

Flagellated but Not Hyperfimbriated *Salmonella enterica* Serovar Typhimurium Attaches to and Forms Biofilms on Cholesterol-Coated Surfaces^{∇†}

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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 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 to and biofilm formation on cholesterol-coated Eppendorf tubes but not on glass and plastic surfaces. We identified 49 mutants with this phenotype. The results indicate that genes involved in flagellum biosynthesis and structure primarily mediated attachment to cholesterol. Subsequent analysis suggested that the presence of the flagellar filament enhanced binding and biofilm formation in the presence of bile, while flagellar motility and expression of type 1 fimbriae were unimportant. Purified *Salmonella* flagellar proteins used in a modified enzyme-linked immunosorbent assay (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.

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. *S. enterica* serovar Typhi is the etiologic agent of typhoid fever, a severe illness characterized by sustained bacteremia and a delayed onset of symptoms that afflicts approximately 20 million people each year (14, 19). 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 (47). Antibiotic regimens are typically ineffective in carriers with gallstones (47), and these patients have an 8.47-fold-higher risk of developing hepatobiliary carcinomas (28, 46, 91). Elimination of chronic infections usually requires gallbladder removal (47), 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 facilitate enhanced colonization of and persistence in the gallbladder. Over the past 2 decades, bacterial biofilms

have been increasingly implicated as burdens for food and public safety worldwide, and they are broadly defined as heterogeneous communities of microorganisms that adhere to each other and to inert or live surfaces (17, 22, 67, 89, 102). A sessile environment provides selective advantages in natural, medical, and industrial ecosystems for diverse species of commensal and pathogenic bacteria, including *Streptococcus mutans* (40, 92, 104), *Staphylococcus aureus* (15, 35, 100), *Escherichia coli* (21, 74), *Vibrio cholerae* (39, 52, 107), and *Pseudomonas aeruginosa* (23, 58, 73, 105). Bacterial biofilms are increasingly associated with many chronic infections in humans and exhibit heightened resistance to commonly administered antibiotics and to engulfment by professional phagocytes (54, 55, 59). The bacterial gene expression profiles for planktonic and biofilm phenotypes differ (42, 90), and the changes are likely regulated by external stimuli, including nutrient availability, the presence of antimicrobials, and the composition of the binding substrate.

Biofilm formation occurs in sequential, highly ordered stages and begins 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 are continuously shed from the sessile, matrix-bound population, 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 and account for much of the person-to-person transmission in underdeveloped countries.

Our laboratory has previously reported that bile is required

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TABLE 1. Bacterial strains and relevant characteristics

S. Typhimurium strain	Characteristics	Source and/or reference
JSG210	ATCC 14028s (CDC6516-60); wild type	ATCC
JSG526	<i>flhC98::Tn10</i> (TH2934)	Gift from K. Hughes
JSG1174	Δ (<i>fim-aph-11::Tn10</i>)-391 <i>lpfC::Kan agfB::Cam pefC::Tet</i>	Gift from A. Baumler
JSG1178	<i>hin108::Tn10d-Cam</i> (FljB locked off; FljB ⁻ FliC ⁺)	Gift from B. Cookson
JSG1179	<i>hin108::Tn10d-Cam fliC::Tn10</i> (FljB locked on; FljB ⁺ FliC ⁻)	Gift from B. Cookson
JSG1190	<i>hin108::Tn10d-Cam fliC::Tn10</i> (FljB locked off; FljB ⁻ FliC ⁺)	Gift from B. Cookson
JSG1547	<i>motA595::Tn10</i>	Gift from T. Lino
JSG3089	<i>fimW::Kan</i>	Gift from S. Clegg
JSG3055	<i>fimW::Tn10d-Tet</i> ; site 1, 17 insertions	This study
JSG3058	<i>fimW::Tn10d-Tet</i> ; site 2, 1 insertion	This study
JSG3059	<i>flhA::Tn10d-Tet</i> ; 9 insertions	This study
JSG3060	<i>fliF::Tn10d-Tet</i> ; 3 insertions	This study
JSG3061	<i>fliA::Tn10d-Tet</i> ; 2 insertions	This study
JSG3062	<i>fliJ::Tn10d-Tet</i> ; 1 insertion	This study
JSG3063	<i>fliL::Tn10d-Tet</i> ; 1 insertion	This study
JSG3099	<i>fimW::Tn10-Tet fim::Kan</i>	This study, gift from A. Baumler
JSG3214	ATCC 14028s/pISF101	This study, gift from A. Baumler
JSG3064	<i>ompC::Tn10d-Tet</i> ; 5 insertions	This study
JSG3065	<i>sseI::Tn10d-Tet</i> ; 1 insertion	This study
JSG3073	<i>ompC::Tn10d-Tet</i> ; 9 insertions	This study

for formation of mature biofilms with characteristic EPS production by *S. enterica* serovars Typhimurium, Enteritidis, and Typhi on human gallstones and cholesterol-coated Eppendorf tubes (18, 78). Cholesterol is the primary constituent of human cholesterol gallstones, and use of cholesterol-coated tubes creates an *in vitro* uniform surface that mimics human gallstones (18). It was also demonstrated that *Salmonella* biofilms that formed on different surfaces had unique phenotypes and required expression of specific EPS (18, 77), yet the factors mediating *Salmonella* binding to gallstones and 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 to during later stages of biofilm growth. Elucidation of 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.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and molecular biology techniques. The *Salmonella* strains used in this study are listed in Table 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: ampicillin, 50 µg/ml; kanamycin, 25 µg/ml; chloramphenicol, 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 (85).

Transposon mutagenesis and screening. Cholesterol-binding-deficient, tetracycline-resistant serovar Typhimurium strains were created by random transposon mutagenesis using an established method (85). Briefly, Tn10d-Tet transposons were introduced into wild-type serovar Typhimurium. Strains containing Tn10d 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). Colonies were confirmed to have a non-biofilm-forming (on cholesterol) phenotype by back-transduction using P22 HT *int-105*.

Cholesterol, glass, and plastic surface biofilm assays. *Salmonella* strains were tested to determine their abilities to form biofilms in the tube biofilm assay (TBA) and on cholesterol-coated glass and plastic coverslips as described previously (18). In brief, log-phase *Salmonella* strains grown with or without 3% crude ox bile were added to cholesterol-coated Eppendorf tubes or 24-well plastic plates (Fisher Scientific, Pittsburgh, PA) containing Chromerge-cleaned glass, plastic, and cholesterol-coated coverslips. 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 three 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-1× phosphate-buffered saline [PBS] [1:1:18]) was then added to stain cells for 5 min at room temperature. The tubes were washed with 1× PBS, and the dye was extracted using 33% acetic acid and quantified by determining the optical density at 570 nm.

DNA sequencing and bioinformatics. Sequencing of transposon mutant genomic DNA from serovar Typhimurium cholesterol-binding-deficient colonies with primer JG1787 (5'-CCTTTTCCGTGATGGTA-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 (43).

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 1× PBS, staining of wells with a 0.1% crystal violet solution (gentian violet in isopropanol-methanol-1× PBS [1:1:18]) for 5 min, and five more washes with 1× PBS. The dye retained by bound cells was extracted using 33% acetic acid and was quantified by determining the optical density at 570 nm.

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- μ l were aliquots 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 Δ *flhC* serovar Typhimurium strains were added to the tubes, and biofilm formation was examined using the TBA as described above.

Purification of serovar Typhimurium flagellin. Serovar Typhimurium wild-type, phase-1-locked (FlhC; H:*i*) mutant, phase-2-locked (FljB; H:*1,2*) mutant, and flagellin (FlhC) mutant strains were grown to late log phase in LB medium at 37°C. The resulting cultures (500 ml) were centrifuged at 8,000 \times g for 15 min. The cell pellets were washed once, resuspended in 15 ml 1× 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 \times g. The flagellum-containing supernatants were collected and centrifuged at 100,000 \times g for 1 h. Filaments were gently resuspended overnight in 1× PBS with slow shaking at 4°C and then centrifuged at 100,000 \times g. This 24-h washing cycle was repeated twice, and the final pellets of purified serovar Typhimurium flagellin were resuspended in 1× 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).

Western blotting. Monoclonal antibodies against *Salmonella* species flagella (Maine Biotechnology Services, Portland, ME) and FlhC subunit protein (BioLegend, San Diego, CA) were used to probe purified flagellin from serovar Typhimurium wild-type, FljB mutant, and FlhC mutant strains in a Western blot analysis. For each sample, 10 μ l containing 1 μ g 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 anti-flagellum antibody (diluted 1:200 in PBS; Maine Biotechnology Services, Portland, ME) or anti-FlhC antibody (5 μ g 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 1× PBS.

Subunit binding ELISA. Purified flagellin from serovar Typhimurium wild-type, FljB mutant, and FlhC 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- μ l aliquots were added to polystyrene wells in 96-well Microtest tissue culture plates (Becton Dickinson Labware, Franklin Lakes, NJ). Six 1- μ g 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 1× PBS and blocked overnight with 3% BSA. Wells were emptied, washed, and incubated with anti-flagellum antibody (diluted 1:20 in 0.3% BSA) or anti-FlhC antibody (diluted 1:100 in 0.3% BSA) for 2 h, each in triplicate. 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.

RESULTS

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

The molecular genetic mechanisms that mediate initiation of biofilm formation vary with the growth medium and substrate and are beginning to be elucidated for microorganisms such as *E. coli* (21, 74, 75), *S. aureus* (15, 35, 100), *Enterococcus faecalis* (38, 97), and *Pseudomonas* spp. (58, 72, 73, 84). As described previously, *S. enterica* serovars Typhimurium, Enteritidis, and Typhi form mature biofilms on cholesterol gallstone surfaces and cholesterol-coated Eppendorf tubes (tube biofilm assay [TBA]) but not on uncoated tubes when they are grown in the presence of 3% crude ox bile extract (18). To identify novel genes that regulate binding to cholesterol, Tn10d-Tet transposon mutagenesis was performed with serovar Typhimurium wild-type strain ATCC 14028s. A total of 40,000 colonies were pooled to generate a mutant library of random Tn10d transposon insertions. The mutant pool was grown to log phase in LB broth containing Tet with 3% crude ox bile extract, and 100- μ l portions were added to siliconized Eppendorf tubes coated with 1 mg of cholesterol. Following incubation for 24 h, 10 μ l of the planktonic, nonadherent culture was removed and added to 90 μ l of fresh LB broth containing Tet with 3% bile in new cholesterol-coated tubes. The panning for cholesterol-binding-deficient bacteria was repeated every 24 h for 10 days, and the final planktonic cells were diluted and plated.

To determine if the ability of these mutants to form biofilms in the TBA, as well as on other surfaces, was affected, 500 individual colonies were assayed in the presence of bile to determine their 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 that a specific loss of cholesterol binding was mediated by the mutations. To rule out the possibility of second-site mutations, mutants were transduced back into the parent strain, and their biofilm-forming phenotypes were confirmed.

Sequencing of Tn10d transposon insertion sites. Direct genomic DNA sequencing from the transposon 5' end and sequence analyses revealed that the Tn10d insertions mapped to several genes corresponding to the serovar Typhimurium loci *fimW*, *ompC*, *flhA*, *fliF*, *fliA*, *fliJ*, *fliL*, and *sseI* (Table 2). Eighteen Tn10d insertion sites were in the *fimW* open reading frame; one was at bp 98 (JSG3058) in the open reading frame (ORF), and 17 others were at bp 125 (JSG3055). The colonies with transposon insertions in *fimW* that were recovered in the cholesterol-binding screen were more numerous than the colonies with transposon insertions in any other gene that were recovered. *FimW* negatively regulates production of type 1 fimbriae by interacting with *FimY* (83) and possibly with *FimZ* (96); the latter two proteins independently activate the P_{fimA} promoter, which controls the expression of the *fim* structural genes (83). Serovar Typhimurium chromosomal *fimW* mutants expressed 4- to 8-fold more type 1 fimbriae than the parent strain (96). Therefore, serovar Typhimurium strains carrying Tn10d insertions in *fimW* exhibit hyperfimbriate phenotypes, suggesting that overproduction of type 1 fimbriae inhibited adherence to cholesterol.

Nine identical Tn10d transposon insertions in *flhA* were

TABLE 2. Transposon insertion sites, frequencies, and functions

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

^a A total of 49 mutants were identified from the 500 mutant colonies screened for the lack of an ability to form cholesterol (but with the ability to form a biofilm on glass or plastic surfaces).

located at bp 1917 (JSG3059). The cytoplasmic membrane protein FlhA mediates type III export of axial components of the *Salmonella* flagellum and the scaffolding proteins for flagellum assembly (45, 82). All three *Tn10d* insertions in the *fliF* open reading frame were located downstream of the start site at bp 872 (JSG3060). Structural subunits of the transmembrane protein FliF comprise the membrane-supramembrane ring (MS ring) and proximal rod of the flagellar basal body in serovar Typhimurium and have been shown to interact with the chemotactic motor-switch protein FliG (94, 95, 99). Two *Tn10d* insertion sites in *fliA* (JSG3061) were located at bp 532 relative to the start codon. The conserved alternative sigma factor σ^{28} encoded by the class 2 regulatory gene *fliA* directs transcription from class 3 promoters of flagellar filament late assembly genes in *E. coli* (16, 57) and serovar Typhimurium (16, 32, 71). The *Tn10d* insertion in JSG3062 was located in the *fliJ* open reading frame at bp 142 relative to the start codon. 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 and hook (FlgD) substrates (31, 61, 66). One *Tn10d* transposon insertion site was located downstream of the *fliL* start site at bp 252 (JSG3063). The function of the membrane-associated component of the flagellar basal body encoded by

fliL is unknown (86). Recent evidence suggests that FliL prevents rod fracture caused by torsional stress during swarming motility (4).

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 on cholesterol-coated surfaces, whereas overproduction of fimbriae is not important for establishment of a biofilm on cholesterol. Figure 1A shows a schematic diagram of the transposon insertion sites in fimbria- and flagellum-related genes found by random mutagenesis.

Overexpression of type 1 fimbriae in serovar Typhimurium has a negative effect on biofilm formation on cholesterol. Fimbriae have been shown to mediate adherence during initiation of biofilm formation and cell-cell interactions during biofilm growth for a variety of microorganisms (22, 73). The serovar Typhimurium genome contains 13 putative fimbrial operons, some of which are not expressed *in vitro* (70). 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 (9, 51) but not on human gallstones incubated with bile (78). 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. 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 ATCC 14028s (Fig. 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 with mutations affecting type 1 fimbriae could form mature biofilms, strains having *Tn10d* transposon insertions in *fimW* were added to cholesterol-coated Eppendorf tubes in the TBA as described previously (18). As shown in Fig. 2, a *fimW*::*Tn10d*-Tet mutation rendered serovar Typhimurium deficient for biofilm formation in the presence of 3% bile. 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 (96). This *fimW*::Kan mutant was deficient for biofilm formation at levels similar to those of the strain having a transposon insertion in *fimW* (Fig. 2). To further test whether overexpression of

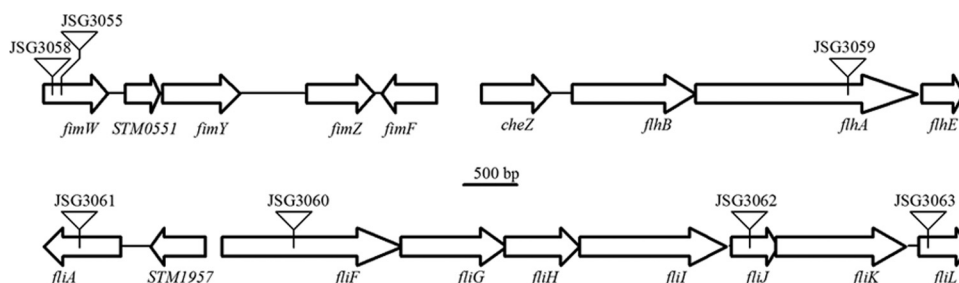


FIG. 1. Schematic diagram of *Tn10d* transposon insertion sites in serovar Typhimurium cholesterol biofilm mutants with mutations affecting type 1 fimbria and flagellum structure and biogenesis. The arrows indicate the sizes and orientations of neighboring genes, and the triangles indicate the locations of transposon insertions.

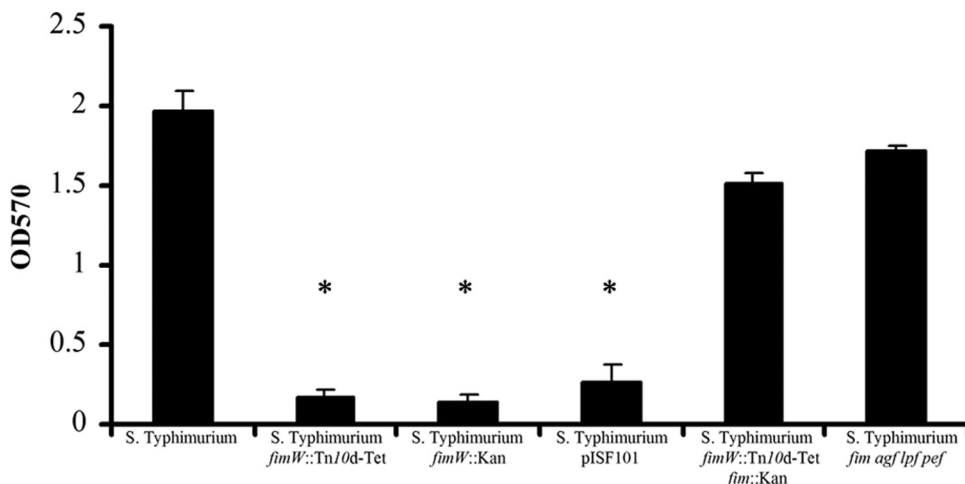


FIG. 2. Overexpression of type 1 fimbriae inhibits biofilm formation on cholesterol-coated surfaces, while normal or decreased expression of type 1 fimbriae has no effect. Biofilm formation by serovar Typhimurium strains grown with 3% crude ox bile extract on cholesterol-coated Eppendorf tubes was examined. Crystal violet-stained TBA biofilms were extracted with acetic acid, and absorbance at 570 nm was measured. *, statistically significantly different ($P < 0.005$) based on a two-tailed Student *t* test. OD570, optical density at 570 nm.

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, did not form a biofilm in the TBA (Fig. 2).

As mentioned above, FimW negatively regulates FimY, a multifunctional protein that positively regulates production of fimbriae by activating the *fimA* promoter (83). 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::Tn10d-Tet* background. The resulting strain lacking *fimW* and type 1 fimbrial genes (including *fimA*) exhibited wild-type levels of biofilm formation in the TBA (Fig. 2). Collectively, these results demonstrate that overexpression of type 1 fimbriae has a negative effect during *Salmonella* binding to cholesterol and that the interference prevents subsequent maturation of a cholesterol biofilm formed by the bacterium.

Flagellum-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 (32, 44). Therefore, it is no surprise that flagella make various contributions to biofilm formation in members of the *Enterobacteraceae*, such as *V. cholerae* (50, 68, 102, 103), *E. coli* (48, 60, 74, 75), and *P. aeruginosa* (26), depending on the environmental conditions, such as binding substrate material, nutrient limitation, temperature, medium flow rate, and other factors (6, 63, 73). Expression of the serovar Typhimurium flagella has been shown to inhibit biofilm formation on polystyrene wells (93) but to promote biofilm development on human gallstones when bile is added to the growth medium (77, 78). 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 gene at the apex of transcriptional regulation (*flhC*) in serovar Typhimurium was created. The

resulting mutant strain did not form a mature biofilm on cholesterol, providing direct evidence of the importance of flagella during biofilm development (Fig. 3).

The presence of, but not the motility mediated by, the serovar Typhimurium flagella is important for biofilm formation on cholesterol surfaces. As demonstrated above, 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 were tested. In the TBA, a serovar Typhimurium *motA* mutation (which eliminates flagellar motility but not synthesis) (24) did not reduce the 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

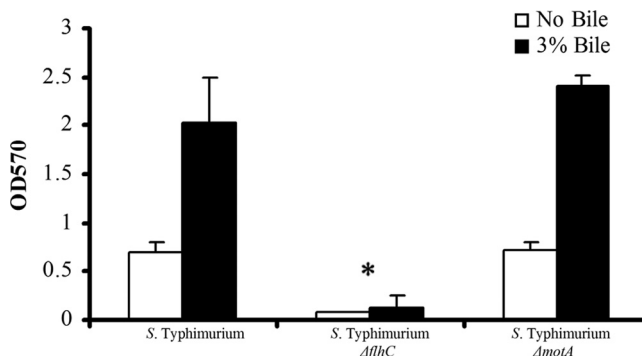


FIG. 3. Flagella are necessary for biofilm formation on cholesterol-coated surfaces, whereas motility is dispensable. Serovar Typhimurium strains were grown with and without 3% crude ox bile extract and added to cholesterol-coated Eppendorf tubes. Biofilms were stained with crystal violet and extracted with acetic acid, and the absorbance at 570 nm was measured. *, statistically significantly different ($P < 0.005$) based on a two-tailed Student *t* test. OD570, optical density at 570 nm.

TABLE 3. Binding quantification assay^a

Strain	Growth conditions	Live	Dead
Serovar Typhimurium wild type	LB medium	+	+
	LB medium + 3% bile	+	+
Serovar Typhimurium <i>flhC</i> mutant	LB medium	–	–
	LB medium + 3% bile	–	–

^a A 3-h binding quantification assay was performed with cholesterol-coated wells containing live or dead serovar Typhimurium wild-type and *flhC* mutant strains. Crystal violet-stained material was extracted with acetic acid, and the optical density at 570 nm was determined for quantification. +, binding equivalent to wild-type binding under appropriate conditions; –, severe defect in or complete loss of adherence (values that were 0 to 10% of the wild-type values).

development of serovar Typhimurium biofilms on cholesterol-coated surfaces (Fig. 3).

The number of surface-expressed serovar Typhimurium flagella is not regulated by bile. The presence of bile was shown to modestly downregulate serovar Typhimurium motility and flagellar gene expression in β -galactosidase assays using MudJ fusions to *flhC*, *flgC*, and *fliC* (76). 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 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 (see Fig. S1 in the supplemental material). Therefore, while exposure to bile may transcriptionally downregulate flagellar genes, bile has no effect on the number of flagella.

Formalin-fixed and heat-killed salmonellae expressing intact flagella bind to cholesterol. If flagella mediate attachment to cholesterol, then dead salmonellae expressing intact flagella should bind to cholesterol in the TBA. Wild-type and Δ *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). 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.

Dead salmonellae bound to cholesterol provide a scaffold for biofilm formation by live cells. 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 1 \times 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. 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

TABLE 4. Biofilm formation on cholesterol or on formalin-fixed bacteria bound to cholesterol

Organism grown with 3% bile	Biofilm development on ^a :	
	Cholesterol	Dead cells
Serovar Typhimurium wild type	+	++
Serovar Typhimurium <i>flhC</i> mutant	–	+++

^a Biofilms were grown on cholesterol-coated Eppendorf tubes or on a substrate consisting of inactivated, bound cells in the TBA. The dye was extracted with acetic acid, and the optical density at 570 nm was determined for quantification. +, formation of a robust, mature biofilm (wild-type amount); ++, amount of biofilm that was 1.5- to 1.99-fold larger than the wild-type amount; +++, amount of biofilm that was \geq 2.0-fold larger than the wild-type amount; –, severe defect in or complete loss of biofilm formation (amount that was 0 to 10% of the wild-type amount).

biofilms were larger than the amounts of the biofilms for salmonellae grown in the TBA without this scaffold (Table 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 a scaffold of bound, dead wild-type cells, and a TBA was performed. The *flhC* mutant, while deficient in biofilm formation on cholesterol-coated surfaces (Fig. 3), 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 4). 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 4).

Serovar Typhimurium flagellar subunit FliC is critical for binding to cholesterol. The flagellar filament of *S. enterica* is approximately 10 μ m long and is comprised of two antigenically distinct flagellin proteins, FliC (H:i) and FljB (H:1,2) (16, 25). During the well-characterized phase variation process, these subunits are alternatively expressed by a posttranscriptional control mechanism (1, 10, 87). 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, Δ *fliC*, Δ *fliB*, and Δ *fliC* Δ *fliB* strains using a mechanical shearing protocol adapted from the protocol of Andersen-Nissen et al. (3). Wild-type and phase-locked mutant flagellin preparations (1 μ g each) were separated by 10% SDS-PAGE. Based on the amino acid sequences, the predicted molecular masses of the FliC and FljB proteins were 51.6 and 52.5 kDa, respectively (98), and the approximately 1-kDa difference was detected by Coomassie blue staining (see Fig. S2A in the supplemental material) and Western blotting. Monoclonal anti-flagellin antibodies (see Fig. S2B in the supplemental material) that recognized a peptide that is present in both FliC and FljB or only in FliC were used. The identity of each band was confirmed by comparing the wild-type and mutant lanes (see Fig. S2 in the supplemental material). Purified proteins from all samples were added to cholesterol-coated wells of a 96-well tissue culture plate and analyzed by a modified ELISA. Equal amounts of the flagella from the wild-type and Δ *fliB* strains bound to cholesterol, and the amounts were signifi-

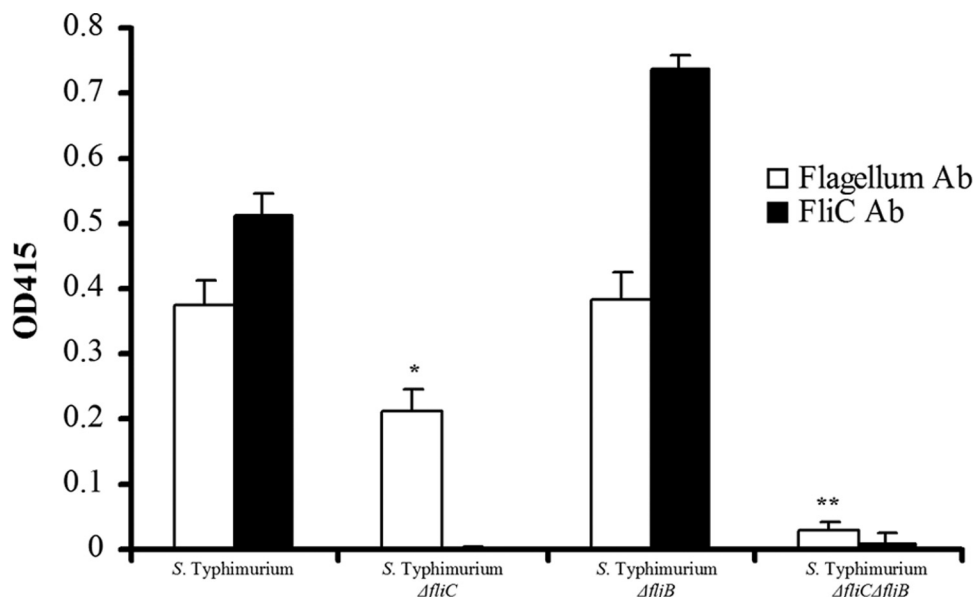


FIG. 4. Expression of purified flagellum proteins from serovar Typhimurium wild-type, *fliC* mutant, *fliB* mutant, and *fliC fliB* mutant strains adhering to cholesterol-coated wells. An ELISA was performed using anti-flagellum or anti-FliC monoclonal antibodies, and binding to cholesterol was quantified by measuring the bound secondary HRP-conjugated substrate using the optical density at 415 nm (OD415). Asterisks indicate statistically significant differences based on a two-tailed Student *t* test (*, $P < 0.05$; **, $P < 0.005$). Ab, antibody.

cantly larger than the amounts observed for an *fliC* mutant, suggesting that FliC is the critical serovar Typhimurium flagellar subunit that mediates binding to cholesterol (Fig. 4).

DISCUSSION

Approximately 5% of patients suffering from typhoid fever carry *Salmonella* in the gallbladder (53), and evidence indicates that there is a high correlation between individuals who become carriers and individuals who have gallstones (28, 47). Because antibiotic treatments are ineffective at removing chronic infections in such cases (47), we hypothesized and have demonstrated that the asymptomatic presence of serovar Typhi is frequently a result of formation of biofilms on cholesterol gallstone surfaces. In this work we show that the flagellar filament, specifically the FliC subunit, of serovar Typhimurium is necessary for and specific for 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 the growth medium and the 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* (18, 78). To determine the critical genes for early biofilm events, specifically the genes involved in attachment, a randomly mutagenized serovar Typhimurium strain was screened using the TBA for loss of adherence. Genomic DNA from mutants that did not bind to cholesterol but formed biofilms on glass and plastic surfaces were sequenced to locate the Tn10d insertion site. Disruptions in genes affecting type 1 fimbria and flagellum structure and biogenesis were the disruptions observed most frequently, and *fimW* disruptions were prevalent.

Tn10d insertions were also found in genes encoding an outer membrane protein (*ompC*) and a translocated effector of the *Salmonella* pathogenicity island 2 (SPI-2) type III secretion system (*sseI*). *OmpC* is necessary for binding and biofilm formation in the presence of bile, possibly because it mediates electrostatic interactions between salmonellae and the cholesterol surface, promotes overall biofilm health as a nutrient channel, or regulates cell-cell interactions, as it does in *E. coli* biofilms (79). *SseI* is a secreted effector protein belonging to the GDSL family of lipases that colocalizes with the polymerizing actin cytoskeleton (65). SPI-1 has been implicated in directing cholesterol distribution during entry into mammalian cells (33), and GDSL proteins associated with SPI-2 have been shown to hydrolyze phospholipids and esterify cholesterol intracellularly (11, 69). The lambdoid phage Gifsy-2 encodes *SseI* (29, 65), and the receptor for Gifsy-2 is *OmpC* (36). Although the Gifsy bacteriophages and SPI-2 secreted effectors are generally virulence factors of serovar Typhimurium in mice, the association between *SseI* and *OmpC* and the known interaction of *SseI* with cholesterol suggest a possible role for adherence to or modification of cholesterol during biofilm formation.

The presence of fimbriae is attributed in part to serovar Typhimurium biofilm formation on HEp-2 tissue culture cells and the intestinal epithelia of mice and chickens (7, 9, 51). In studies by Prouty et al., a serovar Typhimurium SR11 strain having mutations in four fimbrial operons was observed to adhere to and form biofilms on cholesterol gallstones in a manner similar to the manner observed for wild-type serovar Typhimurium (78). Similarly, the amount of biofilm formed by a quadruple-mutant SR11 strain was the same as the amount formed by wild-type serovar Typhimurium both in the presence and in absence of bile in the TBA, suggesting that fim-

briae encoded by the *fim*, *agf*, *lpf*, and *pef* operons do not affect biofilms 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 *fim* structural genes by activating the *fimA* promoter (83), and chromosomal *fimW* mutants expressed 4- to 8-fold more type 1 fimbriae than the parent strain (96). The *fimW::Kan* and *fimW::Tn10d-Tet* serovar Typhimurium strains and a separate type 1 fimbria-overexpressing serovar Typhimurium strain did not form a biofilm on cholesterol-coated Eppendorf tubes, and introduction of a mutation in the type 1 fimbrial gene cluster into a *fimW* mutant background restored the amount of biofilm formation to that by wild-type serovar Typhimurium. These data suggest that overexpression of type 1 fimbriae has a negative effect during *Salmonella* binding to cholesterol and subsequent biofilm formation by the bacterium.

Flagella promote surface binding during biofilm formation (73, 74), as well as adhesion during colonization of tissue culture cells and mucus by *P. aeruginosa* (56, 81), *Campylobacter jejuni* (62), *E. coli* (34), *Yersinia enterocolitica* (45a), and *S. enterica* (2, 12, 27, 49). To examine whether the physical presence of the flagellar filament was necessary for biofilm formation on cholesterol, a serovar Typhimurium *flhC* mutant was examined using the TBA and shown not to form a mature biofilm on cholesterol. These results provide direct evidence that flagella are important during biofilm development.

Flagellum-mediated motility and chemotaxis differentially contribute to colonization and biofilm formation depending on environmental cues (37, 64, 73, 74, 77). Bile, for example, increases motility and is a chemical attractant at physiological concentrations for *V. cholerae* (13), acts as a repellent for *Helicobacter pylori* (106), and enhances tumbling frequency while modestly downregulating motility in *S. enterica* (76). Serovar Typhimurium mutant strains lacking a motor function (*motA*) were analyzed to determine whether they formed a biofilm using the TBA. Loss of flagellar motility had no significant effect on biofilm development in LB broth with or without bile. These results indicate that flagellar motility is dispensable for biofilm formation on cholesterol surfaces.

Bile has also been shown to affect transcription of the serovar Typhimurium flagellum structural genes *flhC*, *flgC*, and *fliC* (76). We report here that wild-type salmonellae grown with and without bile and visualized by TEM express the same amount of flagella, suggesting that prolonged exposure to bile does not alter the ultimate production of flagella. Thus, the reduced levels of *flhC*, *flgC*, and *fliC* transcription in the presence of bile, while slightly less than the levels in the absence of bile, are still above the threshold level necessary for normal flagellum production under these conditions and allow flagellum-mediated binding to cholesterol.

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 strains were added to cholesterol-coated wells. 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 by live, wild-type cells in the TBA, and the amounts of the biofilms were larger than the

amounts of the biofilms produced by salmonellae grown without this scaffold in the TBA. The serovar Typhimurium flagellum (*FlhC*) mutant was added to a layer of adherent, dead cells, and biofilm development was examined. There was an increase in biofilm formation by the *flhC* mutant on a substrate consisting 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 flagellar filament of *S. enterica* is comprised of two antigenically distinct subunit proteins, FliC (H:i) and FljB (H:1,2), and this is a pathogen-associated molecular pattern detected by mammalian cells via surface-expressed Toll-like receptor 5 and cytosolic Nod-like receptors (30, 80). Secretion of interleukin-1 β by mouse cell lines is activated comparably by the FliC and FljB flagellins (88), but memory CD4⁺ T cells preferentially recognize FliC during adaptive immunity and *Salmonella* committed to expressing only FljB is attenuated *in vivo* (8, 41). To determine which flagellar subunit mediated adherence to cholesterol, ELISA was performed using antiflagellum or anti-FliC antibodies against purified flagellum proteins from serovar Typhimurium wild-type, *fliC* mutant, *fljB* mutant, and *fliC fljB* mutant strains bound to cholesterol-coated wells. The FliC subunit was shown to be a critical factor for mediating attachment to cholesterol. Interestingly, *S. Typhi* is monophasic, harboring only the *fliC* gene (5), and FliC expression was anatomically restricted to certain tissues during systemic serovar Typhimurium infections in mice (20).

In this work, we identified a number of *Salmonella* factors that mediate binding to and subsequent biofilm development on cholesterol-coated surfaces. Specifically, we showed that overexpression of fimbriae is detrimental, while expression of the serovar Typhimurium flagellar filament mediates adherence to cholesterol but not to glass or plastic surfaces primarily with the FliC subunit, which initiates biofilm formation, but inhibits subsequent biofilm development. It could be hypothesized that flagellar production is downregulated as a biofilm develops. This new understanding of the events during *Salmonella* biofilm formation on cholesterol-coated surfaces provides therapeutic targets 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.

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