Pathoadaptive Alteration of *Salmonella* Biofilm Formation in Response to the Gallbladder Environment

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ABSTRACT Typhoid fever, a human-specific disease, is primarily caused by the pathogen *Salmonella enterica* serovar Typhi. It is estimated that 3 to 5% of people infected with typhoid fever become chronic carriers. Studies have demonstrated that a mechanism of chronic carriage involves biofilm formation on gallstone surfaces. In the course of a previous study using a chronic carriage mouse model, a *Salmonella enterica* serovar Typhimurium isolate was recovered from a mouse gallstone that exhibited a 2-fold increase in biofilm formation over the wild type. In order to identify the gene(s) responsible for the phenotype, the genomic sequences of this isolate and others were determined and compared. These sequences identified single nucleotide polymorphisms (SNPs) in 14 genes. Mutations in the most promising candidates, *envZ* and *rcsB*, were created, but neither showed increased biofilm-forming ability separately or in combination. The hyperbiofilm isolate did, however, present variations in cellular appendages observable using different techniques and a preferential binding to cholesterol. The isolate was also examined for systemic virulence and the ability to colonize the gallbladder/gallstones in a mouse model of chronic infection, demonstrating a systemic virulence defect and decreased gallbladder/gallstone colonization. Finally, to determine if the appearance of hyperbiofilm isolates could be replicated *in vitro* and if this was a common event, wild-type *Salmonella* spp. were grown long term *in vitro* under gallbladder-mimicking conditions, resulting in a high proportion of isolates that replicated the hyperbiofilm phenotype of the original isolate. Thus, *Salmonella* spp. acquire random mutations under the gallbladder/gallbladder-simulating conditions that may aid persistence but negatively affect systemic virulence.

IMPORTANCE Chronic carriers are the main reservoirs for the spread of typhoid fever in regions of endemicity. *Salmonella* Typhi forms biofilms on gallstones in order to persist. A strain with enhanced biofilm-forming ability was recovered after a nine-month chronic-carriage mouse study. After sequencing this strain and recreating some of the mutations, we could not duplicate the phenotype. The isolate did show a difference in flagella, a preference to bind to cholesterol, and a systemic virulence defect. Finally, gallbladder conditions were simulated *in vitro*. After 60 days, there was a 4.5-fold increase in hyperbiofilm isolates when a gallstone was present. These results indicate that *Salmonella* spp. can undergo genetic changes that improve persistence in gallbladder albeit at the cost of decreased virulence.

KEYWORDS *Salmonella*, biofilms, chronic carriage, gallbladder

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Typhoid fever, a human-specific disease, affects approximately 21 million people each year, resulting in about 200,000 deaths (1). The primary causative agent of typhoid fever is *Salmonella enterica* subsp. *enterica* serovar Typhi. Typhoid primarily affects underdeveloped areas of South Central Asia, Southeast Asia, Latin America, and southern Africa (2). The disease is commonly spread by food or water contaminated with feces or urine of acutely infected people or carriers of *S. Typhi* (3). Once ingested, the bacteria cross the intestinal epithelial barrier, are phagocytosed by macrophages, and spread throughout the body in the bloodstream. From the blood, macrophages carrying the bacteria often result in infection of the liver, spleen, bone marrow, and gallbladder (GB) (4).

It is estimated that 3 to 5% of people who recover from typhoid fever become chronic carriers, and of those, 25% experience no clinical symptoms during the acute phase of the disease, resulting in greater spread of the organism (3, 5). Carriage in humans occurs primarily in the GB (6). Studies have shown a correlation between the typhoid fever carrier state and the presence of gallstones (GS), with 90% of carriers presenting GS (7). This correlation has been shown to be the result of the formation of *Salmonella* GS surface biofilms, which are aggregations of bacteria encased in an extracellular matrix adhering to each other and the GS surface (8, 9). Biofilm formation allows the organism to withstand inhospitable environments, including the bile-filled GB, resulting in persistence within the host. Bacteria are occasionally shed from the biofilm into the feces and urine. Because typhoid fever is a human-specific disease, the carrier state plays a crucial role in its dissemination. Currently, the exact mechanisms of biofilm formation are not completely understood, and the only effective treatment is GB removal, which is rarely a viable option in countries where typhoid fever is prevalent.

Salmonella enterica subsp. *enterica* serovar Typhimurium infection of mice serves as a model for typhoid infection of humans (8). In the course of a previous long-term study of GS-bearing mice infected with *S. Typhimurium*, an isolate that exhibited increased biofilm formation was recovered from a mouse GS at 9 months postinfection (10). Overall, the goal of this study was to gain a greater understanding of the mechanisms that result in the carrier state, specifically focusing on potential changes to *Salmonella* spp. caused by host-pathogen interactions (4, 11–13). By better understanding these mechanisms, it may be possible to develop new nonsurgical techniques to resolve the chronic carrier state, thereby alleviating the spread of typhoid fever. We hypothesize that within the GB, *Salmonella* spp. acquire mutations that enhance biofilm formation and persistent GB colonization. In this study, we identify several genes that may be related to the hyperbiofilm phenotype and demonstrate that prolonged growth under GB-like conditions favors a shift toward greater biofilm production.

RESULTS

***Salmonella* spp. isolated from GS at 9 months postinfection show increased biofilm formation.** *S. Typhimurium* strains isolated from various organs following a long-term infection study (10) displayed different biofilm formation abilities (Fig. 1a). Strains obtained from feces and organs including the liver and pancreas showed a diminished biofilm-forming capacity. Interestingly, isolates from the GB showed contrasting phenotypes, with strains isolated from bile exhibiting decreased biofilm-forming ability, while those isolated from GS had >2-fold-increased biofilm-forming ability. Furthermore, the phenotype persisted when one of the isolates (JSG3538) was passaged 10 times (10P), and additional unique colonies isolated from GS presented the same hyperbiofilm phenotype (Fig. 1b).

Genome sequencing revealed 14 SNPs in the hyperbiofilm-forming strains isolated from mouse GS. The first step in identifying the gene or genes responsible for the formation of the hyperbiofilm state was whole-genome sequencing of both our wild-type *S. Typhimurium* as well as the hyperbiofilm isolate (JSG3538) recovered from the mouse model. The genomes were mapped and compared to the reference strain (*S. Typhimurium* 14028S) in order to generate a list of single nucleotide polymorphisms

FIG 1 Testing of hyperbiofilm isolates recovered from long-term mouse carriage experiments. (a) Biofilm capacity of isolates recovered from the different sites of a chronically infected mouse after 9 months of infection compared to the initial wild-type inoculated strain (JSG210). GS isolate, JSG3538; GS10P, JSG3538 passed 10 times. A one-way analysis of variance (ANOVA) was used to compare the wild type with each isolate (*, $P < 0.05$; ns, nonsignificant). (b) Biofilm production of various isolates recovered from a mouse GS at 9 months postinfection with *S. Typhimurium*. (c) Comparison of motility for hyperbiofilm isolates compared to the wild type. Strain JSG1190 is a mutant lacking crucial flagellar proteins and incapable of motility and was included as a negative control (FljB⁻-FliC⁻ double-knockout mutant). A one-way ANOVA followed by Dunnett's multiple-comparison test were used to compare the wild type versus each isolate (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$).

(SNPs). A second round of sequencing was performed, comparing additional isolates recovered from the mouse GS that originally yielded the hyperbiofilm mutant (see Table S1 in the supplemental material). Although these isolates also displayed enhanced biofilm formation, they exhibited differing motility phenotypes (Fig. 1b and c). Three additional strains were chosen, as follows: a low-motility isolate (no. 3, JSG3752), a wild-type (WT)-level-motility isolate (no. 9, JSG3753), and an isolate obtained from bile that did not show a hyperbiofilm phenotype (JSG3700). After these two rounds of sequencing, we eliminated any silent mutations and those found in our lab wild-type (JSG210) or JSG3700 bile isolate. Genes found to be mutated in hyperbiofilm isolates (regardless of motility phenotype) and not in the wild type or the bile isolate were given highest priority, as these genes were the most likely to be involved in biofilm formation and not simply survival in the harsh bile environment of the GB (Table 1). Because of their regulatory importance and known involvement with the stress response, the following genes that are part of two-component systems were given

TABLE 1 Mutations identified by sequencing^a

Functional annotation	Gene	Reference position	Reference allele	Allele variation	Frequency (%)	Amino acid change
Two-component system	<i>rcsB</i>	2421991	A	G	99	p.Lys149Glu
	<i>envZ</i>	3672981	C	T	86.2	p.Trp176 ^b
Hypothetical proteins	STM14_1478	1328473	T	A	95.2	p.Asn657Lys
	STM14_1618	1422034	A	G	100	p.Asn27Asp
Flagella	<i>fliB</i>	2058074	C	T	98	p.Trp304 ^b
	<i>fliT</i>	2062923	C	T	92.9	p.Arg98Cys
	<i>flgG</i>	2070069	G	A	94.8	p.Glu151Lys
	<i>flgI</i>	1222458	C	A	97.4	p.Gln314Lys
	STM14_0647	614465	C	T	91.2	p.Arg4Lys
DNA rearrangements	<i>malT</i>	3691714	T	C	96.6	p.Leu306Ser
	<i>melR</i>	4554902	C	T	95.5	p.Arg257His
	<i>upgE</i>	3738919	C	T	95.2	p.Gly65Ser
	STM14_0844	791641	G	A	93.4	p.Gly87Asp
O-antigen synthesis	<i>rfbG</i>	2224116	T	C	94	p.Asn261Ser

^aFourteen SNPs unique to the hyperbiofilm isolate JSG3538. Of these *envZ*, *rcsB*, STM14_1478, and STM14_1618 were the most consistently identified variants among all three hyperbiofilm isolates examined. The genes were sorted based on their known functions; unnamed genes are listed according to their position in the genome.

^bIndicates that the amino acid change resulted in a termination of the amino acid sequence.

priority: *rcsB*, which codes for a response regulator in the RcsCDB phosphorelay signaling pathway, which regulates the expression of a large number of genes (14); and *envZ*, which codes for a sensor protein in an important two-component system with OmpR, which directs the cell's response to changes in osmolarity (15). Mutations were also identified in several genes related to flagellar formation. Flagella have been shown to play a key role in the initial surface attachment of *Salmonella* spp. to cholesterol (16, 17). Importantly, two genes coding for hypothetical proteins, STM14_1478 and STM14_1618, were identified in both rounds of sequencing, highlighting their potential role in the hyperbiofilm phenotype. Another highly consistent variant was in *rfbG*, which is part of the lipopolysaccharide (LPS) biosynthesis cluster involved in O-antigen synthesis. The other genes on our list have little to no known connection to biofilm formation or the stress response, but their potential roles in the hyperbiofilm phenotype cannot be excluded.

Mutations in the *envZ* or *rcsB* genes are not enough to replicate the hyperbiofilm phenotype. Different techniques were used in order to generate mutations in the two highest priority candidate genes discovered in the above-mentioned sequencing. The first step in this process was the deletion of the gene of interest. Clean-deletion mutants of the *envZ* and *rcsB* genes were generated by λ red mutagenesis. These mutants were then tested to determine their biofilm-forming capability. The assay showed no significant alteration to biofilm formation following the deletion of *envZ*, *rcsB*, or a double mutant lacking both genes (Fig. 2A). Due to the possibility that the observed mutations would result in an alteration of protein function as opposed to a complete loss of function (as would be seen in the deletion mutant), further mutagenesis work was performed. Wild-type copies of *envZ* and *rcsB* were inserted into plasmid pWSK29, a low-copy-number plasmid, to limit the effect of gene overexpression. Site-directed mutagenesis was performed on the plasmid-borne copy of the gene, replicating the exact SNPs observed from sequencing the hyperbiofilm isolate. The expression of *envZ* and *rcsB* in all complemented strains was verified with quantitative reverse transcription-PCR (RT-qPCR) (Fig. S1). Upon transformation into their respective deletion strains, biofilm assays were performed using these mutants complementing the chromosomal deletions (including empty vector controls). The results of these assays are shown in Fig. 2b and c. In summary, none of the created mutants displayed significant alterations to biofilm formation compared to the deletion strains, suggesting that the hyperbiofilm phenotype might be caused by a combination of mutations or other identified variants.

Hyperbiofilm isolates form robust biofilms on cholesterol surfaces but not on glass. The first goal in characterizing the biofilm formed by our mouse isolate was

FIG 2 Biofilm-forming capacity of *rcsB* and *envZ* mutants. (a) Biofilm production of *S. Typhimurium* mutants lacking potential hyperbiofilm-related genes (WT, JSG210; Δ*envZ* mutant, JSG3687; Δ*rcsB* mutant, JSG3689; Δ*envZ* Δ*rcsB* mutant, JSG3690). (b) Biofilm production of *S. Typhimurium* *rcsB* deletion mutants, including complemented wild type (JSG3746/pWSK-*rcsB*:149K), complemented mutant copy of *rcsB* (JSG3747/pWSK-*rcsB*:149E), and an empty vector control (JSG3748/pWSK29). (c) Biofilm production of *S. Typhimurium* *envZ* deletion mutants, including complemented wild type (JSG3743/pWSK-*envZ*:176W), complemented mutant copy of *envZ* (JSG3744/pWSK-*envZ*:176*), and an empty vector control (JSG3745/pWSK29). For point mutations, abbreviations are K, lysine; E, glutamic acid; W, tryptophan; and *, stop. A one-way ANOVA followed by Dunnett's multiple-comparison test were used to compare differences between strains (*, $P < 0.05$).

determining whether it formed a biofilm with characteristics phenotypically similar to the wild-type strain. To answer this question, biofilms from wild-type *S. Typhimurium* and the hyperbiofilm mutant JSG3538 were visualized via scanning electron microscopy (SEM) on different surfaces in order to compare morphologies. This experiment provided us with two interesting results. First, our hyperbiofilm isolate showed little to no biofilm formation on a glass surface, indicating that the mutant biofilm-forming ability is surface dependent, with increased biofilm formation occurring only on GS-like surfaces. Second, the hyperbiofilm mutant appeared to form a much thicker, more robust biofilm with a significantly different morphology on a cholesterol surface (Fig. 3A). This experiment suggested that the hyperbiofilm mutant forms a phenotypically different biofilm from that in the wild type. In order to find differences in the composition and properties between biofilms from the wild-type and hyperbiofilm strains, we examined the presence of curli (Congo red staining) and cellulose (using a calcofluor assay), and resistance to dispersion through treatment with DNase, proteinase, or cellulase. No significant differences were found for any of these characteristics (Fig. S2).

The hyperbiofilm isolate demonstrates altered flagella. Because of the flagellar mutations found in the hyperbiofilm strain and the importance of cellular appendages in surface attachment, a phenotypic difference in flagellar appendages could be a significant cause of the hyperbiofilm phenotype. Previous experiments had demonstrated that most tested hyperbiofilm isolates displayed a deficiency in motility, with only two isolates displaying motility comparable to that of the WT (Fig. 1c). Cellular appendages of wild-type *S. Typhimurium* were visualized and compared to those of hyperbiofilm isolates with different motility phenotypes (JSG3752 and JSG3753). Using transmission electron microscopy (TEM), we observed long, smooth, and thick flagella present on wild-type *Salmonella* spp., with clearly observable differences occurring in the hyperbiofilm isolates (Fig. 3b). While the exact morphology of the hyperbiofilm isolates differed, both in general appeared to have thinner and more numerous appendages than did the wild type. These differences could be unique surface components, differences in the amount of flagellin expressed, or phase variation of the major flagellar subunit. Western blots were performed using whole-cell lysates or supernatants of the wild-type and hyperbiofilm mutants (JSG3538, JSG3752, and

FIG 3 Evaluation of cellular appendages. (a) SEM pictures of WT (JSG210) and hyperbiofilm (JSG3538) isolates on glass and cholesterol-coated glass. (b) TEM pictures of wild-type *S. Typhimurium* and two hyperbiofilm isolates representing the relative low-motility (JSG3752) and high-motility (JSG3753) phenotypes. (c) Western blot comparing supernatant proteins of WT (JSG210), original hyperbiofilm (JSG3538), low-motility hyperbiofilm (JSG3752), normal-motility hyperbiofilm (JSG3753), and FliC^+ - FljB^- (JSG1178), FljB^+ - FliC^- (JSG1179), and FljB^- - FliC^- double-knockout mutant (JSG1190) strains. Top, anti-*Salmonella* antibody (Ab) was used; bottom, anti-FliC was used.

JSG3753) to address this issue, comparing expression levels of the two flagellin subunit proteins FljB and FliC (Fig. 3c and S4). The wild-type and low-motility hyperbiofilm isolates JSG3538 and JSG3752 displayed similarly high levels of FljB and no detectable FliC . The high-motility isolate (JSG3753) displayed a complete inversion of this phenotype, with low expression of FljB and high expression of FliC . The total amounts of flagellar protein between the three isolates appeared fairly stable. Interestingly, the anti-*Salmonella* serum, which also identifies LPS O antigen, showed the typical ladder appearance of the repeating O antigen to be missing from all three hyperbiofilm-producing isolates (JSG3538, JSG3752, and JSG3753).

The hyperbiofilm isolate showed a marked virulence defect. Given the key role biofilm formation plays in the carrier state of typhoid fever, the larger, more robust biofilm formed by the hyperbiofilm strain is hypothesized to result in increased persistence within the host. In order to evaluate the role of hyperbiofilm production in virulence and colonization abilities, an *in vitro* biofilm competition assay was first performed. Equal amounts of the two strains were mixed to form a biofilm, and after 24 h, bacteria were recovered from the wells for CFU enumeration. A ratio of 1.04:1 (wild type to hyperbiofilm JSG3538) CFU recovery from the wells indicated that the

FIG 4 Virulence properties of the hyperbiofilm and complemented strains. (a) *Nramp1*^{+/+} 129 × 1/SvJ mice were infected with wild-type (JSG210) or hyperbiofilm-forming (JSG3752) *S. Typhimurium* by the intraperitoneal route (10^4 CFU). (b) The wild-type and hyperbiofilm strains were complemented with *envZ* (generating strains JSG3843 and JSG3844, respectively) on a low-copy-number plasmid (pWSK29envZ).

wild-type and hyperbiofilm strains equally colonized the cholesterol surface *in vitro* (Fig. S3).

To compare the virulence of the strains, *Nramp1*^{+/+}(*Slc11A1*) 129 × 1/SvJ mice fed a lithogenic diet (LD) to produce GS (or a normal diet for no GS) were infected with the wild type or a hyperbiofilm strain (JSG3752, which is phenotypically identical in all tests to JSG3538) by the intraperitoneal route. Mice were sacrificed at 8 days postinfection, after which samples were taken from the liver, GB, bile, and GS (LD mice only), homogenized, and enumerated. Bacterial counts from the various organs of the hyperbiofilm strain were below detection level, indicating a severe systemic virulence defect (Fig. 4a). Because we had previously demonstrated that EnvZ does not alter biofilm formation but is known to affect virulence, a second experiment was performed, augmenting the hyperbiofilm strain JSG3752 with the addition of a wild-type *envZ* on a low-copy-number plasmid to test the virulence of the hyperbiofilm state without an *envZ* defect (Fig. 4b). Although this strain was able to invade the host GB, recovery was still inconsistent and below the values seen in the wild type. Additionally, the hyperbiofilm mutant was still absent from all liver samples, indicating that even with a functional EnvZ, there is still a systemic virulence defect of the hyperbiofilm isolate.

***In vitro* modeling of hyperbiofilm pathoadaptive changes.** To determine if the formation of the recovered hyperbiofilm isolate could be recreated *in vitro*, a long-term growth experiment was performed. Wild-type *S. Typhimurium* was grown in LB medium supplemented with 3% bile with or without the presence of a human GS. The medium was replaced daily while the culture was allowed to grow for 60 days. After the 60 days, 100 individual bacterial colonies from the GS were recovered and tested for their biofilm-forming ability (Fig. 5a). In total, 27 isolates from the GS displayed hyperbiofilm properties, compared to 6 isolates from the media lacking a GS (Fig. 5b and $P < 0.001$, determined via a chi-square contingency test).

DISCUSSION

In the course of a previous long-term study (10), an *S. Typhimurium* isolate that exhibited enhanced biofilm-forming ability was recovered from a biofilm on a mouse GS at 9 months postinfection. The persistence of this hyperbiofilm phenotype after several rounds of passage suggested that it was due to a genetic mutation and not a transient change in gene expression or DNA modification (Fig. 1a and b). The discovery of this mutant strain may represent a random mutation that is selected in this environment, which enhances colonization and persistence in the host GB, providing a unique insight into the *in vivo* process of biofilm formation on cholesterol GS.

The purpose of this study was to further identify and characterize the hyperbiofilm

FIG 5 *In vitro* hyperbiofilm model. (a) Biofilms were grown for 60 days on LB supplemented with 3% bile with or without the presence of GS. The medium was changed daily. After 60 days, CFU were isolated from the GS and tested individually for biofilm formation using the crystal violet assay. (b) Percentage of colonies in each population exhibiting a normal biofilm versus hyperbiofilm phenotypes.

Salmonella isolate in the hope of gaining a greater understanding of the mechanisms that result in GB colonization and establishment of the carrier state. We hypothesized that within the GB, *Salmonella* spp. undergo genetic modifications in response to the local microenvironment that enhance GS biofilms and persistent GB colonization. This hypothesis was examined via the following three major approaches: identification of mutations in genes responsible for the hyperbiofilm state, determination of the relationship of the identified gene mutations to the mechanism of hyperbiofilm formation, and examination of biofilm and virulence-related properties of the hyperbiofilm strain.

Pathogenic adaptations (pathoadaptations), representing a mechanism whereby an organism adapts to a new pathogenic niche, have been documented in various bacteria, including *Salmonella* spp. (18). Previous studies have shown that *Salmonella* spp. are known to undergo an *in vivo*-induced alteration of the lipopolysaccharide (LPS) component of their outer membrane that aids their ability to avoid detection by the immune system (19–24). *Pseudomonas aeruginosa*, a pathogen of the cystic fibrosis lung, acquires a genetic change in which it becomes mucoid, fostering a biofilm phenotype and lung persistence (25). Uropathogenic *Escherichia coli* alters its type 1 fimbriae, dramatically increasing its colonization ability (26). Given these examples, it is possible that such a mechanism resulted in the observed hyperbiofilm phenotype in *Salmonella* spp. Because the hyperbiofilm state and long-term chronic carriage have been the focus of few studies, limited data are available on bacteria from carriers (mouse or human) and their biofilm-forming ability.

Through full-genome sequencing, we identified a list of 14 genes with single nucleotide polymorphisms (SNPs) in the hyperbiofilm strains. These genes were ranked based on their currently known association with biofilm growth and development, as well as comparisons to additional isolates of the original mouse GB. This list includes two genes involved in two-component regulatory systems, two hypothetical genes, several flagellar genes, and *rfbG*. The *rcs* family of genes encodes an important regulator in *Enterobacteriaceae* (14). The system has been shown to be involved in cell wall formation, cell division, and several biofilm-related products, including colonic acid (27), curli fimbriae (28), and flagella (29). Thus, a mutation in the *rcsB* gene, which serves primarily as a regulator for the function of the Rcs family, could potentially have a significant impact on biofilm development and morphology. EnvZ and OmpR have also been connected to several signaling pathways involving biofilm development, and OmpR/EnvZ affect the synthesis of the Vi antigen in *S. Typhi* (but absent in *S. Typhimurium*), an important biofilm matrix element and a surface component that aids in the avoidance of immune system killing (30). Given these factors, a mutation in the *envZ* gene could also cause a major change in biofilm production. Mutants for both *rcsB* and *envZ* were constructed. This included full gene deletions, point mutations matching those found through sequencing, and combinations of the two full deletions. None of the mutants showed any effect on biofilm production. As such, we can conclude that

individually, they do not play a crucial role in the creation of the hyperbiofilm phenotype or in biofilm formation in general (Fig. 2). Previous studies have linked both of these genes to biofilm formation, although given the results shown here, it appears that *Salmonella* spp. have sufficient biofilm-forming compensatory mechanisms.

The identification of genes producing proteins of unknown function in both rounds of sequencing gives a strong indication of their potential link to the hyperbiofilm state. The function of the hypothetical protein STM14_1478 is unknown, but there are other genes present within the *Salmonella* genome that contain some of the same highly conserved regions, including STM14_1190, which is 97% identical. As a result, a mutation that affects the function of STM14_1478 could potentially be compensated for by other genes with the same conserved regions. STM14_1618 had a 100% frequency of mutation, and it is a gene encoding a hypothetical protein with no currently known function. The protein is not predicted to be secreted from the cell but may have a short transmembrane domain. It has no known orthologs outside the *Salmonella* genus. Further studies will focus on the potential role of STM14_1618 in *Salmonella* biofilm formation and GB pathoadaptation.

The experiments performed here demonstrated phenotypic differences between wild-type *S. Typhimurium* and the hyperbiofilm isolates. Observed via SEM, biofilms of these two strains (wild-type JSG210 and JSG3538) have drastic morphological differences on cholesterol-coated surfaces, while the hyperbiofilm mutant is unable to form significant biofilms on glass surfaces (Fig. 3a). These findings indicate that the enhanced biofilm phenotype is surface dependent, favoring cholesterol-coated surfaces like those of GS, though the reason for this preference is not yet known. Additional experiments comparing the composition of the biofilm showed no differences in the prominent extracellular matrix components curli or cellulose or in colony morphology on Congo red plates. Similarly, wild-type and hyperbiofilm isolates showed no differences in biofilm dispersal when treated with cellulase, DNase, or protease (see Fig. S2 in the supplemental material). There were, however, differences in the morphologies of cellular appendages. TEM imaging showed significantly different cellular appendage phenotypes even between two hyperbiofilm isolates (JSG3752 and JSG3753) and the wild type (Fig. 3b). Given that surface attachment is the first stage of biofilm development on GS and the previous demonstration of a role for flagella in this process (16), mutations in flagellar genes could have a large effect on the affinity of *Salmonella* spp. for the cholesterol surface, thereby altering biofilm formation. Western blots confirmed similar amounts of total flagellar proteins between the strains, although the expression of the flagellar subunits differed (Fig. 3c and S4). Flagellar phase variation in *Salmonella* is thought to play a role in immune evasion (31), and the finding that one of the hyperbiofilm mutants (JSG3753) shifted to a majority FlhC flagellin is consistent with it playing a role in chronic carriage, as the human typhoid pathogen *S. Typhi* only possesses FlhC. Furthermore, FlhC was demonstrated to be the primary flagellar subunit interacting with cholesterol (16). However, it is unclear why flagellar mutations were identified in the hyperbiofilm isolate, because they play a positive role in biofilm formation and binding to cholesterol. It is possible that the mutations enhance binding. Analysis of the flagellar mutations identified and their role in the hyperbiofilm phenotype await further study.

The identification of an *rfbG* variant suggested a potential alteration in the LPS O antigen, as *rfbG* encodes a CDP-glucose 4,6-dehydratase involved in O-antigen biosynthesis. Strikingly, all three hyperbiofilm strains examined by Western blotting demonstrated a defect in LPS O-antigen laddering (Fig. 3c). Mutations in *rfbG* have been shown to cause a rough colony morphology and loss of LPS O antigen (32). During a screen for *Salmonella* genes required for long-term systemic infection after intraperitoneal infection, mutants in O-antigen biosynthesis were negatively selected (33). This may suggest that the O-antigen defect is responsible for the observed virulence deficiency of the hyperbiofilm isolate. However, such a defect also renders the bacterium more sensitive to bile salts (34) and negatively affects its ability to form a biofilm on gallstone surfaces (35). These data suggest that an O-antigen defect is a surprising

trait for a GB hyperbiofilm isolate to possess, yet it is one that could explain the observed virulence defect.

In vitro competition experiments demonstrated that the hyperbiofilm strain does not outcompete the wild type when colonizing a surface in a 24-h assay (Fig. S3). This might indicate that it is a feature that develops over time in the harsh GB environment. *In vivo*, the hyperbiofilm strain demonstrates a clear systemic virulence defect in the mouse typhoid fever model (Fig. 4a). We initially believed that this was likely due to the mutation in *envZ*, encoding a protein crucial to survival and escape from the host phagosome (38). An *envZ*-complemented strain was able to weakly enter and invade the host GB, but recovery was still inconsistent and below the values seen in the wild type (Fig. 4b). Additionally, the hyperbiofilm mutant complemented with *envZ* on a plasmid was still absent from most tissues, indicating that even with a functional EnvZ, there is still a systemic virulence defect of this isolate. We speculate that while the pathoadaptation increases biofilm-forming ability, it limits systemic virulence and spread, with the mutations causing the virulence attenuation still yet to be confirmed.

Following prolonged incubation under GB-resembling conditions, 27 of 100 isolates recovered from a GS displayed hyperbiofilm characteristics as opposed to 6 of the 100 isolates grown without GS (Fig. 5). This significant difference supports our hypothesis of random mutation followed by a selection mechanism resulting in a shift in the biofilm phenotype in response to the host environment. Analysis of the genomic sequences of the *in vitro* hyperbiofilm isolate mutations will further define the mutations involved and the mechanism(s) resulting in the phenotype-causing mutations. The presence of some hyperbiofilm isolates in the group without GS suggests that bile/stress may play a role in the process, but the significant increase in the GS-containing samples further suggests that prolonged biofilm growth is critical to the pathoadaptive process.

Because typhoid fever is a human-specific disease, carriers represent a crucial reservoir, occasionally shedding *Salmonella* spp. through feces and urine, contaminating food and water supplies, thereby perpetuating the spread of the disease. Biofilm formation within the GB has previously been shown to be a key mechanism of persistence within the host. The presented experiments demonstrate that there may be specific mutations that are selected for in the gallbladder environment that provide *Salmonella* spp. a hyperbiofilm phenotype, which increases persistence and allows further spread of the disease. Although the hyperbiofilm state is advantageous in persistence, *in vivo* experimentation revealed that it is extremely detrimental to systemic virulence, which is at odds with current data suggesting that carriers are responsible for significant spread of disease in the world. However, given the results of the biofilm competition assay, we know that wild-type and hyperbiofilm mutants are capable of cocolonization of cholesterol surfaces. This may provide an advantage to *S. Typhimurium* over favoring a single phenotype. Following initial colonization of the GS surface by wild-type *S. Typhimurium*, some colonies may shift toward the hyperbiofilm state, while others remain in the wild-type state. The prevalence of the hyperbiofilm bacteria will allow for the formation of stronger, more robust biofilms on the GS surface, thereby increasing persistence and survival within the host. At the same time, the survival of the wild-type phenotype ensures that *Salmonella* spp. that are shed can still infect additional hosts, thereby allowing continued propagation of the disease via contaminated food and water supplies.

MATERIALS AND METHODS

Bacterial growth conditions. Bacterial strains used in this study are listed in Table S1 in the supplemental material. Luria-Bertani (LB) was used for cultures, biofilm assays, and creation of mutants, unless otherwise stated. When necessary, antibiotics were used in the following concentrations: ampicillin, 100 μ g/ml; kanamycin, 45 μ g/ml; and chloramphenicol, 25 μ g/ml. For long-term growth experimentation, cultures were grown in LB broth supplemented with 3% ox bile (Sigma-Aldrich, St. Louis, MO), with or without the presence of a human GS for 60 days. The medium was replaced daily. GS were primarily composed of cholesterol and were obtained from Wayne Schwesinger at the University of Texas Health Science Center (HSC) at San Antonio. These samples were determined to be exempt from institutional review board (IRB) approval.

Biofilm growth on microtiter plates. Glass-bottom 12-well plates (14-mm-diameter microwells, glass, no 1.5; MatTek Corp., MA) uncoated or coated by evaporation with 4 mg of cholesterol (diluted in ether, anhydrous; J. T. Baker, NJ) were inoculated with 2×10^8 bacteria in 2 ml of LB with or without 3% ox bile. The plates were incubated for 24 h at 37°C in a GyroMini nutating mixer at 24 rpm (LabNet International, Inc., NJ). Isolates from the long-term growth experiment were tested in plastic-bottom 24-well plates (Thomas Scientific, NJ). Overnight cultures were grown in LB broth, normalized to an optical density at 600 nm (OD_{600}) of 0.8, diluted 1:100 in tryptic soy broth (TSB; diluted 1:20), and grown at 30°C under static conditions.

Crystal violet assays. Biofilms attached to the microtiter walls or cholesterol-coated coverslips were washed with $1 \times$ phosphate-buffered saline (PBS) and heat fixed at 60°C for 1 h. Following fixation, the biofilms were stained with 0.25% crystal violet for 5 min. Microtiter wells were then washed three times with $1 \times$ PBS, after which a 33% acetic acid solution was used to extract the dye. Crystal violet retention was measured by the optical density at 570 nm (OD_{570}) in a SpectraMax microplate reader (Molecular Devices, Sunnyvale, CA). Biofilms for each strain were grown in triplicate to ensure the consistency of the results. To compensate for background absorbance, OD_{570} values for noninoculated cholesterol-coated coverslips were averaged and subtracted. Hyperbiofilm isolates were defined as those with OD_{570} readings statistically significantly higher than those of the wild-type strain.

Biofilm disruption assays. Biofilms of various *S. Typhimurium* strains were initially grown for 24 h as listed above. Following this incubation period, medium was removed from each well and replaced with new LB supplemented with DNase (56 units/well), proteinase K (14 μ l/well), or cellulase (50 units/well). The plates were then incubated for 20 h at 37°C in a GyroMini nutating mixer (LabNet International, Inc., NJ) prior to bacterial enumeration or the crystal violet assay.

Motility assay. Overnight cultures of *S. Typhimurium* strains were diluted 1:100 and incubated on a rotating drum at 37°C to reach mid-to-late-exponential-phase growth (OD_{600} , 0.6 to 0.8). Cultures were then normalized, and 3 μ l of each culture was inoculated into the center of a 0.3% agar LB plate. These plates were incubated at 37°C for 24 h, after which the radius of growth was measured.

Cellulose production assay. Cellulose production was assessed via a modified broth calcofluor-binding assay. Overnight cultures were grown in LB broth without salt (LBNS) and normalized by optical density. These cultures were then diluted 1:100 in LBNS broth containing calcofluor (20 μ g/ml; Sigma) in black 96-well microtiter plates (Corning Costar, Cambridge, MA) and incubated in the dark at 22°C for 6 days (39). The fluorescence (excitation, 366 nm; emission, 565 nm) of each well was measured using a SpectraMax M2 plate reader (Molecular Devices, Sunnyvale, CA).

Electron microscopy. For SEM observations, biofilms grown on glass or cholesterol were fixed overnight at 4°C with 2.5% glutaraldehyde in 0.1 M phosphate buffer-0.1 M sucrose (pH 7.4), rinsed twice with 0.1 M phosphate buffer, and dehydrated by the addition of a graded series of ethanol (35%, 50%, 70%, 80%, 95%, and 100%). Samples were then chemically dried with consecutive washes of 25%, 50%, 75%, and 100% hexamethyldisilazane (Ted Pella, CA). Samples were dried overnight in a fume hood, mounted on aluminum stubs, and sputter coated with gold for observation using an FEI Nova NanoSEM. For TEM observations, overnight cultures of *S. Typhimurium* strains were rinsed with $1 \times$ PBS and resuspended in 200 μ l of 1.5% paraformaldehyde–1.5% glutaraldehyde in 0.1 M phosphate buffer to fix the cells. Cultures were stained using 2% uranyl acetate and visualized using an FEI Technai G2 Spirit transmission electron microscope.

Western blotting. Monoclonal antibodies against *Salmonella* species flagella (FlhC and FlhB; Maine Biotechnology Services, Portland, ME), anti-FlhC (BioLegend, San Diego, CA), or polyclonal anti-*Salmonella* (Thermo Fisher, Waltham, MA) were used to detect flagellin in whole-cell lysates or supernatants. Overnight cultures were grown in LB broth and normalized to an OD_{600} of 1.5. Cells were pelleted, and the supernatant was collected. Supernatant proteins were precipitated by trichloroacetic acid (TCA) and pelleted. Cell or protein pellets were resuspended in 75 μ l of Laemmli loading buffer. Samples were boiled for 15 min, and 15 μ l was loaded onto a 15% SDS-PAGE gel for separation. Proteins were transferred for 1 h at 60 V to a methanol (MeOH)-activated polyvinylidene difluoride (PVDF) membrane and blocked overnight in 5% bovine serum albumin (BSA) in Tris-buffered saline (TBS). The membrane was washed in TBS-Tween 20 (TBST) (3×10 min), incubated with antibody (1:10,000 in 5% BSA-TBST for 2 h, 22°C) washed in TBST (3×10 min), and incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (1:12,000 in 5% BSA-TBST for 2 h, 22°C; Bio-Rad, Hercules, CA). Membranes were visualized using a Bio-Rad ChemiDoc system.

Generation of mutants and cloning procedures. Mutations of various genes of interest were performed by using the λ red mutagenesis method (40). To create a complemented strain of the deletion mutants, the wild-type genes were cloned in pWSK29. In order to mimic the mutations observed from sequencing various hyperbiofilm strains, the wild-type genes were altered via site-directed mutagenesis with a Phusion kit (Thermo Fisher Scientific Inc., Waltham, MA) to match the observed single nucleotide polymorphisms (SNPs). Oligonucleotide primers used to perform gene deletion, cloning, and site-directed mutagenesis are listed in Table S2.

Mouse infections. Because *S. Typhi* is a human-specific pathogen, a mouse model of infection using *S. Typhimurium* was employed (8). Naturally resistant *Nramp1*^{+/+} 129 $\times$ 1/SvJ mice (Jackson Laboratory, ME) were fed a lithogenic diet (1% cholesterol and 0.5% cholic acid; Sigma) or normal chow (Harlan Laboratory, IN). After 6 weeks, mice were infected intraperitoneally with 10^4 *S. Typhimurium* bacteria and sacrificed at 8 days postinfection. Liver, GB, bile, and GS (where appropriate) were homogenized and/or diluted for bacterial enumeration using LB agar.

Sequencing. Two rounds of sequencing were performed to identify mutations in the hyperbiofilm strains. The first round, where strains JSG210 (WT) and JSG3558 (hyperbiofilm) were sequenced, was

performed at the Ohio State University Program in Pharmacogenetics using the Ion Torrent sequencer and software. The assembled sequences were compared to those of a reference strain (*S. Typhimurium* 140285), a previously sequenced complete genome for *S. Typhimurium*, in order to identify SNPs. In a second round of sequencing, strains JSG3752 (no. 3), JSG3753 (no. 9), JSG3700 (bile) were sequenced along with the original WT (JSG210) and hyperbiofilm (JSG3538) strains. This sequencing was performed at the Nationwide Children's Hospital Biomedical Genomics Core using Illumina MiSeq technology with 151-bp paired-end reads. The average sequence coverage ranged from 375 to 472 reads, and the variants were identified by the Churchill method (36) and were annotated using Annovar (37). The mutation frequencies identified relate to the number of times the allele variant occurred in the sequence versus the reference (wild type) sequence.

Statistical analysis. Statistical significance testing was performed using GraphPad Prism 7. All *P* values of <0.05 were considered significant.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/JB.00774-18>.

SUPPLEMENTAL FILE 1, PDF file, 0.9 MB.

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