* species includes many different serovars, including Typhi and Typhimurium. *S.* Typhimurium causes gastroenteritis in humans, symptoms of which include cramping, nausea, vomiting, and diarrhea (Coburn, et al., 2007). *S.* Typhi is the etiological agent of typhoid fever, a systemic infection resulting in high fever, bradycardia, and bacteremia (Tsolis, et al., 2008;). It is also a human-specific pathogen (Tsolis, et al., 2008). *S.* Typhimurium is well understood at the genetic level, induces an enteric fever-like illness in murine mammals, and is thus used in the laboratory as a model for *S.* Typhi (Crawford, et al., 2008; Prouty and Gunn, 2003; Prouty et al., 2002).

      S. Typhi and *S.* Typhimurium infections are global health concerns. *S.* Typhimurium-induced gastroenteritis is generally cleared within 7-10 days, but can cause death in immunocompromised individuals (Crawford, et al., 2008). *S.* Typhi infections cause approximately 17 million cases of typhoid fever and 600,000 deaths worldwide every year (Crump, et al., 2004). The chronic carrier state of *S.* Typhi, occurring in approximately 5 – 10% of infected individuals, is dangerous because carriers can still infect others and are often unaware that they are carrying a deadly organism (Prouty and Gunn, 2003). The bacteria persist in the gallbladder, despite the caustic nature of bile in the gallbladder and the gallbladder's continuous cycle of bile excretion (Lai, et al., 1992). Antibiotic therapy is often ineffective in treating chronic gallbladder carriage of *S.* Typhi

(Lai, et al., 1992). Additionally, there is an increased risk of gallbladder cancer for chronic carriers (Lai, et al., 1992).

It has been shown that gallstones are the most significant risk factor for becoming a chronic carrier of *S.* Typhi (Lai, et al., 1992). Human gallstones are primarily comprised of either cholesterol or calcium bilirubinate, and previous research in our laboratory has demonstrated that salmonellae form mature biofilms specifically on cholesterol human gallstones *in vitro* (Prouty, et al., 2002) and on cholesterol mouse gallstones *in vivo* (Crawford, et al., 2010b). A mature biofilm, as previously defined, is a multilayer bacterial community over 15 times thicker than a monolayer of cells (~31 μm compared to ~2 μm) and possessing a copious extracellular matrix (Prouty, et al., 2002). These results were confirmed with the Tube Biofilm Assay (TBA), an assay used to study biofilm formation in an *in vitro*, standardized environment (Crawford, et al., 2008). The TBA utilizes siliconized Eppendorf tubes evenly coated with cholesterol in order to study *Salmonella* growth and biofilm formation in a bile-rich environment on cholesterol surfaces, serving as a model for cholesterol gallstones in the human gallbladder. Detailed information about the design of the TBA can be found in the Materials and Methods section.

A biofilm is defined as a bacterial colony adhered to a solid surface that secretes a self-initiated, protective exopolysaccharide matrix (Fig. 1.1) (Costerton, et al., 1995). After initial colonization on the solid surface, cells recruit and replicate until a microcolony forms, which then progresses to a mature biofilm as the exopolysaccharide matrix is formed. Biofilms allow the continual shedding and reattachment of individual cells, contributing to the spread of bacteria, particularly in the human host (Levine, et al.,

1982). Many human pathogens have been shown to form biofilms, including *Escherichia coli* (Pratt and Kolter, 1998), *Vibrio cholerae* (Yildiz and Schoolnik, 1999), and *Staphylococcus aureus* (Vaudaux et al., 1995). The protective nature of biofilms also confers resistance to antibiotics (Lai, et al., 1992). As a result, biofilms are a major health concern and have been implicated in food- and water-borne illnesses, hospital-acquired infections, and asymptomatic colonization in the human host (Davey and O'Toole, 2000).

1.3 Objectives

This project was part of an ongoing effort to characterize genes that mediate biofilm formation on cholesterol, specifically genes that promote initial binding to cholesterol-coated surfaces. Since the mechanisms of *Salmonella* biofilm formation on cholesterol surfaces are not fully understood, any investigation into this area serves to further our understanding and characterization of the *Salmonella* chronic carrier state in the gallbladder. These studies could also have implications for *Salmonella* biofilm formation on a variety of other surfaces, as well as biofilm formation in other pathogenic bacteria, including *E. coli*, *V. cholerae*, and *S. aureus*. The ultimate goal for this project is to determine the genes responsible for bile-induced biofilm formation and pave the way for targeted gene or drug therapies based on these findings. Such therapies have the potential to reduce or eliminate the chronic carrier state of *Salmonella* Typhi, thus alleviating the global health burden of typhoid fever.

Chapter 2

Materials and Methods

2.1 Bacterial strains, growth conditions, and molecular biology techniques

The *Salmonella* strains used in this study are listed in Table 2.1. Luria-Bertani (LB) broth and agar were used for bacterial growth, creation of mutants, biofilm assays, and flagellum 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 [OD600], 0.6 to 0.8). When necessary, antibiotics were added at the following concentrations: kanamycin (Kan), 25 µg/ml; chloramphenicol (Cam), 25 µg/ml; and tetracycline (Tet), 15 µg/ml. Molecular cloning and PCR were performed using established protocols. Plasmids were purified using QIAprep spin miniprep kits (Qiagen, Valencia, CA) and were transformed by electroporation as previously described (Schmid and Roth, 1983).

2.2 Transposon mutagenesis and screening

Cholesterol-binding-deficient, tetracycline-resistant serovar Typhimurium strains were created by random transposon mutagenesis using an established method (Schmid and Roth, 1983). Tn*10*d-Tet transposons were introduced into wild-type serovar Typhimurium. Strains containing Tn*10*d transposon insertions were selected on plates containing LB agar with Tet, and 40,000 individual colonies were pooled in LB broth containing Tet. The serovar Typhimurium transposon mutant pool was grown to log phase (OD600, 0.6) in LB broth containing Tet with 3% crude ox bile extract, and 100-µl

portions were added to siliconized Eppendorf tubes (Fisher Scientific, Pittsburgh, PA) coated with 1 mg of chromatography-grade cholesterol (Sigma, St. Louis, MO). Following incubation for 24 h at room temperature on a Nutator shaker (Labnet International, Edison, NJ), 10 µl of the planktonic, nonadherent culture was removed and added to 90 µl of fresh LB broth containing 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 h for 10 days. Serial dilutions of the final planktonic culture were plated on LB agar containing Tet, and 500 individual colonies were screened for loss of or a defect in biofilm formation on cholesterol-coated surfaces (values that were 0 to 25% of wild-type values) and preservation of biofilm formation on glass and plastic coverslips (values that were 75 to 100% of wild-type values). 49 colonies were confirmed to have a non-biofilm-forming (on cholesterol only) phenotype by backtransduction using P22 HT *int*-105. Direct genomic DNA sequencing from the transposon 5' end and sequence analyses revealed that the Tn*10*d insertions mapped to several genes corresponding to the serovar Typhimurium loci *fimW*, *ompC*, *flhA*, *fliF*, *fliA*, *fliJ*, *fliL*, and *sseI* (Table 2.2). These strains provided the foundation of the studies outlined in this work.

2.3 Cholesterol, glass, and plastic surface biofilm assays

Salmonella strains were tested to determine their abilities to form biofilms in the tube biofilm assay (TBA) 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. The resulting cultures were incubated on a Nutator

shaker at room temperature for 6 days. Every 24 h, the medium was removed, the tubes

were washed two times with LB medium, and fresh medium (LB medium with or without

3% bile) was added. Bound bacterial samples were fixed at 60°C for 1 h, and a solution

of 0.1% crystal violet (gentian violet in isopropanol-methanol-1x phosphate-buffered

saline [PBS] [1:1:18]) was then added to stain cells for 5 min at room temperature. The

tubes were washed with 1x PBS, and the dye was extracted using 33% acetic acid and

quantified by determining the optical density at 570 nm.

2.4 DNA sequencing and bioinformatics

Sequencing of transposon mutant genomic DNA from serovar Typhimurium

cholesterol-binding-deficient colonies with primer JG1787 (5'-

CCTTTTTCCGTGATGGTA-3') was performed using an Applied Biosystems 3730

DNA capillary analyzer and BigDye cycle fluorescent terminator chemistry at 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).

2.5 Assay of adherence 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 grown to an optical density at 600 nm of 0.6. Bacteria in

these cultures were killed by incubation for 40 min in 10% formalin or by heat fixation

for 20 min at 65°C. Triplicate 100-μl aliquots of live and dead salmonellae were added to

24-well polystyrene tissue culture plates (Becton Dickinson Labware, Franklin Lakes,

NJ) coated with 1 mg of cholesterol per well, and the plates were centrifuged at 165

relative centrifugal force (RCF) for 5 min to initiate contact between the bacteria and

cholesterol. Three hours of incubation at room temperature for adherence was followed

by five washes in 1x PBS, staining of wells with a 0.1% crystal violet solution (gentian

violet in isopropanol-methanol-1x PBS [1:1:18]) for 5 min, and five more washes with 1x

PBS. The dye retained by bound cells was extracted using 33% acetic acid and was

quantified by determining the optical density at 570 nm.

2.6 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 100-μl aliquots were added to siliconized Eppendorf tubes coated

with 1 mg of chromatography-grade cholesterol. Following incubation at room

temperature on a Nutator shaker for 24 h, cultures were removed, and the tubes were

washed three times in LB broth to remove nonadherent bacteria. Live cultures of late-log-

phase wild-type or *flhC* serovar Typhimurium strains were added to the tubes, and

biofilm formation was examined using the TBA as described above.

2.7 Purification of serovar Typhimurium flagellin

Serovar Typhimurium wild-type, phase 1 (*fliC*; H:i) mutant, phase 2 (*fljB*; H:1,2)

mutant, and flagellin (*fliCfljB*) mutant strains were grown to late log phase in LB medium

at 37°C. The resulting cultures (500 ml) were centrifuged at 8,000 x g for 15 min. The

cell pellets were washed once, resuspended in 15 ml 1x PBS, and sheared mechanically

for 3 min at 30,000 rpm using a Power Gen 125 tissue homogenizer (Fisher Scientific, Pittsburgh, PA). Flagellar filaments were separated from cellular debris by centrifugation for 10 min at 8,000 x g. The flagellum-containing supernatants were collected and centrifuged at 100,000 x g for 1 h. Filaments were gently resuspended overnight in 1x PBS with slow shaking at 4°C and then centrifuged at 100,000 x g. This 24-h washing cycle was repeated twice, and the final pellets of purified serovar Typhimurium flagellin were resuspended in 1x PBS and stored at -20°C. The protein concentrations of isolated flagellin were determined with a bicinchoninic acid (BCA) assay kit (Pierce Biotechnology Inc., Rockford, IL), and the purity was confirmed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and staining with GelCode Blue reagent (Pierce Biotechnology Inc., Rockford, IL).

2.8 Western blotting

Monoclonal antibodies against *Salmonella* species flagella (Maine Biotechnology Services, Portland, ME) and FliC subunit protein (Bio-Legend, San Diego, CA) were used to probe purified flagellin from serovar Typhimurium wild-type, *fljB* mutant, and *fliC* mutant strains in a 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 min. Preparations were separated by 10% SDS-PAGE and transferred to Hybond-ECL nitrocellulose (Amersham Biosciences, Pittsburgh, PA) using a Trans-Blot semidry transfer apparatus (Bio-Rad, Hercules, CA). Membranes were blocked overnight in 5% bovine serum albumin (BSA) (Sigma, St. Louis, MO) and incubated with antiflagellum antibody (diluted 1:200 in PBS; Maine Biotechnology Services, Portland,

ME) or anti-FliC antibody (5 µg diluted in 5 ml PBS; BioLegend, San Diego, CA) for 4 h. Goat anti-mouse horseradish peroxidase (HRP) conjugate (Bio-Rad, Hercules, CA) diluted 1:5,000 in PBS (with 2 h of incubation) and enhanced chemiluminescence (ECL) reagents (GE Healthcare, Pittsburgh, PA) were used to detect bound antibodies. Bands were visualized after exposure and development on HyBlot CL autoradiography film (Denville Scientific Inc., Metuchen, NJ). All washes were performed with 1x PBS.

2.9 Subunit binding ELISA

Purified flagellin from serovar Typhimurium wild-type, *fljB* mutant, and *fliC* mutant strains was assayed to determine its ability to bind cholesterol using a modified enzyme-linked immunosorbent assay (ELISA). Chromatography-grade cholesterol was dissolved in anhydrous ether (J. T. Baker, Phillipsburg, NJ) at a concentration of 25 mg/ml, and 100-µl aliquots were added to polystyrene wells in 96-well Microtest tissue culture plates (Becton Dickinson Labware, Franklin Lakes, NJ). Six 1-µg replicates of each purified flagellin sample were then added to the cholesterol-coated wells. Following 3 h of binding and incubation at room temperature, the plates were washed three times in 1x PBS and blocked overnight with 3% BSA. Wells were emptied, washed, and incubated with antiflagellum antibody (diluted 1:20 in 0.3% BSA) or anti-FliC antibody (diluted 1:100 in 0.3% BSA) for 2 h, both in triplicate for a total of six replicates. Another washing step was followed by addition of goat anti-rabbit HRP conjugate (Bio-Rad, Hercules, CA) diluted 1:5,000 in 0.3% BSA and incubation for 1 h. 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 to determine the optical density at 415 nm.

Chapter 3

Results and Discussion

3.1 Results

3.1.1. Fimbriae

Fimbriae have been shown to mediate adherence during initiation of biofilm formation and cell-cell interactions during biofilm growth for a variety of microorganisms (Davey and O'Toole, 2000; O'Toole and Kolter, 1998). The serovar Typhimurium genome contains 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 the chicken intestinal epithelium (Boddicker, et al., 2002; Ledeboer and Jones, 2005) but not on human gallstones incubated with bile (Prouty, et al., 2002).

A serovar Typhimurium SR11 strain having mutations in four fimbrial operons (*fim*, *agf*, *lpf*, and *pef*) was added to the TBA to examine biofilm formation on cholesterol-coated surfaces. To test whether serovar Typhimurium cholesterol-binding deficient mutants with mutations affecting type 1 fimbriae could form mature biofilms, strains having Tn*10*d transposon insertions in *fimW* were added to cholesterol-coated Eppendorf tubes in the TBA. FimW negatively regulates FimY, a multifunctional protein that positively regulates production of fimbriae by activating the *fimA* promoter (Saini, et al., 2009). To determine whether the *fimW*-mediated effects on biofilm formation were a direct result of disruption of type 1 fimbriae, a strain having a deletion of the type 1 fimbrial operon marked with a kanamycin cassette was transduced in the *fimW*::Tn*10*d-

Tet background. To further test whether over-expression of type 1 fimbriae could affect biofilm formation in an otherwise wild-type background, a strain with pISF101, which contains the type 1 fimbrial gene cluster and results in a hyperfimbriate phenotype, was added to the TBA.

The results of the TBA are detailed in Fig. 3.1. In the presence of bile, the amount of biofilm formed by the quadruple-mutant SR11 strain equaled the amount of biofilm formed by serovar Typhimurium wild-type strain 14028s, suggesting that fimbriae encoded by the *fim*, *agf*, *lpf*, and *pef* operons do not contribute to biofilm formation in this static assay. A *fimW*::Tn*10*d-Tet mutation rendered serovar Typhimurium deficient for biofilm formation in the presence of 3% bile. Complementation of this mutation with a plasmid-borne copy of *fimW* resulted in near WT biofilm formation (data not shown). When *fimW* was disrupted by insertion of a kanamycin cassette, the resulting serovar Typhimurium mutant expressed 4- to 8-fold more type 1 fimbriae than the parent strain (Tinker, et al., 2001). This *fimW*::Kan mutant was deficient for biofilm formation at levels similar to those of the strain having a transposon insertion in *fimW* (Fig. 3.1). The strain with the pISF101 plasmid, demonstrating a hyperfimbriate phenotype, did not form a biofilm in the TBA (Fig. 3.1). The strain lacking *fimW* and type 1 fimbrial genes (including *fimA*) exhibited wild-type levels of biofilm formation in the TBA (Fig. 3.1). Collectively, these results demonstrate that over-expression of type 1 fimbriae plays a negative role during *Salmonella* binding to cholesterol and that the inhibition prevents subsequent maturation of a cholesterol biofilm formed by the bacterium.

3.1.2 Flagella

The highly ordered transcriptional hierarchy controlling expression of flagella is comprised of three classes of genes and is regulated by many global signals (Apel and Surette, 2007). Therefore, it is no surprise that flagella make various contributions to biofilm formation depending on the environmental conditions, such as binding substrate material, nutrient limitation, temperature, medium flow rate, and other factors (Barken, et al., 2008; Merritt, et al., 2007; O'Toole and Kolter, 1998). Expression of the serovar Typhimurium flagella has been shown to inhibit biofilm formation on polystyrene wells (Teplitski, et al., 2006) but to promote biofilm development on human gallstones when bile is added to the growth medium (Prouty and Gunn, 2003; Prouty et. al, 2002). However, the stage at which this appendage positively or negatively impacts *Salmonella* biofilms has not been defined. To examine whether production of the flagellar filament is necessary for biofilm formation on cholesterol, a mutation in the flagellar transcriptional activation gene (*flhC*) in serovar Typhimurium was created and tested in the TBA. As demonstrated above (Table 2.2), mutations in serovar Typhimurium flagellum structural and biosynthesis genes affected binding to and biofilm formation on cholesterol. To determine if the physical presence of the flagellar filament or flagellum-mediated motility was required for biofilm formation, mutants that expressed flagella but could not swim (*motA*), demonstrated a smooth-swimming phenotype only (*cheA*, *cheR*, *cheY*), and demonstrated a hyper-tumbling phenotype only (*cheB*, *cheZ*) were also tested in the TBA.

The TBA results are detailed in Figs. 3.2 and 3.3. The *flhC* mutant strain did not form a mature biofilm on cholesterol, providing direct evidence of the importance of flagella during biofilm development. A serovar Typhimurium *motA* mutation (which eliminates flagellar motility but not synthesis) (Dean, et al., 1984) did not reduce the

18

levels of biofilm on cholesterol surfaces in the presence or absence of bile compared to the results obtained for the parent strain, suggesting that motility is not critical for development of serovar Typhimurium biofilms on cholesterol-coated surfaces. Chemotaxis mutants demonstrating either smooth swimming and hyper-tumbling phenotypes (Pratt and Kolter, 1998) showed increased biofilm formation in the absence of bile, but biofilm formation in presence of bile was comparable to wild-type, suggesting that functional chemotaxis is inhibitory for biofilm formation in the absence of bile, but not in the presence of bile.

The presence of bile has been shown to modestly downregulate serovar Typhimurium motility and flagellar gene expression in β-galactosidase assays using MudJ fusions to *flhC*, *flgC*, and *fliC* (Prouty, et al., 2004). Interestingly, bile is required for formation of mature biofilms on cholesterol-coated Eppendorf tubes, and flagellum biosynthesis mediates, at least in part, attachment to this surface for biofilm development. 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 posttranscriptional level, wild-type and *flhC* mutant strains of serovar Typhimurium were grown to late exponential phase with or without 3% bile and examined by transmission electron microscopy (TEM). For the wild-type strain TEM analysis, the average number of flagella was nearly 6 flagella per bacterium regardless of the growth conditions, whereas no flagella were observed for the *flhC* mutant (Fig. 3.4). Therefore, while exposure to bile may transcriptionally down-regulate flagellar genes, bile has no effect on the number of flagella.

If flagella mediate attachment to cholesterol, then dead salmonellae expressing intact flagella should bind to cholesterol in the TBA. Wild-type and *flhC* strains of serovar Typhimurium were fixed in 10% formalin for 40 min or heat killed at 65°C for 20 min and added to cholesterol-coated wells of a 24-well tissue culture plate. Live and dead wild-type cells bound to cholesterol following 3 h of incubation, and the association was only modestly enhanced by bile (Table 3.1). Strains lacking FlhC production did not bind to cholesterol under any of the conditions tested, further suggesting that the serovar Typhimurium flagellar filament mediates binding to cholesterol in the early stages of biofilm formation.

Furthermore, to determine if flagellum-mediated binding could provide a scaffold for serovar Typhimurium biofilm formation, formalin-fixed salmonellae were incubated in cholesterol-coated Eppendorf tubes for 24 h and washed vigorously with 1x PBS. A late-logarithmic culture of wild-type serovar Typhimurium grown in 3% bile was added on top of the bound cells, and the standard 6-day TBA was performed. To test whether the presence of flagella contributed to later events during biofilm development, a live serovar Typhimurium *flhC* mutant culture was added to a scaffold of bound, dead wild-type cells, and a TBA was performed.

When formalin-fixed salmonellae were added, these killed, bound cells were able to support biofilm formation by live wild-type serovar Typhimurium, and the amounts of the biofilms were larger than the amounts of the biofilms for salmonellae grown in the TBA without this scaffold (Table 3.2). The *flhC* mutant, while deficient in biofilm formation on cholesterol-coated surfaces (Fig. 3.2), was able to form a biofilm on the dead cell scaffold, suggesting that serovar Typhimurium flagellar filaments, while

necessary for binding to cholesterol, do not contribute to subsequent biofilm development (Table 3.2). Furthermore, the amount of biofilm formed by an *flhC* mutant on dead cells was significantly larger the amount of biofilm formed by the wild-type strain under the same conditions, suggesting that after mediating the initial binding, flagella may inhibit biofilm growth (Table 3.2).

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:1,2) (Chilcott and Hughes, 2000; de Vries, et al., 1998). During the well-characterized phase variation process, these subunits are alternatively expressed by a genetic control mechanism (Aldridge, et al., 2006; Bonifield and Hughes, 2003; Silverman, et al., 1979). To determine which subunit protein mediated binding of the serovar Typhimurium flagellar filament to cholesterol, antibody to *Salmonella* whole flagella and FliC was used in a quantitative binding ELISA. Briefly, flagella were isolated and purified from serovar Typhimurium wild-type, *fliC*, *fljB*, and *fliCfljB* strains using a mechanical shearing protocol adapted from the protocol of Andersen-Nissen et al. (2007). Wild-type and phase-locked mutant flagellin preparations (1 μg each) were separated by 10% SDS-PAGE. Monoclonal antiflagellin antibodies (Fig. 3.5B) that recognized a peptide that is present in both FliC and FljB or only in FliC were used. Purified proteins from all samples were added to cholesterol-coated wells of a 96-well tissue culture plate and analyzed by a modified ELISA.

Based on the amino acid sequences, the predicted molecular masses of the FliC and FljB proteins were 51.6 and 52.5 kDa, respectively (Uchiya and Nikai, 2008), and the approximately 1-kDa difference was detected by Coomassie blue staining (Fig. 3.5A) and

21

Western blotting. The identity of each band was confirmed by comparing the wild-type

and mutant lanes (Fig. 3.5). Flagella from the wild-type and *fljB* strains bound equally

well to cholesterol ELISA, while flagella from the *fliC* mutant showed decreased binding,

suggesting that FliC is the critical serovar Typhimurium flagellar subunit that mediates

binding to cholesterol (Fig. 3.6).

3.1.3 Porin

OmpC is an outer membrane porin that allows for passage of nutrients and

antibiotics between the extra- and intra-cellular milieu via passive diffusion (Dorman, et

al., 1989). It has been well characterized in many members of *Enterobacteriaceae*,

including *Salmonella* (Puente, et al., 1991). OmpC, OmpD, and OmpF are the three

major osmoregulatory porins in *Salmonella*, and they are controlled by the two-

component regulatory system EnvZ-OmpR based on the osmolarity of the environment

(Dorman, et al., 1989). During periods of high osmolarity, OmpF expression is reduced,

but OmpC is expressed in equal amounts in both high and low osmolarity (Puente, et al.,

1991).

Bacteria within biofilms are thought to encounter higher osmolarity than in the

liquid phase due to the higher gradient of ions and ionized molecules near the liquid-solid

interface (Prigent-Combaret, et al., 1999). Studies in *E. coli* have shown that OmpC

expression is upregulated in biofilm cells (Prigent-Combaret, et al., 1999). Since OmpF

expression is reduced in high osmolarity, a mutant lacking a functional *ompC* gene may

be unable to persist in the high osmolarity environment of the biofilm with fewer porins

to regulate the osmolarity of the cell. To test whether biofilm formation of *ompC* mutants

is affected by variations in solution osmolarity, a modified TBA was performed with LB-3% bile medium of varying osmolarity (0.11 M – 0.41 M). Osmolarity was artificially altered in solution by varying the amount of sodium chloride added to the broth.

The TBA results are detailed in Fig. 3.7. Compared to wild-type *Salmonella*, the *ompC* mutant strain showed decreased biofilm formation, but biofilm formation was unaffected by variations in osmolarity. At this time, the exact cause of decreased biofilm formation in *ompC* mutants is unclear, but it appears that the defect is the result of a phenotype other than osmolarity alteration.

3.1.4 Secreted Effector Protein

SseI is a secreted effector protein belonging to the GDSL family of lipases that colocalizes with the polymerizing actin cytoskeleton during host invasion (Miao, et al., 2003). It is a member of the effector proteins secreted by the SPI-2 Type III Secretion System (Miao, et al., 2003). GDSL proteins associated with SPI-2 have been shown to hydrolyse phospholipids and esterify cholesterol intracellularly (Miao, et al., 2003; Ehrbar and Hardt, 2005). It has also been demonstrated that *sseI* is required to establish long-term *Salmonella* infection in a murine model (McLaughlin, et al., 2009). To examine whether production of the secreted effector protein is necessary for biofilm formation on cholesterol, the *sseI* mutation recovered in the transposon mutagenesis and related SPI-2 genes were analyzed in the TBA. These related genes included another SPI-2 secreted effector protein gene, *sseJ*, and a SPI-2 knockout strain.

The TBA results are detailed in Fig. 3.8. When compared to wild-type, the *sseI* mutant strain recovered from the initial transposon mutagenesis formed increased

biofilms on cholesterol in the absense of 3% bile but decreased biofilms in the presence

of bile. Similar results were obtained for a second *sseI* strain obtained from a different

serovar Typhimurium background, as well as the secreted effector gene *sseJ* and the SPI-

2 knockout mutant. Together, these results suggest that SPI-2 genes are important for the

formation of biofilms in the presence of bile, and SPI-2 mutants show enhanced biofilm

formation in the absence of bile.

3.2 Discussion

Based on previous demonstrations that *Salmonella* persistence in bile and chronic

carriage is mediated by biofilm formation on cholesterol surfaces and cholesterol

gallstones (Crawford, et al., 2008; Prouty et al., 2002), we have isolated several *S.*

Typhimurium genes crucial to cholesterol binding. These genes were divided into four

categories: fimbrial genes (*fimW*), flagellar genes (*flhA*, *fliF*, *fliA*, *fliJ*, and *fliL*), porin

genes (*ompC*), and secreted effector protein genes (*sseI*).

FimW has been shown to down-regulate production of type 1 fimbriae by

inhibiting FimY, a protein that enhances expression of the *fim* structural genes by

activating the *fimA* promoter (Saini, et al., 2009), and chromosomal *fimW* mutants

demonstrate a hyper-fimbriate phenotype (Tinker, et al., 2001). The *fimW*::Kan,

fimW::Tn*10*d-Tet and Type I fimbriae overexpressing serovar Typhimurium strains did

not form a biofilm on cholesterol. Mutation of *fimA* in a *fimW* mutant background

restored biofilm formation to that of wild-type serovar Typhimurium. These data suggest

that over-expression of type 1 fimbriae plays a negative role during *Salmonella* binding

to cholesterol and subsequent biofilm formation.

Flagella have been shown to promote surface binding during biofilm formation in *E. coli* (Giron, et al., 2002) and *Pseudomonas* spp. (Lillehoj, et al., 2002), as well as adhesion during colonization of tissue cultured cells and mucus by *Salmonella* spp. (Dibb-Fuller, et al., 1999). To examine whether the physical presence of the flagellar filament was necessary for biofilm formation on cholesterol, a serovar Typhimurium mutant lacking flagella (*flhC*) 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.

Past studies have suggested that flagellar motility is impacted by environmental cues, such as bile enhancing tumbling frequency while modestly down-regulating motility in *S. enterica* (Prouty, et al., 2004), so *S.* Typhimurium mutant strains lacking motor function (*motA*) and functional chemotaxis (*cheA*, *cheB*, *cheR*, *cheY*, and *cheZ*) were analyzed for biofilm formation in the TBA. Loss of flagellar motility had no significant effect on biofilm development in LB broth with or without bile. Similarly, loss of functional chemotaxis was unimportant in LB broth with bile. These results indicate that flagellar motility is dispensable to biofilm formation on cholesterol surfaces in the presence of bile.

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 *flhC* mutant serovar Typhimurium strains were added to cholesterol-coated wells. The results of these experiments indicate that surface expression of intact flagella is 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. The *flhC* mutant showed a greater increase in biofilm formation on a substrate of bound bacteria when compared to wild-type, 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 flagellar filament of *S. enterica* is comprised of two antigenically distinct subunit proteins, FliC (H:*i*) and FljB (H:*1,2*). To determine which flagellar subunit mediated adherence to cholesterol, a modified 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 has been shown to be anatomically restricted to certain tissues during systemic serovar Typhimurium mouse infections (Cummings, et al., 2006).

The investigation of the flagellar and fimbrial genes recovered in the original transposon mutagenesis is largely complete, but further examination of the *ompC* and *sseI* insertions will be required in the future. The lambdoid phage Gifsy-2 encodes SseI (Ehrbar and Hardt, 2005; Miao, et al., 2003), and the receptor for Gifsy-2 is OmpC (Ho and Slauch, 2001). The association between SseI and OmpC and the known interaction of SseI with cholesterol suggest a role for adherence and/or modification of cholesterol during biofilm formation.

Preliminary studies of the *ompC* mutant demonstrated that biofilm formation in the presence of bile was decreased when compared to wild-type. No significant

difference was observed in *ompC* biofilm formation at varying osmolarities in the presence of bile. Future studies for *ompC* will include growth curve analyses and MIC assays to determine the nature of the biofilm and/or growth defects shown in the TBA (Fig. 3.7). Of the fourteen Tn*10*d insertions found within *ompC*, five were located downstream of the start codon at bp position 252 (JSG3064) and nine others were found at position 346 (JSG3073), suggesting potential genomic hotspots crucial to cholesterol binding. The *ompC* studies described above utilized only JSG3064, so studies comparing the two *ompC* strains will also be performed to assess any potential differences in the mutations.

Preliminary studies of *sseI* and other SPI-2 genes showed that SPI-2 mutant strains demonstrated increased biofilm formation in the absence of bile, suggesting that SPI-2 genes are important for biofilm formation in the presence of bile, but unimportant for biofilm formation in the absence of bile. SPI-1 genes have been shown to be down-regulated by the presence of bile (Prouty and Gunn, 2000), so it is possible that SPI-2 genes show a similar sensitivity to the presence or absence of bile. Future studies for *sseI* will include detailed analyses of *ssrA* and *ssrB*, members of the two-component regulatory systems that controls SPI-2 expression (Miao and Miller, 2000) to determine if SPI-2 expression impacts biofilm formation.

These studies showed that several genes are required to mediate successful salmonellae adherence specifically to cholesterol. These and future studies will help to elucidate the temporal requirements of factors involved in biofilm formation on cholesterol-coated surfaces, which will lead to further understanding of the chronic carrier state of *Salmonella* Typhi. As these studies progress, the cholesterol-impaired

mutants will be examined in the mouse model of *Salmonella* carriage to establish an *in vivo* correlation to the *in vitro* TBA results. Studies will also be performed to study the pharmaceutical implications of the bacterial proteins involved in cholesterol attachment. Currently, a study to determine the chemical binding site between cholesterol and *Salmonella* flagella is being arranged, with the hopes that a targeted drug could be developed to block this binding site. As the roles of fimbriae, outer membrane porins, and secreted effector proteins are fully characterized, these too will be examined for potential therapeutic applications. Overall, it is our hope that the findings outlined in this thesis will lead to a better understanding of the chronic carrier state of *S.* Typhi, and may one day yield effective and novel therapies to eliminate chronic *Salmonella* carriage.

Strain	Characteristic(s)	Source or reference
S. Typhimurium		
JSG210	ATCC 14208s (CDC6516-60); wild-type	ATCC
JSG526	*flhC98*::Tn*10* (TH2934); (LT2 background)	Gift of K. Hughes
JSG1174	Δ(*fim-aph-11*::Tn*10*)-*391 lpfC*::Kan *agfB*::Cam *pefC*::Tet; (SR11 background)	Gift of A. Baumler
JSG1178	*hin108*::Tn*10*d-Cam (FljB locked off; FljB-FliC+); (14208s background)	Gift of B. Cookson
JSG1179	*hin108*::Tn*10*d-Cam *fliC*::Tn*10* (FljB locked on; FljB+FliC-); (14208s background)	Gift of B. Cookson
JSG1190	*hin108*::Tn*10*d-Cam *fliC*::Tn*10* (FljB locked off; FljB-FliC-); (14208s background)	Gift of B. Cookson
JSG1547	*motA595*::Tn*10*; (14208s background)	Gift of T. Lino
JSG3024	*cheZ*::Tn*10*; (14208s background)	Gift of B. Ahmer
JSG3041	*cheA*::Tn*10*; (14208s background)	Gift of B. Ahmer
JSG3042	*cheB*::Tn*10*; (14208s background)	Gift of B. Ahmer
JSG3043	*cheR*::Tn*10*; (14208s background)	Gift of B. Ahmer
JSG3044	*cheY*::Tn*10*; (14208s background)	Gift of B. Ahmer
JSG3055	*fimW*::Tn*10*d-Tet; Site 1, 17 insertions; (14208s background)	This study
JSG3058	*fimW*::Tn*10*d-Tet; Site 2, 1 insertion; (14208s background)	This study
JSG3059	*flhA*::Tn*10*d-Tet; 9 insertions; (14208s background)	This study
JSG3060	*fliF*::Tn*10*d-Tet; 3 insertions; (14208s background)	This study
JSG3061	*fliA*::Tn*10*d-Tet; 2 insertions; (14208s background)	This study
JSG3062	*fliJ*::Tn*10*d-Tet; 1 insertion; (14208s background)	This study
JSG3063	*fliL*::Tn*10*d-Tet; 1 insertion; (14208s background)	This study
JSG3064	*ompC*::Tn*10*d-Tet; Site 1, 5 insertions; (14208s background)	This study
JSG3065	*sseI*::Tn*10*d-Tet; 1 insertion; (14208s background)	This study
JSG3073	*ompC*::Tn*10*d-Tet; Site 2, 9 insertions; (14208s background)	This study
JSG3186	*fimW*::Kan; (LB5010 background)	Gift of S. Clegg
JSG3188	*fimW*::Tn*10*-Tet *fimA*::Kan; (14208s background)	This study, Gift of S. Clegg
JSG3189	ATCC 14028s, pISF101	This study, Gift of S. Clegg
JSG3327	*sseI*::Tn*10*d-Tet back-transduced into wild-type; (14208s background)	This study
JSG3328	*sseI*::Kan; (14208s background)	Gift of M. McClelland
JSG3329	*sseJ*::Kan; (14208s background)	Gift of M. McClelland
JSG3330	SPI-2::Kan; (14208s background)	Gift of M. McClelland

Table 2.1: Bacterial strains and relevant characteristics.

Gene	No. Colonies	No. Unique Insertions	Function
fimW	18	2	Inhibitor of type 1 fimbriae regulator FimZ
ompC	14	2	Outer membrane protein; passive diffusion of ions, hydrophilic solutes
flhA	9	1	Flagellar export apparatus, biosynthesis membrane protein
fliF	3	1	Flagellar cytoplasmic anchor MS-ring protein
fliA	2	1	Flagellar biosynthesis sigma factor
fliJ	1	1	Rod/hook and filament biosynthesis chaperone
fliL	1	1	Basal body protein; stability of MotAB complexes of MS-ring
sseI	1	1	Secreted effector; colocalizes with host polymerizing actin cytoskeleton

Table 2.2: Transposon insertion sites, frequencies, and functions. Of the 500 mutant colonies screened for lack of cholesterol biofilm forming ability (but sufficient biofilm forming ability on glass or plastic surfaces), these 49 mutants were identified.

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 3.1: 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).

Organism grown in 3% bile	Biofilm development on[a]	
	Cholesterol	Dead Cells
Serovar Typhimurium	+	++
Serovar Typhimurium *flhC* mutant	-	+++

Table 3.2: Summary of biofilm formation on cholesterol or on formalin fixed bacteria bound to cholesterol. 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 – 1.99-fold higher than wild-type values); +++, increased biofilm present (2.0-fold or greater above wild-type values); -, severe defect in or complete loss of biofilm formation (0 to 10% of wild-type values).
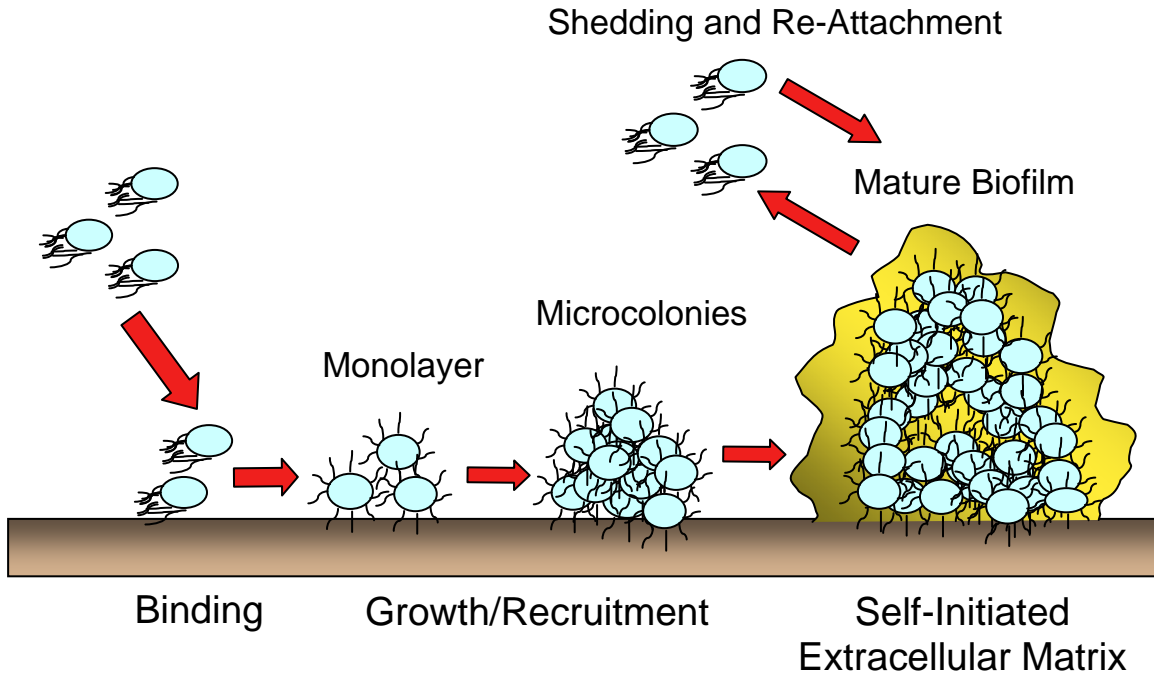
Fig. 1.1: Mechanism of biofilm formation. Bacteria initiate biofilm formation by binding to a biotic or abiotic surface/substrate. Subsequent biofilm development requires growth and recruitment of cells from a monolayer, to a larger microcolony, and eventually to a mature biofilm with a characteristic extracellular matrix (ECM). The self-initiated ECM is frequently comprised of polysaccharides, proteins, or extracellular DNA, and is thought to provide protection for bacteria embedded within.
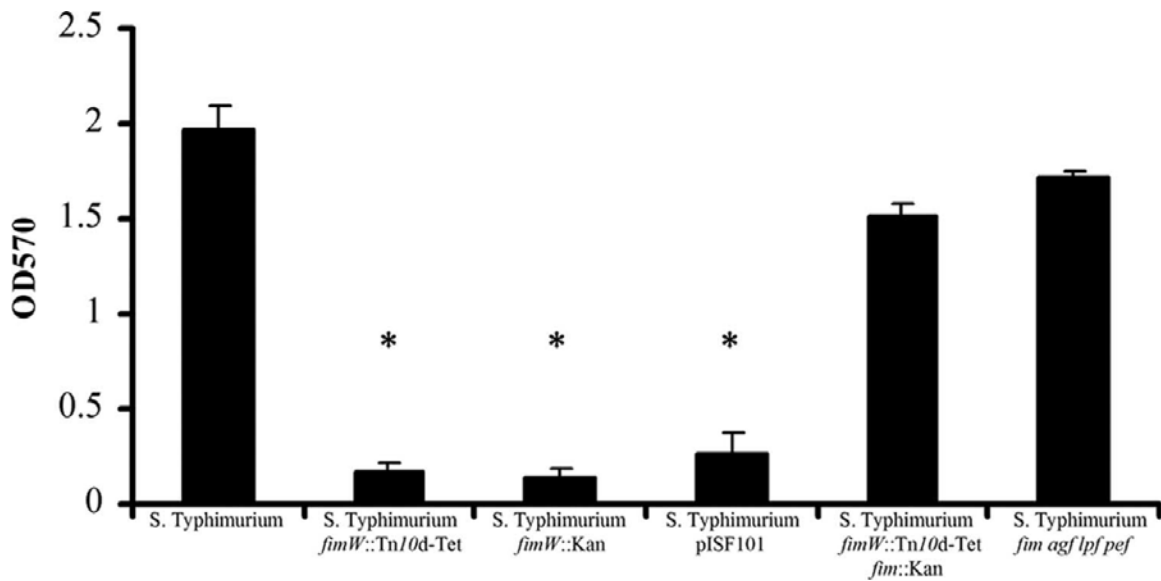
Fig. 3.1: Tube Biofilm Assay. Over-expression of type 1 fimbriae inhibits biofilm formation on cholesterol-coated surfaces while normal or decreased expression of type 1 fimbriae has no effect.. Biofilm formation of serovar Typhimurium strains grown with 3% crude ox bile extract on cholesterol-coated Eppendorf tubes. Crystal violet-stained TBA biofilms were extracted with acetic acid, and absorbance was measured at 570 nm. *, statistical significance ($P < 0.005$) based on a two-tailed Student t test. OD_{570}, optical density at 570 nm.
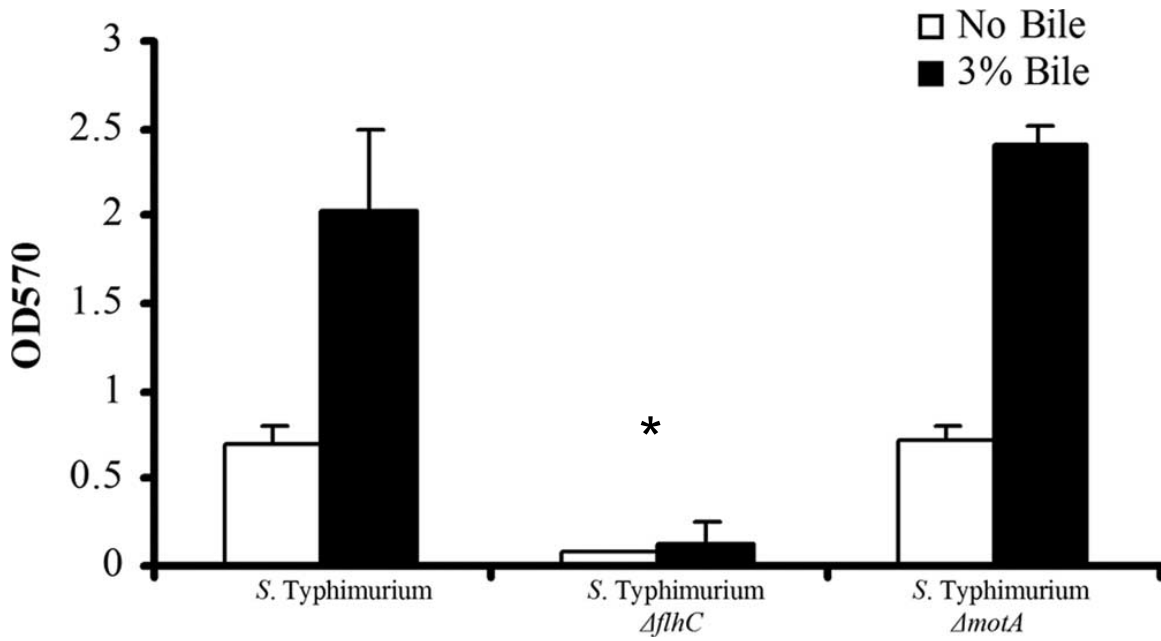
Fig. 3.2: Tube Biofilm Assay. The presence of flagella is necessary for biofilm formation on cholesterol-coated surfaces whereas motility is dispensable. Serovar Typhimurium strains were grown with and without 3% crude ox bile extract and added to cholesterol-coated Eppendorf tubes. Biofilms were stained with crystal violet, extracted with acetic acid, and absorbance was measured at 570 nm. *, statistical significance ($P < 0.005$) based on a two-tailed Student t test. OD$_{570}$, optical density at 570 nm.
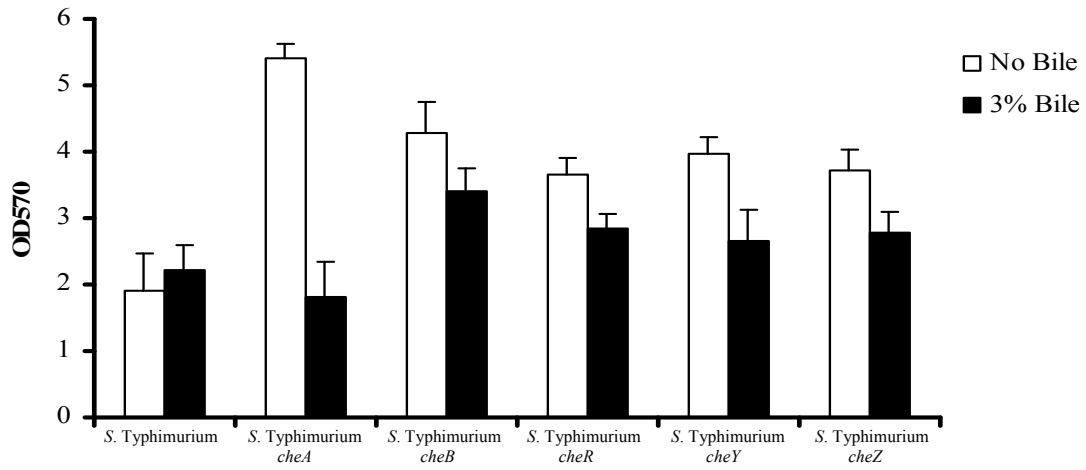
Fig. 3.3: Tube Biofilm Assay. Both smooth swimming and hyper-tumbling phenotypes showed increased biofilm formation in the absence of bile, whereas biofilm formation in presence of bile was comparable to wild-type. These data suggest that functional chemotaxis is important for biofilm formation in the absence of bile, but not in the presence of bile.
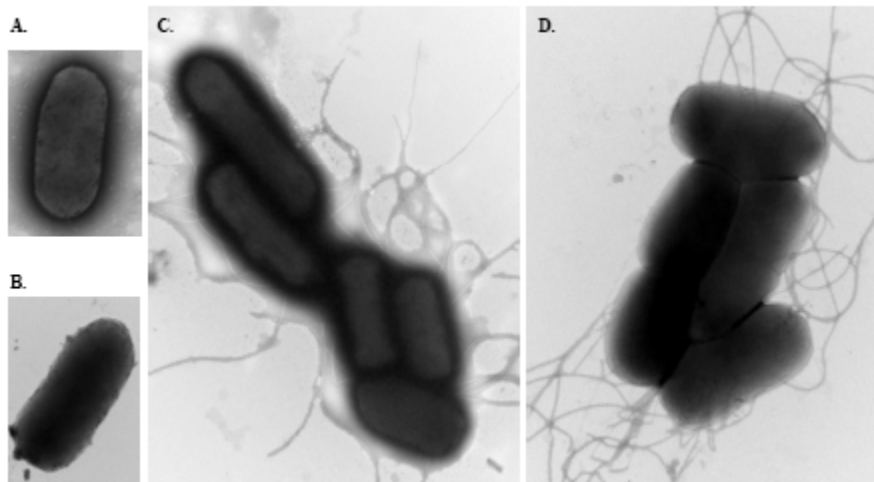
Fig. 3.4: Expression of flagella on the surface of serovar Typhimurium strains grown in the presence or absence of bile visualized by negative staining under transmission electron microscopy. (A) Serovar Typhimurium *flhC* mutant. (B) Serovar Typhimurium *flhC* mutant + 3% bile. (C) Serovar Typhimurium wild-type. (D) Serovar Typhimurium wild-type + 3% bile.
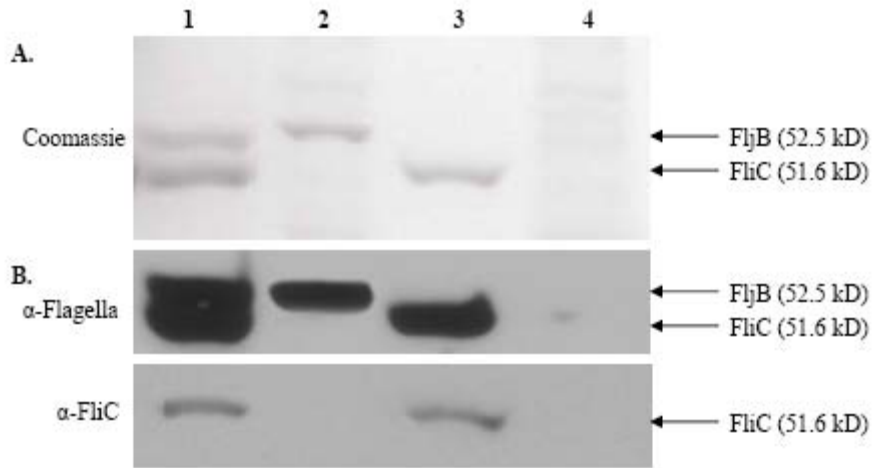
Fig. 3.5: Identification of flagellin proteins from wild-type and mutant serovar Typhimurium strains. Flagella were isolated from late log phase bacterial cultures using mechanical shearing, purified, separated by 10% SDS-PAGE gel electrophoresis, and analyzed by GelCode Blue staining (A) and Western 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, *fliC* mutant strain; 3, *fljB* mutant strain; 4, *fliC fljB* double mutant strain. FliC; 51.6 kD, FljB; 52.5 kD.
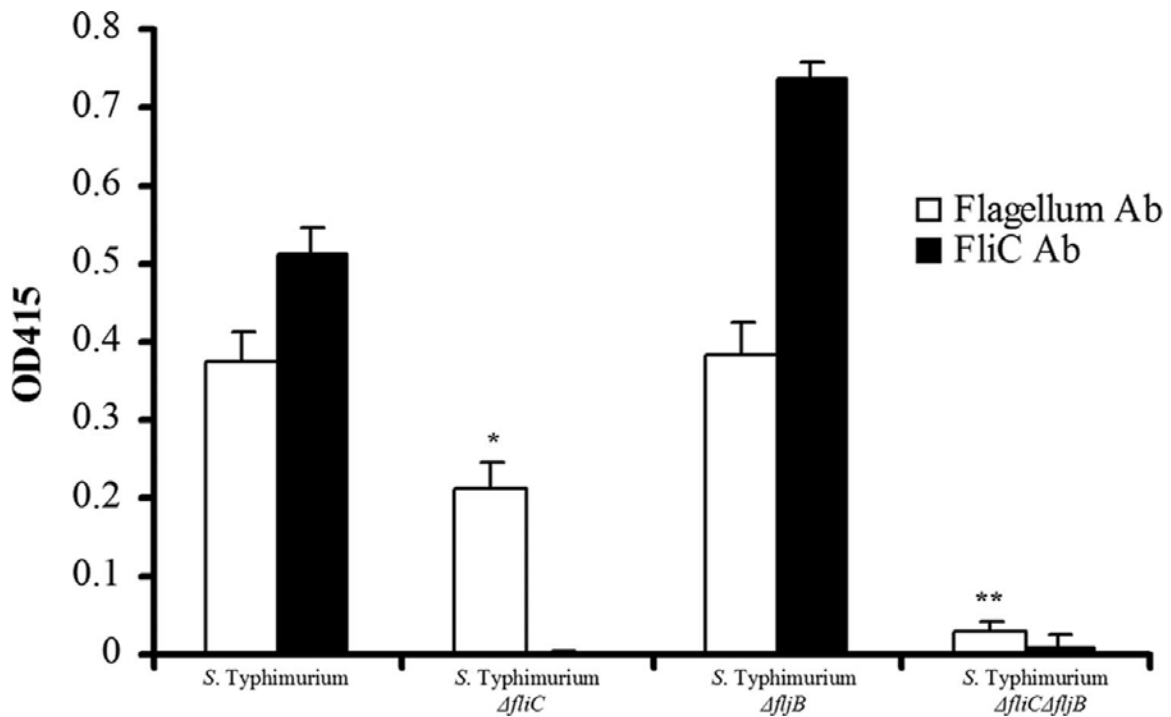
Fig. 3.6: Expression of purified flagella proteins from serovar Typhimurium wild-type, *fliC*, *fljB*, and *fliC fljB* mutant strains adhered to cholesterol-coated wells. A modified ELISA was performed using anti-flagella or anti-FliC monoclonal antibodies and binding to cholesterol was quantified by measuring bound secondary conjugated HRP substrate at optical density 415 nm. Statistical significance based on a two-tailed Student *t* test, *****; $P < 0.05$, ******; $P < 0.005$.
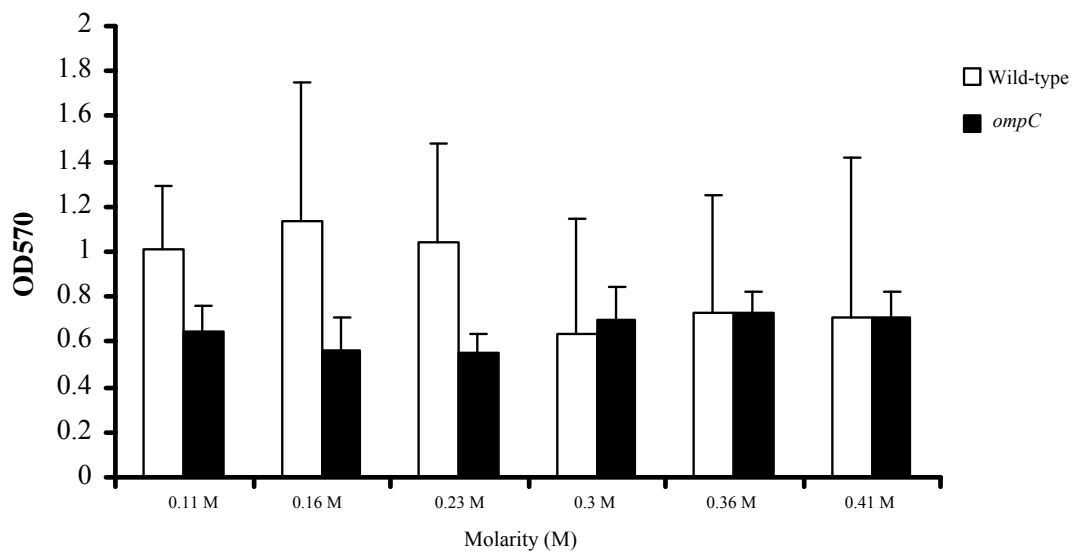
Fig. 3.7: Tube Biofilm Assay. Wild-type *S.* Typhimurium and *ompC* mutants were compared in LB/bile solution of variable molarity. The molarity was changed by altering the salt content of LB. The results suggest that biofilm defects in *ompC* mutants are due to a phenotype other than osmoregulation defect.
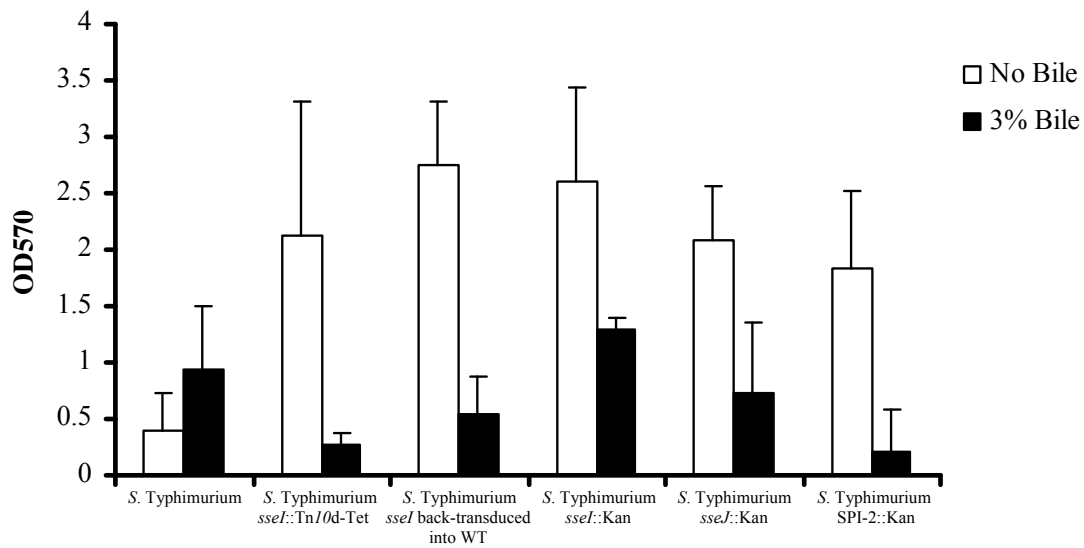
Fig. 3.8: Tube Biofilm Assay. Strains containing mutations in genes found in the SPI-2 pathogenicity island, as well as genes regulated by SPI-2, showed decreased biofilm formation in the absence of bile. These results suggest that SPI-2 genes are important for the formation of biofilms in the presence of bile, and show enhanced biofilm formation in the absence of bile. (This assay was performed twice more in triplicate, showing similar trends in the biofilm formation for all three assays.)

List of References:

1. **Aldridge, P.D., C. Wu, J. Gnerer, J.E. Karlinsey, K.T. Hughes, and M.S. Sachs.** 2006. Regulatory protein that inhibits both synthesis and use of the target protein controls flagellar phase variation in Salmonella enterica. Proc. Natl. Acad. Sci. U. S. A. **103**:11340–11345.

2. **Andersen-Nissen, E., K.D. Smith, R. Bonneau, R.K. Strong, and A. Aderem.** 2007. A conserved surface on Toll-like receptor 5 recognizes bacterial flagellin. J. Exp. Med. **204**:393–403.

3. **Apel, D., and M.G. Surette.** 2007. Bringing order to a complex molecular machine: the assembly of the bacterial flagella. Biochim Biophys Acta. **9**:1851-8.

4. **Baker, S., K. Holt, S. Whitehead, I. Goodhead, T. Perkins, B. Stocker, J. Hardy, and G. Dougan.** 2007. A linear plasmid truncation induces unidirectional flagellar phase change in H:z66 positive Salmonella Typhi. Mol. Microbiol. **66**:1207–1218.

5. **Barken, K.B., S.J. Pamp, L. Yang, M. Gjermansen, J.J. Bertrand, M. Klausen, M. Givskov, C.B. Whitchurch, J.N. Engel, and T. Tolker-Nielsen.** 2008. Roles of type IV pili, flagellum-mediated motility and extracellular DNA in the formation of mature multicellular structures in Pseudomonas aeruginosa biofilms. Environ. Microbiol. **10**:2331–2343.

6. **Boddicker, J.D., N.A. Ledeboer, J. Jagnow, B.D. Jones, and S. Clegg.** 2002. Differential binding to and biofilm formation on, HEp-2 cells by Salmonella enterica serovar Typhimurium is dependent upon allelic variation in the fimH gene of the fim gene cluster. Mol. Microbiol. **45**:1255–1265.

7. **Bonifield, H.R., and K.T. Hughes.** 2003. Flagellar phase variation in Salmonella enterica is mediated by a posttranscriptional control mechanism. J. Bacteriol. **185**:3567–3574.

8. **Chilcott, G.S., and K.T. Hughes.** 2000. Coupling of flagellar gene expression to flagellar assembly in Salmonella enterica serovar typhimurium and Escherichia coli. Microbiol. Mol. Biol. Rev. **64**:694–708.

9. **Coburn, B., G.A. Grassl, and B.B. Finlay.** 2007. Salmonella, the host and disease: a brief review. Immunol Cell Biol. **85:**112-8.

10. **Costerton, J. W., Z. Lewandowski, D. E. Caldwell, D. R. Korber, and H. M. Lappin-Scott.** 1995. Microbial biofilms. Annu Rev Microbiol. **49:**711-45.

11. **Crawford, R.W., D.L. Gibson, W.W. Kay, and J.S. Gunn.** 2008. Identification of a Bile-Induced Exopolysaccharide Required for *Salmonella* Biofilm Formation on Gallstone Surfaces. Infect Immun. **76**:5341-9.

12. **Crawford, R.W., K.E. Reeve, and J.S. Gunn.** 2010. Flagellated but not hyperfimbriated Salmonella enterica serovar Typhimurium attach to and form biofilms on cholesterol-coated surfaces. J Bacteriol. [Epub ahead of print.]

13. **Crawford, R.W., R. Rosales-Reyes, L. Ramírez-Aguilar Mde, O. Chapa-Azuela, C. Alpulche-Aranda, and J.S. Gunn.** 2010. Gallstones play a significant role in Salmonella spp. gallbladder colonization and carriage. Proc Natl Acad Sci U. S. A. **107**:4353-8.

14. **Crump, J.A., S.P. Luby, and E.D. Mintz.** 2004. The global burden of typhoid fever. Bull. W. H. O. **82**:346–353.

15. **Cummings, L.A., W.D. Wilkerson, T. Bergsbaken, and B.T. Cookson**. 2006. In vivo, fliC expression by Salmonella enterica serovar Typhimurium is heterogeneous, regulated by ClpX, and anatomically restricted. Mol Microbiol. **61**:795-809.

16. **Davey, M. E. and G. A. O'Toole.** 2000. Microbial biofilms: from ecology to molecular genetics. Microbiol Mol Biol Rev. **64:**847-67.

17. **Dean, G.E., R.M. Macnab, J. Stader, P. Matsumura, and C. Burks.** 1984. Gene sequence and predicted amino acid sequence of the motA protein, a membrane-associated protein required for flagellar rotation in Escherichia coli. J. Bacteriol. **159**:991–999.

18. **de Vries, N., K.A. Zwaagstra, J.H. Huis in't Veld, F. van Knapen, F.G. van Zijderveld, and J.G. Kusters.** 1998. Production of monoclonal antibodies specific for the i and 1,2 flagellar antigens of Salmonella typhimurium and characterization of their respective epitopes. Appl. Environ. Microbiol. **64**:5033–5038.

19. **Dibb-Fuller, M.P., E. Allen-Vercoe, C.J. Thorns, and M.J. Woodward.** 1999. Fimbriae- and flagella-mediated association with and invasion of cultured epithelial cells by Salmonella enteritidis. Microbiology. **145**:1023–1031.

20. **Dorman, C.J., S. Chatfield, C.F. Higgins, C. Hayward, and G. Dougan.** 1989. Characterization of porin and ompR mutants of a virulent strain of Salmonella typhimurium: ompR mutants are attenuated in vivo. Infect Immun. **57**:2136-40.

21. **Ehrbar, K. and W.D. Hardt.** 2005. Bacteriophage-encoded type III effectors in Salmonella enterica subspecies 1 serovar Typhimurium. Infect Genet Evol. **5:**1-9.

22. **Giron, J.A., A.G. Torres, E. Freer, and J.B. Kaper.** 2002. The flagella of enteropathogenic Escherichia coli mediate adherence to epithelial cells. Mol. Microbiol. **44**:361–379.

23. **Ho, T.D. and J.M. Slauch**. 2001. OmpC is the receptor for Gifsy-1 and Gifsy-2 bacteriophages of Salmonella. J Bacteriol. **183**:1495-8.

24. **Johnson, M., I. Zaretskaya, Y. Raytselis, Y. Merezhuk, S. McGinnis, and T. L. Madden.** 2008. NCBI BLAST: a better web interface. Nucleic Acids Res. **36**:W5–W9.

25. **Lai, C.W., R.C. Chan, A.F. Cheng, J.Y. Sung, and J.W. Leung.** 1992. Common bile duct stones: a cause of chronic salmonellosis. Am. J. Gastroenterol. **87**:1198–1199.

26. **Ledeboer, N.A., and B.D. Jones.** 2005. Exopolysaccharide sugars contribute to biofilm formation by Salmonella enterica serovar typhimurium on HEp-2 cells and chicken intestinal epithelium. J. Bacteriol. **187**:3214–3226.

27. **Levine, M. M., R.E. Black, and C. Lanata.** 1982. Precise estimation of the numbers of chronic carriers of Salmonella typhi in Santiago, Chile, an endemic area. J Infect Dis. **146:**724-6.

28. **Lillehoj, E.P., B.T. Kim, and K.C. Kim.** 2002. Identification of Pseudomonas aeruginosa flagellin as an adhesin for Muc1 mucin. Am. J. Physiol. Lung Cell. Mol. Physiol. **282**:L751–L756.

29. **McLaughlin, L.M., G.R. Govoni, C. Gerke, S. Gopinath, K. Peng, G. Laidlaw, Y.H. Chien, H.W. Jeong, Z. Li, M.D. Brown, D.B. Sacks, and D. Monack.** 2009. The Salmonella SPI2 effector SseI mediates long-term systemic infection by modulating host cell migration. PLoS Pathog. **11**:e1000671.

30. **Merritt, J.H., K.M. Brothers, S.L. Kuchma, and G.A. O'Toole.** 2007. SadC reciprocally influences biofilm formation and swarming motility via modulation of exopolysaccharide production and flagellar function. J. Bacteriol. **189**:8154–8164.

31. **Miao, E.A., M. Brittnacher, A. Haraga, R.L. Jeng, M.D. Welch, and S.I. Miller.** 2003. Salmonella effectors translocated across the vacuolar membrane interact with the actin cytoskeleton. Mol Microbiol. **48:**401-15.

32. **Miao, E. A. and S. I. Miller**. 2000. A conserved amino acid sequence directing intracellular type III secretion by Salmonella typhimurium. Proc Natl Acad Sci U. S. A. **97**:7539-44.

33. **Nuccio, S.P., D. Chessa, E.H. Weening, M. Raffatellu, S. Clegg, and A.J. Baumler.** 2007. SIMPLE approach for isolating mutants expressing fimbriae. Appl. Environ. Microbiol. **73**:4455–4462.

34. **O'Toole, G.A., and R. Kolter.** 1998. Flagellar and twitching motility are necessary for Pseudomonas aeruginosa biofilm development. Mol. Microbiol. **30**:295–304.

35. **Pratt, L.A. and R. Kolter.** 1998. Genetic analysis of Escherichia coli biofilm formation: roles of flagella, motility, chemotaxis and type I pili. Mol Microbiol. **30:**285-93.

36. **Prigent-Combaret, C., O. Vidal, C. Dorel, and P. Lejeune**. 1999. Abiotic surface sensing and biofilm-dependent regulation of gene expression in Escherichia coli. J Bacteriol. **181**:5993-6002.

37. **Prouty, A.M., I.E. Brodsky, J. Manos, R. Belas, S. Falkow, and J.S. Gunn.** 2004. Transcriptional regulation of Salmonella enterica serovar Typhimurium genes by bile. FEMS Immunol. Med. Microbiol. **41**:177–185.

38. **Prouty, A. M. and J. S. Gunn.** 2000. Salmonella enterica serovar typhimurium invasion is repressed in the presence of bile. Infect Immun. **68:**6763-9.

39. **Prouty, A.M., and J.S. Gunn.** 2003. Comparative Analysis of *Salmonella enterica* Serovar Typhimurium Biofilm Formation on Gallstones and on Glass. Infect Immun. **71**:7154-8.

40. **Prouty, A.M., W.H. Schwesinger, and J.S. Gunn.** 2002. Biofilm Formation and Interaction with the Surfaces of Gallstones by *Salmonella* spp. Infect Immun. **70**:2640-9.

41. **Puente, J.L., A. Verdugo-Rodriguez, and E. Calva**. 1991. Expression of Salmonella typhi and Escherichia coli OmpC is influenced differently by medium osmolarity; dependence on Escherichia coli OmpR. Mol Microbiol. **5**:1205-10.

42. **Saini, S., J.A. Pearl, and C.V. Rao.** 2009. Role of FimW, FimY, and FimZ in regulating the expression of type I fimbriae in Salmonella enterica serovar Typhimurium. J. Bacteriol. **191**:3003–3010.

43. **Schmid, M.B., and J.R. Roth.** 1983. Genetic methods for analysis and manipulation of inversion mutations in bacteria. Genetics. **105**:517–537.

44. **Silverman, M., J. Zieg, M. Hilmen, and M. Simon.** 1979. Phase variation in Salmonella: genetic analysis of a recombinational switch. Proc. Natl. Acad. Sci. U. S. A. **76**:391–395.

45. **Teplitski, M., A. Al-Agely, and B. M. Ahmer.** 2006. Contribution of the SirA regulon to biofilm formation in *Salmonella enterica* serovar Typhimurium. Microbiology. **152**:3411-24.

46. **Tinker, J.K., L.S. Hancox, and S. Clegg.** 2001. FimW is a negative regulator affecting type 1 fimbrial expression in Salmonella enterica serovar Typhimurium. J. Bacteriol. **183**:435–442.

47. **Tsolis, R.M., G.M. Young, J.V. Solnick, and A.J. Baumler.** 2008. From bench to bedside: stealth of enteroinvasive pathogens. Nat Rev Microbiol. **6:**883-92.

48. **Uchiya, K., and T. Nikai.** 2008. Salmonella virulence factor SpiC is involved in expression of flagellin protein and mediates activation of the signal transduction pathways in macrophages. Microbiology. **154**:3491–3502.

49. **Vaudaux, P. E., P. Francois, R. A. Proctor, D. McDevitt, T. J. Foster, R. M. Albrecht, D. P. Lew, H. Wabers, and S. L. Cooper.** 1995. Use of adhesiondefective mutants of Staphylococcus aureus to define the role of specific plasma proteins in promoting bacterial adhesion to canine arteriovenous shunts. Infect Immun. **63:**585-90.

50. **Yildiz, F. H. and G. K. Schoolnik.** 1999. Vibrio cholerae O1 El Tor: identification of a gene cluster required for the rugose colony type, exopolysaccharide production, chlorine resistance, and biofilm formation. Proc Natl Acad Sci U. S. A. **96:**4028-33.