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MOLECULAR PATHOGENESIS

Analysis of *Shigella flexneri* Resistance, Biofilm Formation, and Transcriptional Profile in Response to Bile Salts

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ABSTRACT The *Shigella* species cause millions of cases of watery or bloody diarrhea each year, mostly in children in developing countries. While many aspects of *Shigella* colonic cell invasion are known, crucial gaps in knowledge regarding how the bacteria survive, transit, and regulate gene expression prior to infection remain. In this study, we define mechanisms of resistance to bile salts and build on previous research highlighting induced virulence in *Shigella flexneri* strain 2457T following exposure to bile salts. Typical growth patterns were observed within the physiological range of bile salts; however, growth was inhibited at higher concentrations. Interestingly, extended periods of exposure to bile salts led to biofilm formation, a conserved phenotype that we observed among members of the *Enterobacteriaceae*. Characterization of *S. flexneri* 2457T biofilms determined that both bile salts and glucose were required for formation, dispersion was dependent upon bile salts depletion, and recovered bacteria displayed induced adherence to HT-29 cells. RNA-sequencing analysis verified an important bile salt transcriptional profile in *S. flexneri* 2457T, including induced drug resistance and virulence gene expression. Finally, functional mutagenesis identified the importance of the AcrAB efflux pump and lipopolysaccharide O-antigen synthesis for bile salt resistance. Our data demonstrate that *S. flexneri* 2457T employs multiple mechanisms to survive exposure to bile salts, which may have important implications for multidrug resistance. Furthermore, our work confirms that bile salts are important physiological signals to activate *S. flexneri* 2457T virulence. This work provides insights into how exposure to bile likely regulates *Shigella* survival and virulence during host transit and subsequent colonic infection.

KEYWORDS *Shigella*, bile salts, biofilm, resistance, EPS matrix, virulence genes, *Escherichia coli*, virulence

The *Shigella* species are Gram-negative, facultative intracellular pathogens that cause illness by invading the colonic epithelium. Shigellosis results in significant morbidity and mortality around the globe each year, predominately in children under the age of 5 years in developing countries (1–3). There is currently no vaccine against *Shigella*, and antibiotic resistance is an increasing complication, particularly in areas with limited medical care (4). In order to invade epithelial cells, the bacteria utilize a type III secretion system (T3SS), the Ipa proteins required for invasion, and other effector proteins required to maintain infection encoded by a 200- to 220-kb virulence plasmid (3).

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While many aspects of the *Shigella* invasion process, its intracellular survival, and the immune response have been thoroughly investigated, there is a significant gap in knowledge regarding how the bacterium successfully reaches the colonic epithelium to establish infection. During host gastrointestinal transit, the bacteria are exposed to numerous stimuli, particularly bile in the small intestine. Bile is an essential component of digestion and is composed of proteins, lipids, carbohydrates, vitamins, mineral salts, and other trace elements (5). In the liver, primary bile acids are synthesized by hepatocytes and are further metabolized by conjugation to glycine or taurine through N-acyl amidation. At physiological pH, conjugated bile acids are almost fully ionized and are termed bile salts (5, 6). Bile salts are maintained in the duodenum, jejunum, and proximal ileum to solubilize, digest, and absorb lipids and lipid-soluble vitamins (5, 6). In the distal ileum, bile salts are absorbed into the bloodstream, complexed to plasma proteins, and returned to the liver (6). The majority of bile (95%) is recycled back into the small intestine and does not enter the colon (7). In the small intestine, concentrations of bile salts range from 0.2% to 2% (wt/vol) depending on time of day, diet, and the individual (8). Bile is bactericidal (9, 10); however, Gram-negative bacteria, particularly enteric pathogens, are known to resist the bactericidal activity of bile and utilize this host component as a localization signal to regulate virulence gene expression and enhance infection (11).

Bile salts have been previously demonstrated to increase the ability of *Shigella flexneri* to adhere to and invade epithelial cells (12–15). Additionally, we have previously identified a mechanism of the bile salt-induced adherence phenotype by demonstrating that bile salts increase the expression of the *ospE1* and *ospE2* genes and subsequently promote the outer membrane localization of the proteins to function as adhesins (16). In order to further understand how *S. flexneri* 2457T survives host transit, we analyzed the ability of the bacteria to resist bile salts under various growth conditions. We identified that prolonged periods of exposure to bile salts led to biofilm formation in *Shigella*, a phenotype that we demonstrate is conserved among other enteric bacteria. Biofilm formation required the presence of glucose, whereas biofilm dispersion required removal of bile salts from the medium. Subsequent experiments demonstrated that *S. flexneri* 2457T released from the biofilm had an induced adherence phenotype relative to bacteria grown in traditional medium. RNA-sequencing analysis identified important changes in gene expression profiles during exposure to bile salts, including transcriptional alterations of key virulence genes and genes encoding products involved in resistance to bile salts. Finally, targeted mutagenesis experiments confirmed the importance of the predicted bile salt resistance genes. This work complements our previous analysis and provides us with a more complete understanding of how *S. flexneri* 2457T resists bile exposure and likely utilizes this host signal to induce virulence factor expression prior to entry into the colon. Insights gained from this work could enhance the development of novel therapeutics or vaccines.

RESULTS

Growth curve analysis of *S. flexneri* in the presence of bile salts. *S. flexneri* 2457T was grown in tryptic soy broth (TSB) or Luria-Bertani broth (LB) in the presence of various concentrations of bile salts (Fig. 1; see also Fig. S1 in the supplemental material). We examined growth at the physiological range of bile salts, 0.2% to 2% (wt/vol) (8), as well as at concentrations above the physiological range, i.e., at 5% and 10% (wt/vol). *S. flexneri* 2457T grew normally within the physiological range of bile salts as determined by CFU per milliliter, but growth was significantly slowed at 5% (wt/vol) bile salts and inhibited (bacteriostatic) at 10% (wt/vol) bile salts. The data indicate that *S. flexneri* 2457T can withstand the normal concentrations of bile salts found in the human gastrointestinal tract (8). For the TSB analysis, longer growth within the physiological range led to the formation of bacterial aggregates and white precipitation in the cultures (see below), which was not observed above the physiological range of bile salts. The aggregation and precipitation phenotype was reflected by sharp increases in the optical density readings relative to those taken in media without bile salts (Fig. 1B).

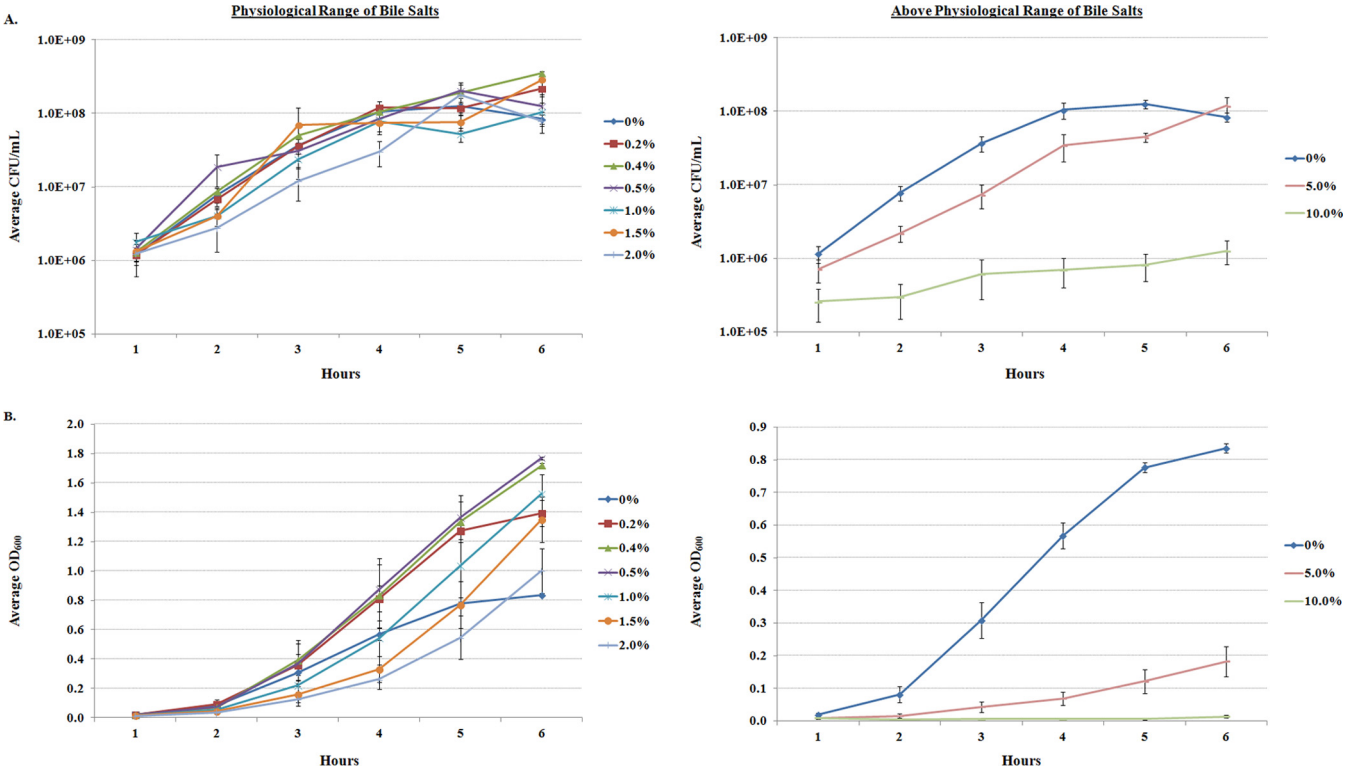

FIG 1 Growth curve analysis of *S. flexneri* 2457T in bile salts. Bacterial growth was monitored in TSB medium or medium containing increasing concentrations of bile salts. (A) Average CFU/ml ± standard errors (SE) for three independent experiments are plotted. *S. flexneri* 2457T grew normally within the physiological range of 0.2% to 2.0% (wt/vol), but growth was slowed and inhibited at 5% and 10% (wt/vol), respectively. Statistical significance relative to the 0% control was detected at 5% and 10% (wt/vol) with *P* values of 0.001. (B) The corresponding average OD_{600} ± SE are plotted. In the physiological range of bile salts, sharp increases in optical density were detected. Statistical significance relative to the 0% control was detected at 0.4%, 5%, and 10% (all wt/vol) with *P* values ranging from <0.05 to 0.0001.

These increases in optical density did not correlate with increases in bacterial growth (Fig. 1A). All subsequent analyses were performed in 0.4% (wt/vol) bile salts, since growth was determined to not be significantly altered at this concentration, the aggregation phenotype was statistically significant (Fig. 1B), and these conditions induced an adherence phenotype as previously described (16).

Prolonged exposure to bile salts induces biofilm formation. The longer periods of bile salt exposure in TSB (3 to 6 h under shaking conditions or overnight static growth) resulted in the formation of bacterial aggregates, clumping, and white precipitation in the culture (Fig. 2A). This phenotype was not detected in cultures exposed to bile salts in LB (data not shown). Scanning electron microscopy (SEM) analysis of static overnight cultures verified the observations (Fig. 2B). Bacteria cultured in TSB were dispersed on glass coverslips and were easily removed during the gentle washing step prior to fixing for SEM analysis. However, bacteria grown in TSB with 0.4% (wt/vol) bile salts adhered to the coverslips and bacterial aggregates were visualized during SEM analysis.

Given that the appearance of bacterial aggregates and the clumping phenotype resembled biofilm formation by other pathogens (17–22), additional assays were performed to verify and quantify the observations. First, crystal violet staining was performed based on previous observations of bile-induced biofilm formation in *Vibrio cholerae* (17). In the absence of bile salts, biofilm formation was minimal; however, there was a significant induction of biofilm formation in the presence of bile salts (Fig. 3). Second, since biofilm formation is characterized by extracellular polymeric substances (EPS) that consist mostly of polysaccharides whose production can be visualized through concanavalin A (ConA) staining (23, 24), we performed this analysis on *S. flexneri* 2457T overnight static cultures. In the absence of bile salts, EPS production and

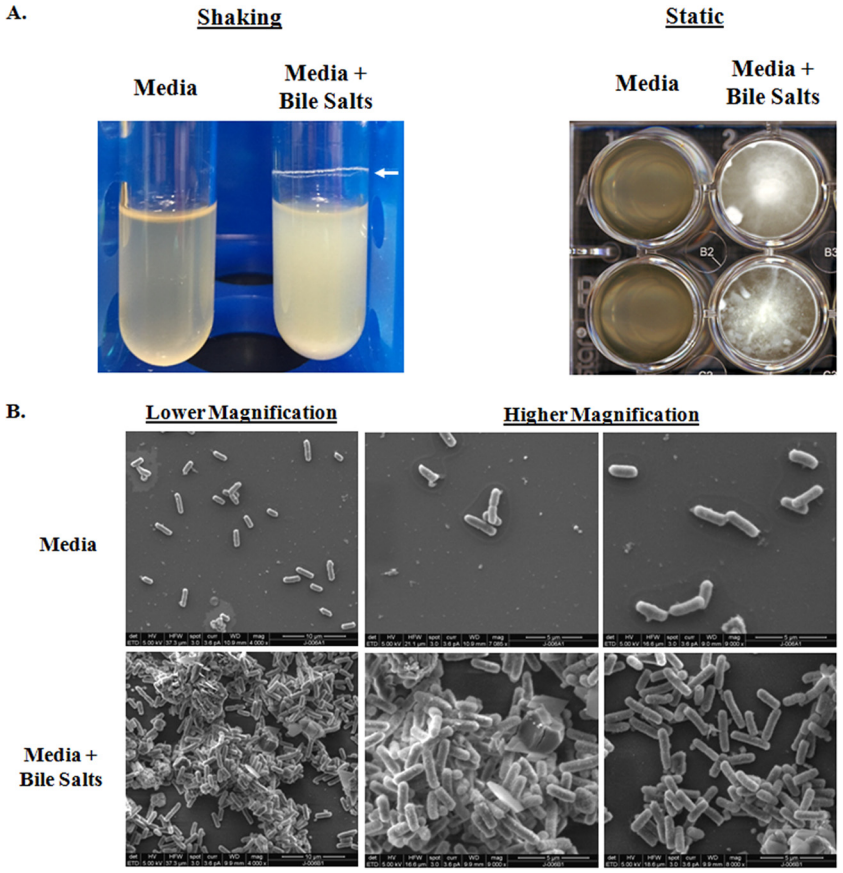

FIG 2 Prolonged exposure to bile salts leads to clumping of the bacteria. (A) Bacteria subcultured for 4 h under shaking conditions at 225 rpm (left) or single colonies inoculated into each well of a 24-well plate and grown statically overnight (right) in medium with or without bile salts. The bacteria exposed to 0.4% (wt/vol) bile salts clumped and formed an adhesive ring (white arrow) around the culture tube or to the bottom of the wells. Images are representative of three independent experiments. (B) Scanning electron microscopy analysis of bile salt-induced clumping. Bacteria were grown on coverslips in medium alone (top) or in the presence of 0.4% (wt/vol) bile salts (bottom), fixed, and processed for SEM analysis. In medium alone, the bacteria were dispersed on the coverslips. Following exposure to bile salts, the bacteria clumped and adhered better to the coverslips. Images are representative of three independent experiments. Lower magnification images (×4,000; scale bars, 10 μm) are on the left, and higher magnification images (×7,000 to ×9,000; scale bars, 5 μm) are in the middle and on the right.

the overall presence of aggregates were minimal. However, in the presence of bile salts, EPS production, aggregate frequency, and thickness significantly increased (Fig. 4). We further demonstrated that EPS production was dependent on the presence of glucose (Fig. 5A), the removal of bile salts led to biofilm dispersion (Fig. 5B), and *S. flexneri* 2457T released from the biofilm had significantly induced adherence to HT-29 cells (Fig. 5C). Finally, we were interested in determining if the bile salt-induced biofilm formation was conserved among the enteric pathogens within the *Enterobacteriaceae* family. We therefore tested isolates of both commensal and pathogenic strains of *Escherichia coli* as well as *Salmonella enterica* serovars Typhi and Typhimurium. As shown in Table 1, most strains of *E. coli* and both *S.* Typhi and *S.* Typhimurium formed a biofilm in this assay in the presence of bile salts.

RNA-seq analysis identifies differentially expressed genes following exposure to bile salts. We performed high-throughput RNA sequencing (RNA-seq) analysis of *S. flexneri* 2457T grown in the presence and absence of bile salts under both shaking and static conditions to identify patterns of gene expression, with differential expression defined as induced or repressed genes with a 2-fold or greater change in expression and a *P* value cutoff of ≤0.05. During shaking growth, there were a total of 276 differentially expressed genes in the cultures grown in the presence of bile salts

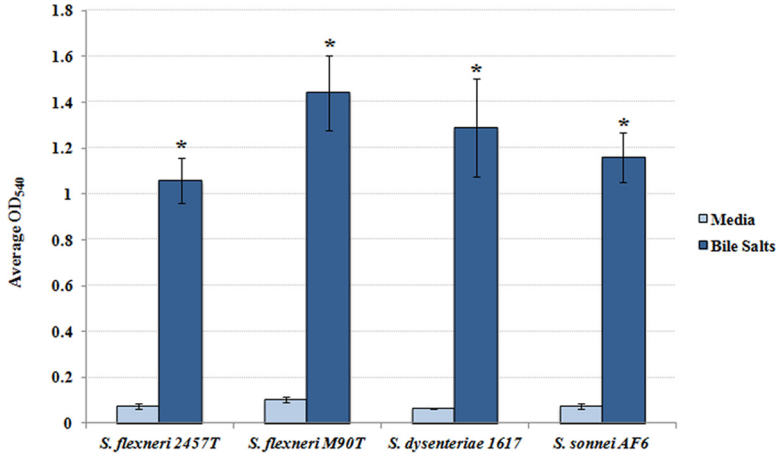

FIG 3 Quantification of biofilm formation. Single colonies of the indicated bacterial strains were inoculated into TSB ± 0.4% (wt/vol) bile salts, grown overnight at 37°C, and subsequently processed with crystal violet staining. In medium without bile salts, biofilm formation was minimal; however, following bile salt exposure, biofilm formation was significantly induced in all strains of *Shigella* tested (*, $P < 0.0001$). The average OD_{540} ± SE values from three independent experiments are presented. There were no significant differences between bacterial strains.

compared to those in media without bile salts, of which 172 genes were induced and 104 genes were repressed (Fig. 6; see also Tables S2 and S3 in the supplemental material). During static growth, there were a total of 156 differentially expressed genes in the presence of bile salts compared to media without bile salts, of which 93 genes were induced and 63 genes were repressed. There were 96 differentially expressed genes that occurred under both shaking and static conditions (Fig. 6B). Overall, induced genes in the presence of bile salts most notably comprised genes involved in central metabolism (*pur*, *glp*, *mgl*, *nik* genes), gene expression (*tdcA*, *yhjC*, and *rhaS*), sugar transporters (*fruAB*, *ptsG*, and *treB*), drug resistance (*acrAB*, *fsr*, and *yegB*), and virulence (several *osp* and *ipaH* genes, including *ospE1* and *ospE2*) (Fig. 6C; Tables S2 and S3). The analysis confirmed many of the microarray findings previously identified by our group (16) and allowed us to extend the gene expression studies to include bile salts under static growth conditions. Furthermore, the increased sensitivity and specificity of RNA-seq allowed us to detect additional transcriptional changes in the presence of bile salts, especially on the virulence plasmid (Tables S2 and S3).

In order to verify the RNA-seq results, we selected several genes to analyze via quantitative reverse-transcription (qRT)-PCR analysis using three biologically independent samples of RNA to compare the expression levels in the presence of bile salts with those in media without bile salts under shaking growth conditions (Fig. 7). We chose two highly induced genes, S2558 and *ospE1-ospE2*, two induced genes, *acrB* and *yhcN*, a repressed gene, *dsdA*, and an independently regulated control gene, *yacG*. In all, qRT-PCR analysis confirmed the induction of S2558, *ospE1-ospE2*, *acrB*, and *yhcN* in the presence of bile salts, the repression of *dsdA*, and the unchanged transcription of *yacG*.

Identification of mutants unable to grow in bile salts. In conjunction with our RNA-seq results and the literature characterizing bile resistance in other enteric pathogens (11), we analyzed a mutant in the AcrAB multidrug efflux pump (Δ*acrB*) and a Δ*galU* mutant that lacks the O-antigen of the lipopolysaccharide (LPS) (25, 26). Both mutants had growth patterns similar to those of the wild type in unsupplemented medium; however, the mutants were unable to grow in either 0.4% or 2.0% (wt/vol) bile salts. Complementation restored the bile salt resistance phenotype (Fig. 8). The data confirm the importance of the AcrAB efflux pump and complete LPS for *S. flexneri* 2457T resistance to bile salts.

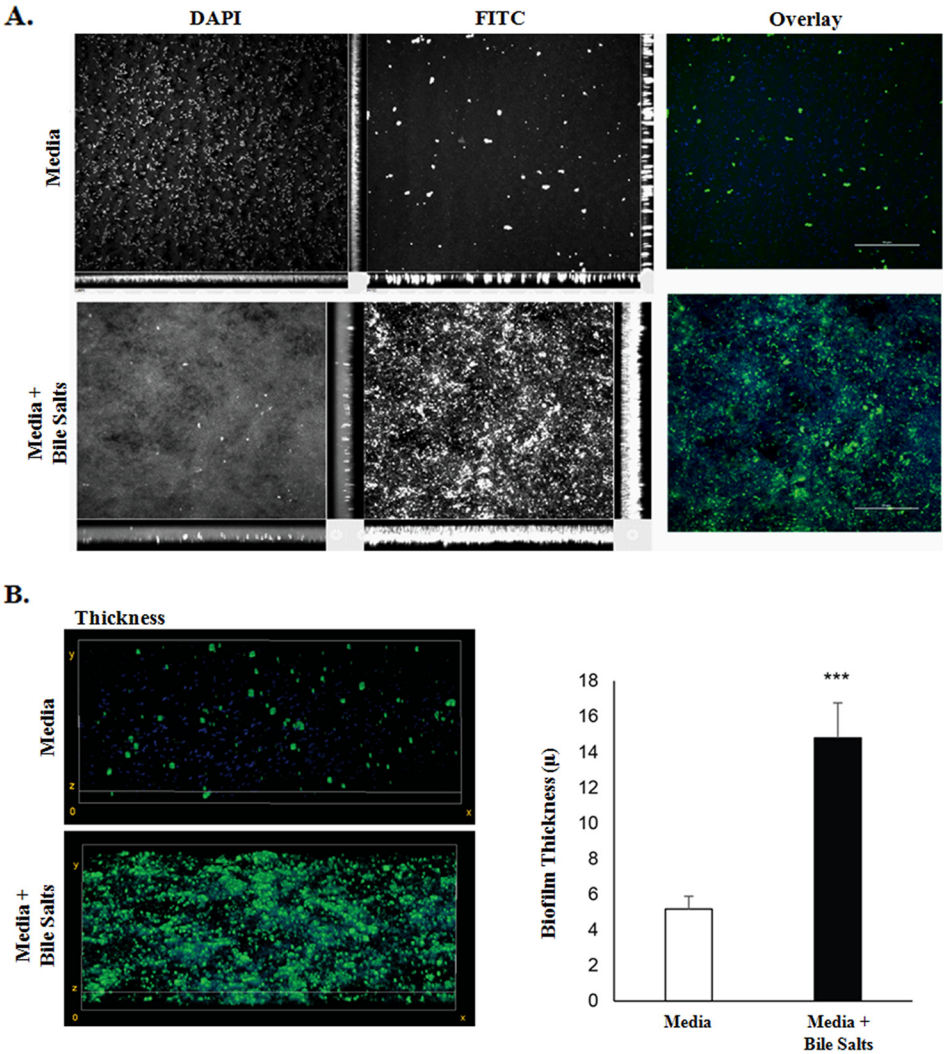

FIG 4 Analysis of exopolysaccharide matrix production. (A) *S. flexneri* 2457T was grown on glass coverslips in TSB ± 0.4% (wt/vol) bile salts, fixed, and stained with DAPI (blue) and 25 μg/ml FITC-conjugated concanavalin A (green) to assess EPS production. Bacteria in medium alone were dispersed and had few areas staining positive for concanavalin A, while bacteria exposed to bile salts clumped and had more concanavalin A staining. The images are representative of three independent experiments. (B) To quantify the thickness of the biofilms, image stacks were taken every 0.25 μm of the biofilm, and quantification was determined from the full thickness of the stack. The image on the left represents 3D reconstructed stacks using ImageJ software. The average thickness (±SE) of each biofilm from three independent experiments is plotted on the right. There was a significant difference in biofilm thickness of *S. flexneri* 2457T following exposure to bile salts (***, $P < 0.01$).

DISCUSSION

Bile is a multifunctional, essential component of digestion. In humans, it is secreted into the duodenum to solubilize and digest fats and lipids as food transits the small intestine (6). Bile also solubilizes lipid-based microbial membranes to function as an endogenous antimicrobial compound (9). Many enteric pathogens have developed multiple strategies to resist the bactericidal effects of bile while also utilizing this host signal to regulate virulence factor expression (11). For the first time, this work identifies bile salt resistance strategies used by *Shigella* that we hypothesize are required to successfully transit the small intestine prior to infection in the colon. Moreover, this work provides further evidence that *Shigella* utilizes bile as a signal to adapt to an intestinal environment and facilitate virulence gene expression prior to infection in the colon.

Resistance to bile is accomplished through several approaches in enteric pathogens (9, 11). One resistance strategy repeatedly observed in *Salmonella*, *Vibrio*, *Campylobacter*, and *E. coli* is the removal of bile compounds through the use of several types of

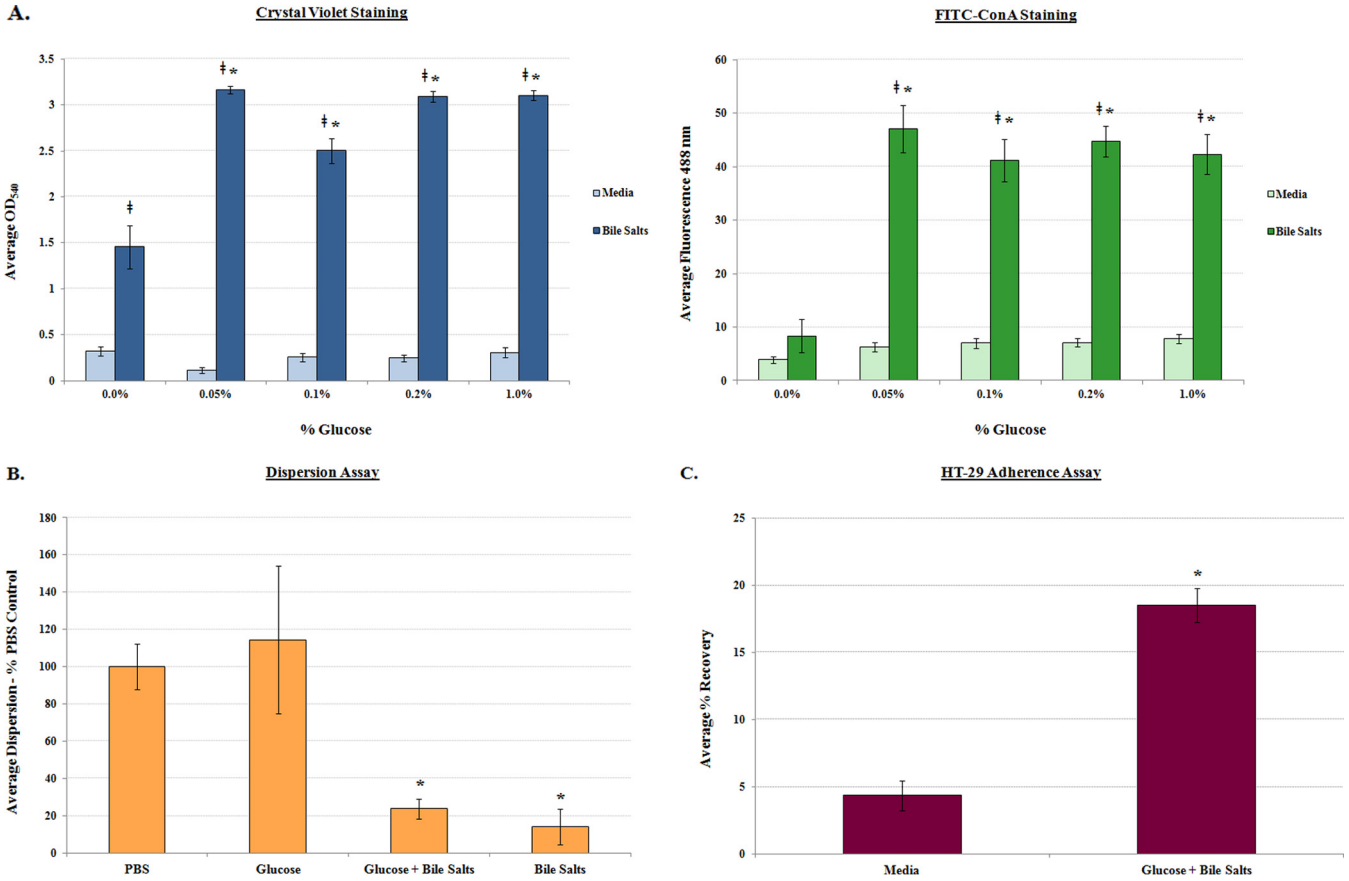

FIG 5 Analysis of biofilm formation, dispersion, and subsequent adherence to HT-29 cells. (A) Analysis of glucose requirement in biofilm formation. Single colonies of *S. flexneri* 2457T were inoculated into medium (LB) with or without 0.4% (wt/vol) bile salts at the indicated percentage (wt/vol) of glucose. Crystal violet (left) and FITC-ConA (right) staining was performed, and the average OD_{540} and fluorescence at 488 nm, respectively, ± SE are presented. Bile salts increased bacterial aggregation, and the addition of glucose further increased this phenotype. However, EPS production was dependent on the presence of glucose. For both the crystal violet and FITC-ConA staining, all differences between medium alone and the corresponding condition with bile salts are indicated with a double dagger (‡, $P < 0.001$). Differences in biofilm formation between each of the glucose plus bile salts concentrations relative to 0.0% glucose plus bile salts concentration are indicated with an asterisk (*, $P < 0.01$). (B) Analysis of bile salt removal in biofilm dispersion. *S. flexneri* 2457T biofilms were gently washed with PBS and resuspended in prewarmed PBS or PBS with the indicated treatments for 30 min at 37°C. The supernatants were subsequently removed and dilution plated, and CFU were determined. Dispersion was minimal in the presence of bile salts (*, $P < 0.01$), regardless of the presence of glucose. The average dispersion ± SE relative to the PBS control is plotted for three independent experiments in which technical triplicates were performed for each assay. For reference, the average recovery for the PBS treatment was 7.6×10^7 CFU while the average recovery for the PBS with bile salts treatment was 7.8×10^5 CFU. (C) Analysis of *S. flexneri* 2457T adherence following dispersion from the biofilm. Bacteria were grown overnight in media (LB) without or with a combination of glucose and bile salts to initiate biofilm formation. On the next day, bacteria were collected, washed with 1× PBS, and analyzed for adherence to HT-29 cells. Strain 2457T consistently had significantly increased adherence following biofilm dispersion (*, $P < 0.0001$) compared to bacteria recovered from medium without glucose and bile salts. The average percent recovery of adherent bacteria ± SE is plotted for three independent experiments, each of which had technical triplicates.

efflux pumps (11, 27–34). Efflux pumps are utilized by bacteria to expel antibiotics and other harmful substances from the bacterial cytosol. The AcrAB efflux pump is a member of the resistance nodulation division (RND) family of efflux pumps, which represents one of the five families associated with multidrug resistance (MDR) (35). The increased expression of the AcrAB efflux pump in the presence of bile salts not only would enable *Shigella* to resist the bactericidal effects of bile but could also potentially "prime" the bacteria for an antibiotic resistance phenotype. In fact, a recent study evaluating the bile salt effects on enterohemorrhagic *E. coli* (EHEC) found that increased expression of *acrAB* transcripts, as well as increased expression of lipopolysaccharide genes, conferred resistance to the antibiotic polymyxin (30). Furthermore, overexpression of AcrB has been linked to MDR resistance in *S.* Typhimurium (36, 37). Therefore, the ability to combat MDR in enteric pathogens is potentially further complicated by the simple fact that these pathogens are exposed to bile during the infection process. This concept is an important consideration for future antimicrobial strategies to combat MDR and treat infections resulting from enteric pathogens.

TABLE 1 Biofilm analysis of additional bacterial strains

Strain[a]	Bile salts	Avg OD_{540}[b]	SE	*P* value[c]
E. coli F18	−	0.156	±0.038	
	+	1.712	±0.108	<0.0001
E. coli HS	−	0.220	±0.018	
	+	0.249	±0.019	Not significant
E. coli MC4100	−	0.201	±0.039	
	+	1.189	±0.116	<0.0001
EHEC 86-24	−	0.015	±0.021	
	+	1.216	±0.070	<0.0001
EHEC 933	−	0.014	±0.013	
	+	1.399	±0.140	<0.0001
STEC 98NK2	−	0.088	±0.035	
	+	0.747	±0.097	<0.0001
EPEC E2348/69	−	0.005	±0.002	
	+	0.323	±0.058	0.0001
EAEC O42	−	0.228	±0.017	
	+	0.257	±0.024	Not significant
EAEC Δ*aaf*	−	0.012	±0.004	
	+	0.282	±0.039	0.0001
S. Typhi Ty2	−	0.053	±0.007	
	+	0.505	±0.165	0.04
S. Typhimurium SL1344	−	0.009	±0.003	
	+	0.210	±0.036	0.0001

[a]EHEC, enterohemorrhagic *E. coli*; STEC, Shiga toxin-producing *E. coli*; EPEC, enteropathogenic *E. coli*; EAEC, enteroaggregative *E. coli*.
[b]The average OD_{540} for the crystal violet quantification of biofilm formation was measured for three independent experiments.
[c]*P* values based on the Student *t* test between media with or without bile salts for each of the strains tested.

The presence of LPS, specifically the O-antigen, is important for bile resistance in enteric pathogens, as various rough mutants or mutations in *galU*, which catalyzes the formation of UDP-glucose and is required for O-antigen synthesis, lead to bile salt sensitivity in *Salmonella*, *E. coli*, and *Vibrio* (9, 32, 38, 39). Likewise, in our study, loss of *galU* and therefore the complete O-antigen (26) abolished *Shigella* growth capability in the presence of bile salts. Analysis in *Salmonella* has demonstrated that both remodeling of lipid A and elongation of the O-chains of LPS are important for bile resistance (40–42). LPS length has also been demonstrated to be important for *Shigella* virulence. The *S. flexneri* Δ*galU* mutant exhibited a defect in adherence to and invasion of polarized intestinal epithelial cells (25) and failed to efficiently spread from cell to cell due to a lack of proper IcsA unipolar localization (26). Future analysis will determine the *Shigella* LPS modifications that occur in the presence of bile and examine the subsequent effects on virulence.

Prolonged exposure of *Shigella* to bile salts resulted in increased biofilm formation, an important resistance mechanism for many bacterial pathogens. Biofilm formation is characterized by a surface-adherent, EPS matrix-protected prosurvival state and is a common approach for bacterial pathogens to resist antimicrobial exposures such as antibiotics, UV radiation, and pH stress (24). We hypothesize that bile salt-induced biofilm formation is an additional bile resistance strategy employed by *Shigella*. We are currently in the process of identifying and performing functional mutagenesis studies in genes required for EPS production to assess their effects on biofilm formation and bile salt resistance. Similar biofilm phenotypes have been described for *Vibrio*, *Campylobacter*, and *Listeria* as well as for *Salmonella* gallbladder colonization (11, 17, 43–46).

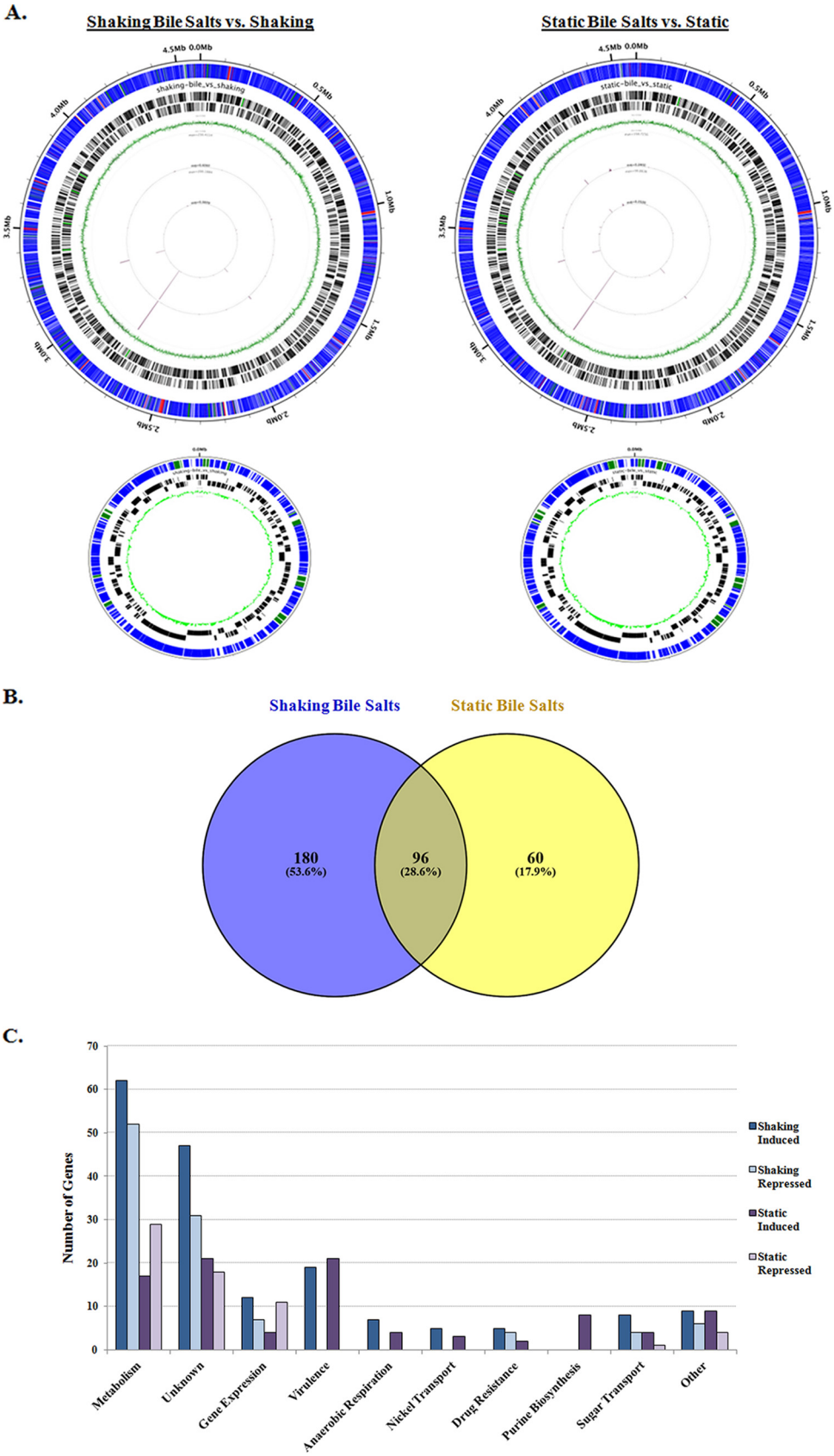

FIG 6 RNA-sequencing analysis of *S. flexneri* 2457T grown in the presence and absence of 0.4% (wt/vol) bile salts. (A) Results of the RNA-seq analysis visualized with the Circleator program (71), in which blue represents unchanged genes, green represents induced genes (fold change $\geq$ 2, $P < 0.05$), and red represents repressed genes (fold

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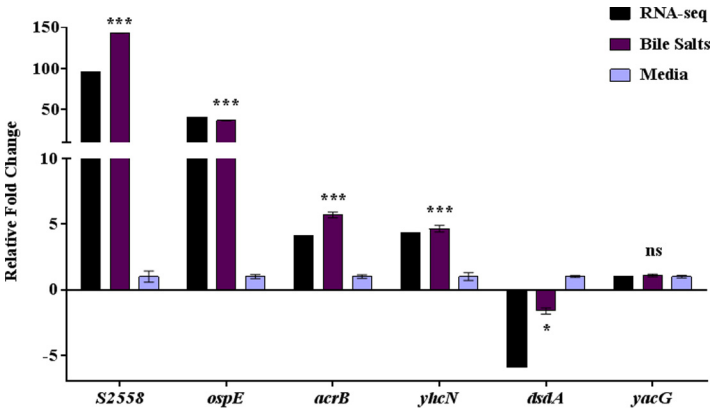

FIG 7 Quantitative reverse transcription-PCR analysis verifies the RNA-sequencing analysis. qRT-PCR was performed on three independent RNA samples isolated from *S. flexneri* 2457T grown with 0.4% (wt/vol) bile salts (Bile Salts) or without (Media). The relative fold changes ± SE from the ΔC_T values are plotted for the 6 genes. The corresponding changes in gene expression from the RNA-seq analysis are provided as a reference in black. *ospE* indicates both *ospE1* and *ospE2*. Significance symbols for the qRT-PCR data of expression of genes in bile salts compared to the expression of genes in media without bile salts: ***, $P < 0.0001$; *, $P < 0.05$; n.s., not significant.

Our work extends this observation to *Shigella* and additional enteric bacteria such as the pathogenic variants of *E. coli* and, as a result, demonstrates that bile salt-induced biofilm formation is conserved among members of the *Enterobacteriaceae* family. In keeping with the accepted paradigm that minimal numbers of *Shigella* are required for establishing an infection (3), we hypothesize that biofilm formation also promotes bacterial cell aggregation to ensure successful transit of the small intestine and entry into the colon for infection. As noted in our analysis here, and in other pathogens such as *Vibrio* (47, 48), virulence factor expression is also regulated during exposure to bile salts. Given the dual control of resistance to bile salts and virulence factor expression in the presence of bile salts, we propose that the enteric bile salt-induced biofilm is a transient phenotype compared to traditional biofilms produced by pathogens like *Pseudomonas* (24). The transient nature would allow a more rapid dispersion as the bacteria reach the epithelial cell surfaces in the small intestine or enter the colon, where bile concentrations are reduced relative to those of the lumen of the small intestine (6). In fact, to highlight this transient nature, our analysis demonstrated that there was significant biofilm dispersion following 30 min of removal of bile salts from the media. Interestingly, biofilm formation has been shown to require purine biosynthesis regulation of the bacterial second messenger cyclic-di-GMP (49), efflux pumps (50), and *galU* for EPS matrix production (38), further connecting our findings of the bile salt-induced gene expression profile and the bile salt Δ*acrB* and Δ*galU* mutant phenotypes to biofilm formation. Future analysis will determine the effects of these mutations on biofilm formation and virulence.

We demonstrated that robust biofilm formation did not occur in LB medium and required the presence of glucose as well as bile salts. The requirement for glucose in

FIG 6 Legend (Continued)
change ≤ -2, $P < 0.05$) in the presence of bile salts for both the chromosome (top) and virulence plasmid (bottom). Barcodes represent forward and reverse genes, while the green circle represents the percent GC skew throughout the genome. (B) Venn diagram (72) of differentially expressed genes identified in the RNA-seq analysis. The numbers of induced and repressed genes for the "shaking + bile salts versus shaking" condition compared to the "static + bile salts versus static" condition are depicted. For both comparisons, there were 96 genes that were induced or repressed in the presence of bile salts. For the "shaking + bile salts versus shaking" analysis, there were an additional 180 genes induced or repressed, for a total of 276 differentially expressed genes in bile salts. For the "static + bile salts versus static" analysis, there were an additional 60 genes that were induced or repressed, for a total of 156 differentially expressed genes in bile salts. (C) Functional categories for differentially expressed genes in the presence of bile salts are plotted with the number of genes induced or repressed under the bile salts condition for both the shaking and static growth conditions. Please refer to Tables S2 and S3 in the supplemental material for details.

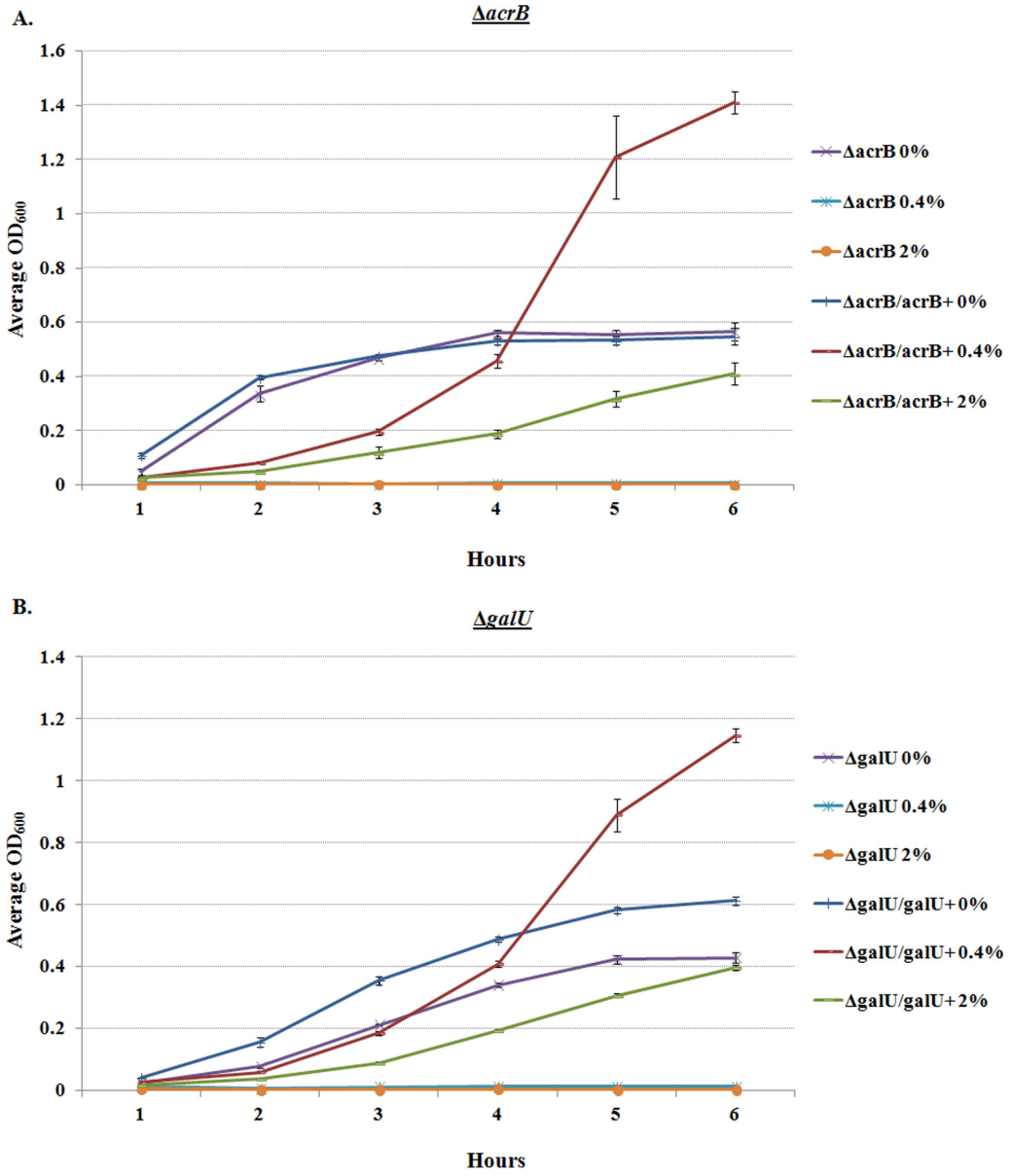

FIG 8 Growth analysis of mutants in the presence of bile salts. The Δ*acrB* (A) and Δ*galU* (B) mutants were analyzed for growth in TSB ± 0.4% and 2% (wt/vol) bile salts. The graphs depict the growth curve analysis of three independent experiments, with the average OD_{600} ± SE plotted. The mutants were unable to grow in either 0.4% or 2% bile salts ($P < 0.001$), and complementation restored growth in both 0.4% and 2% bile salts ($P < 0.001$).

biofilm formation, particularly for EPS production, has been documented for many pathogens, including *Vibrio* (51, 52), *Listeria* (53), *Pseudomonas* (54), *Acinetobacter* (55), and *Staphylococcus* (56). To further highlight the importance of glucose in biofilm formation, the RNA-seq analysis demonstrated that the *ptsG* gene, encoding the glucose-specific phosphoenolpyruvate phosphotransfer system (PTS) IIBC components, was induced in *S. flexneri* 2457T in the presence of bile salts as seen with *Vibrio* biofilms (51, 52). As food is digested in the small intestine, simple sugars such as glucose are available until these sugars are absorbed by host cells in the terminal ileum (57). Therefore, *Shigella* is exposed to both bile salts and glucose as it transits the majority of the small intestine to enable biofilm formation. Given that bile has additional components, including a few bile salts not analyzed here (6), future analysis of biofilm formation with the individual components of bile or bile extract in the presence and absence of glucose will confirm our findings.

Finally, this work provides further insights that bile acts as a host localization signal to prepare *Shigella* for entry into the colon. In conjunction with previous work demonstrating that bile salts induce *Shigella* adherence and invasion to epithelial cells (12–16), *S. flexneri* 2457T recovered from the biofilm had an increased adherence phenotype. This result is confirmed by the RNA-seq analysis in which there was significant induction of *ospE1* and *ospE2* genes that encode the bile salt-induced adhesins (16). Furthermore, induction of additional *osp* and *ipaH* genes in the presence of bile salts was detected, which we hypothesize would prepare the bacteria for an intracellular lifestyle, as many of these genes are important for maintaining infection in epithelial cells (3, 58). Interestingly, no virulence genes were repressed in our analysis; however, it is worth noting that genes encoding the Mxi-Spa T3SS and the Ipa invasion proteins were not induced in the presence of bile salts, using the conservative thresholds outlined. The *mxi-spa* and *ipa* operons are effectively induced at 37°C through VirF activation of the central transcriptional regulator *virB*, with VirB in turn regulating *mxi-spa* and *ipa* transcription (3). We hypothesize that additional induction of invasion genes in the small intestine by bile would be detrimental to *Shigella* by promoting a premature invasion phenotype and possibly preventing the ability of the bacteria to survive in the presence of bile. Bile salts trigger the recruitment of the invasins to the tip of the T3SS needle complex, which would be sufficient for *Shigella* to prepare for invasion (12, 14, 15). In addition to virulence gene inductions, we hypothesize that exposure to bile salts permits *Shigella* to prepare for the anaerobic environment encountered in the colon. The *nikABCDE* operon, which is important for nickel transport and has been shown to be required for survival under anaerobic conditions (59), was induced in bile salts. Also, the *yegB* gene was induced 2.8-fold in the presence of bile salts. This gene is a homolog to the efflux pump component *mdtE*, which has been shown to protect *E. coli* from toxic by-product accumulation during anaerobic respiration (60). In all, the induction of genes that support both virulence and exposure to an anaerobic environment further suggests that *Shigella* utilizes bile as a signal to prepare for infection in the low-oxygen environment of the colon.

In conclusion, this study identifies potential mechanisms by which *Shigella* resists the bactericidal effects of bile and provides further insights into how bile enhances a virulent phenotype in *Shigella*. We propose a model by which *Shigella* induces bile salt resistance and related biofilm formation phenotypes during transit of the small intestinal lumen while at the same time inducing virulence factor expression (Fig. 9). Based on our data, we propose that the reduction of available bile in the terminal ileum promotes *Shigella* biofilm dispersion, enabling subsequent adherence and invasion in the colon. Furthermore, this work highlights the importance of incorporating *in vivo* exposures or aspects of human physiology in studying host-microbe interactions. One of the well-known challenges in studying *Shigella* is the limited animal models available (61). Since *Shigella* is a human-adapted pathogen and bile composition varies from species to species with notable differences in routine laboratory animals such as rats, rabbits, and nonhuman primates (62), significant alterations in *Shigella* gene expression profiles following exposure to bile salts underscore the importance of adaptation to host physiology during pathogenesis analysis. Incorporation of preexposure of bile salts into *in vitro* and *in vivo* infection models for *Shigella* and other enteric pathogens may finally improve our approaches to developing cost-effective, long-lasting therapeutics and vaccines.

MATERIALS AND METHODS

Bacterial strains and growth conditions used in the study. Table 2 lists the bacterial strains and plasmids used in this study. Bacteria were routinely cultured at 37°C in either Luria-Bertani broth (LB) or tryptic soy broth (TSB, which contains an additional 2.5 g/liter glucose relative to LB), with aeration or in petri dishes to represent static growth conditions. Plating for CFU was performed using tryptic soy broth plates with 1.5% agar and 0.025% Congo red (CR; Sigma). Bile salts (Sigma B8756, consisting of an approximate 1:1 mixture of cholate and deoxycholate) were used at a concentration of 0.4%, wt/vol, unless indicated otherwise. For the dose-dependent glucose analysis, the indicated amounts of glucose were added to LB. All media were filter sterilized with a 0.22-μm filter following the addition of bile salts

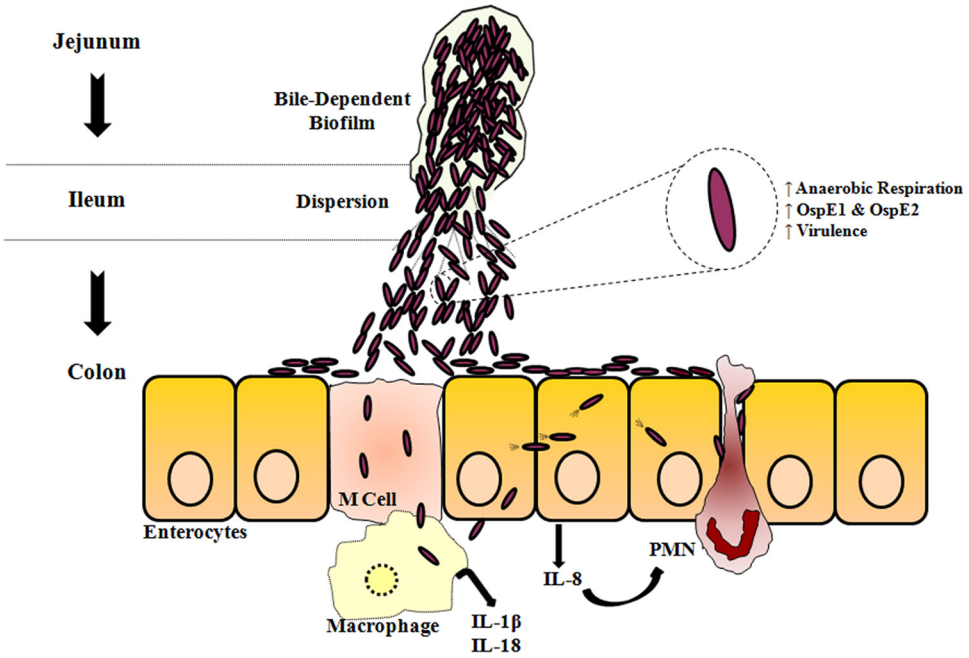

FIG 9 Model of *Shigella* infection following bile exposure. As *Shigella* transits the small intestine, exposure to bile induces transcriptional changes, bile resistance mechanisms, and biofilm formation. In the terminal ileum, where the majority of bile is absorbed, *Shigella* disperses the biofilm and subsequently enters the colon. Transcriptional changes already induced in response to bile, such as increased anaerobic respiration and the induction of the *osp* and *ipaH* virulence genes, enable the bacteria to efficiently adapt to the colonic environment, attach and invade epithelial cells, and establish infection. The bacteria escape macrophages by inducing cell death, invade the basolateral pole of the colonic epithelium, and regulate interleukin-8 (IL-8) secretion to control polymorphonuclear (PMN) cell migration (3, 73–75).

and/or glucose. Chloramphenicol was used at 5 μg/ml, ampicillin at 100 μg/ml, and tetracycline at 10 μg/ml when indicated.

Growth curve analysis. Bile salts were dissolved in sterile medium (LB or TSB) at the specified concentration and sterilized with a 0.22-μm filter. Overnight cultures of *S. flexneri* 2457T were diluted 1:50 into medium with or without the various concentrations of bile salts. Triplicate cultures were grown in a shaking incubator at 225 rpm at 37°C in a 96-well plate. The optical density at 600 nm (OD_{600}) was measured every hour over a period of 6 h using a Spectramax plate reader with samples blanked against medium alone. Aliquots of the cultures were removed for dilution plating from each well after gently mixing at each time point to determine the CFU present in each well. For all assays, statistical significance was determined by a two-way analysis of variance (ANOVA) and a *P* value of $\leq$0.05 was considered significant.

Static growth analysis and biofilm assay. Single colonies of each bacterial strain were inoculated into TSB $\pm$ 0.4% (wt/vol) bile salts in a single well of a 96-well plate. Plates were incubated at 37°C overnight without shaking. On the following day, wells were gently washed twice with 1$\times$ phosphate-buffered saline (PBS) and stained with 0.5% crystal violet for 5 min. Afterwards, the wells were gently washed five times with sterile distilled water (dH_2O) and then set to air dry. Biofilm formation was quantified by adding 95% ethanol to the wells to solubilize the crystal violet stain. After 30 min of incubation at room temperature, absorbance at 540 nm (OD_{540}) was measured with the plate reader (63). Samples were blanked to wells that were incubated with medium without bile salts and underwent the same staining procedure. For the dose-dependent glucose analysis, the indicated percentage of glucose was added to LB medium with or without 0.4% (wt/vol) bile salts, and the assay was performed as described above. Absorbance readings at OD_{600} were taken to ensure that there were no significant differences in growth prior to the washing steps. Statistical significance was determined by Student's *t* test (for comparisons of presence and absence of bile salts for each strain) or an ANOVA, and a *P* value of $\leq$0.05 was considered significant.

For biofilm dispersion analysis, *S. flexneri* 2457T biofilms were seeded as described above. On the following day, wells were gently washed twice with 1$\times$ PBS, and biofilms were resuspended in prewarmed PBS, PBS with 2% glucose, PBS with 2% glucose and 0.4% bile salts, or PBS with 0.4% bile salts and incubated at 37°C for 30 min. Afterwards, supernatants were collected, dilution plated onto CR plates, and incubated overnight to determine the CFU. Experiments were performed in biological triplicates, with technical triplicates for each experiment. Statistical significance was determined using an ANOVA, and a *P* value of $\leq$0.05 was considered significant.

Scanning electron microscopy. In order to analyze the appearance of the outer surface of the bacteria in the presence of bile salts, SEM was performed. Single colonies were grown on coverslips in

TABLE 2 Bacterial strains and plasmids used in this study

Name	Description	Source or reference
Strains		
Shigella		
2457T	*S. flexneri* serotype 2a	76
M90T	*S. flexneri* serotype 5	77
1617	*S. dysenteriae*	78
AF6	*S. sonnei* (recent clinical isolate)	AFRIMS[a]
BS766	2457T transformed with pKM208	70
Δ*acrB*	2457T/*acrB*::*cat*, chloramphenicol resistance	This study
Δ*acrB* (pAcrB)	Δ*acrB* transformed with pAcrB	This study
BS109	2457T/*galU*::Tn*10*, tetracycline resistance	26
BS109 (pGalU)	BS109 transformed with pGalU	This study
E. coli		
DH5α	Laboratory *E. coli*	79
F18	Mouse commensal *E. coli*	80
HS	Human commensal *E. coli*	81
MC4100	*E. coli* K-12 derivative	82
86-24	Enterohemorrhagic *E. coli* O157:H7 (Stx2)	83
933	Enterohemorrhagic *E. coli* O157:H7 (Stx1, Stx2)	83
STEC 98NK2	Shiga toxin-producing *E. coli* O113:H21 strain	84
EPEC E2348/69	Enteropathogenic *E. coli* archetype O127:H6 strain	81
EAEC O42	Enteroaggregative *E. coli* archetype O42	85
Δ*aaf* strain	O42/*aafA*::Tn*phoA*	86
Salmonella		
Typhi	*S. enterica* serovar Typhi strain Ty2	87
Typhimurium	*S. enterica* serovar Typhimurium strain SL1344	88
Plasmids		
pKD3	*oriR6K*, *bla*, *cat*	89
pKM208	Temperature-sensitive *red*-, *gam*-, *lacI*-expressing plasmid driven by P_{Tac} promoter, *bla*	89
pUC19	Cloning vector, *bla*, high copy no.	90
pGEMT	PCR cloning vector, *bla*, high copy no.	Promega
pAcrB	pUC19::*acrB*, wild-type gene cloned into pUC19	This study
pGalU	pGEMT::*galU*, wild-type gene cloned into pGEMT	This study

[a]Department of Enteric Diseases, Armed Forces Research Institute of Medical Sciences (AFRIMS), Bangkok, Thailand.

TSB ± 0.4% (wt/vol) bile salts and incubated at 37°C overnight. The coverslips were gently washed, placed in 3% glutaraldehyde fixative (in PBS), and sent for SEM processing by the Core Imaging Facility, University of Maryland School of Dentistry. Samples were imaged with a FEI Quanta 200 scanning electron microscope.

Analysis of exopolysaccharide matrix formation and confocal microscopy. *S. flexneri* 2457T biofilms (in medium with or without 0.4% [wt/vol] bile salts) grown on glass coverslips were fixed with 3% paraformaldehyde and 0.25% gluteraldehyde in 1× PBS and subsequently stained with 25 μg/ml fluorescein isothiocyanate-conjugated concanavalin A (FITC-ConA; Sigma) for 15 min at room temperature. Stained and control cells were washed in 1× PBS, mounted using prolong antifade with 4′,6-diamidino-2-phenylindole (DAPI; Molecular Probes), and imaged using a Leica confocal microscope. To quantify the thickness of the biofilms, image stacks were taken every 0.25 μm of the biofilm, and quantification was determined from the full thickness of the stack. Three-dimensional (3D) reconstructed images were generated using ImageJ software (https://imagej.nih.gov). The average thicknesses from three independent experiments ± the standard errors (SE) were plotted to quantify differences in thickness. Statistical significance was determined by Student's *t* test, and a *P* value of ≤0.05 was considered significant. For quantification analysis, the biofilm assay was performed in black 96-well immunoplates and performed as described above. After the wash steps following overnight incubation, wells were treated with fixative (3% paraformaldehyde and 0.25% glutaraldehyde in 1× PBS) for 15 min. The wells were subsequently washed and stained with FITC-ConA for 15 min at room temperature. Each well was subsequently washed in 1× PBS, and the fluorescence at 488 nm was measured with a BioTek fluorescence plate reader. This analysis was performed in three independent experiments, and statistical significance was determined using a two-way ANOVA, with a *P* value of ≤0.05 considered significant.

HT-29 adherence assay. The adherence assay was performed as previously described for T84 cells (16) with the following modifications. HT-29 cells (ATCC HTB-38) were seeded in Dulbecco's modified Eagle medium (DMEM) to establish a semiconfluent monolayer of approximately 75%. For bacterial cultures, single colonies of *S. flexneri* 2457T were inoculated into medium (LB) or medium with 2% glucose and 0.4% (wt/vol) bile salts in tissue culture plates and grown statically at 37°C. Following overnight growth, the bacteria were collected, washed with 1× PBS, standardized to an OD_{600} of 0.35,

resuspended in DMEM, and applied to the HT-29 cell monolayers. Cells were incubated at 37°C with 5% CO_2 for 3 h. Afterwards, the monolayers were washed five times with 1× PBS and lysed with 1% Triton X-100. Serial dilutions were made to determine the number of cell-associated bacteria. The average percent recovery was calculated for three independent experiments according to the following formula: (recovered bacterial titer/infecting titer) × 100%. Statistical significance was determined by Student's *t* test, and a *P* value of ≤0.05 was considered significant.

RNA isolation and RNA-seq analysis. RNA was isolated from *S. flexneri* 2457T grown in TSB ± 0.4% (wt/vol) bile salts under shaking or static conditions. Isolation was performed on two independent, biological replicates for each growth condition (a total of 8 samples). All bacteria were grown to an OD_{600} of 0.7, approximately 2.5 h without bile salts and 3.0 h with bile salts for both shaking and static conditions. Bacterial RNA was isolated using the RNeasy kit (Qiagen) according to the manufacturer's protocol. DNase treatment was performed using Invitrogen's Turbo DNase, and concentrations of total RNA were determined using a NanoDrop ND-1000 spectrophotometer. To confirm RNA integrity, RNA was resolved on a Tris-borate-EDTA (TBE) agarose gel to ensure lack of degradation. Next, cDNA was synthesized from total RNA using the RevertAid cDNA first-strand synthesis kit (Thermo Scientific) according to the manufacturer's protocol followed by PCR amplification with 2× *Taq* reaction mix (Denville) of the housekeeping gene *rpoA* (see Table S1 in the supplemental material). All RNA was confirmed to be free of DNA contamination by performing separate cDNA synthesis reactions without reverse transcriptase, followed by PCR amplification of *rpoA*. RNA samples were sent to Genewiz for RNA-seq, with RNA integrity determined prior to sample processing through bioanalyzer analysis (Agilent 2100) by the company. All RNA integrity numbers (RIN) ranged from 9.7 to 10.0, confirming that the RNA was intact and not degraded (64). rRNA was depleted prior to RNA library preparation and multiplexing. Sequencing was performed on the Illumina HiSeq2500 platform, in a 1 × 50 bp single-read configuration.

The number of sequenced reads ranged from 17,044,246 to 20,194,826 with mean Phred quality (Q) scores ranging from 38.28 to 38.36, representing a 99.99% base call accuracy rate (65, 66). The RNA-seq analysis was performed as previously described (67). Briefly, the publicly available *S. flexneri* 2457T genome and *S. flexneri* 2a strain 301 virulence plasmid annotations were used in the data analysis. The transcript reads were aligned to the predicted coding regions, and the differential expression of each gene under the different conditions was determined using DESeq v. 1.5.24 (68). The reads aligned to each gene were normalized and then averaged for each of the two biological replicates, and the fold change and the $\log_2$ of the fold change (LFC) were calculated for each comparison. The expression data were then filtered for further analysis, and genes that had a fold change of ≥2 and ≤−2, a minimum normalized read count of 10, and a *P* value and false discovery rate (FDR) of ≤0.05 were determined to be transcriptionally altered using DESeq v. 1.5.24 and R-2.15.2.

qRT-PCR analysis. To verify the RNA-seq results, qRT-PCR analysis was performed on biologically independent RNA samples isolated from the static or shaking cultures in the presence or absence of 0.4% (wt/vol) as described above. The cDNA was synthesized from total RNA and confirmed to be free of DNA contamination as described above. The various PCRs were performed with the primer sets listed in Table S1 in the supplemental material. Analysis by qRT-PCR was performed in a two-step reaction in which cDNA was synthesized using the RevertAid first-strand cDNA synthesis kit (Thermo), and quantitative PCR followed using the SYBR green detection method with a CFX Connect Real-Time PCR detection system (Bio-Rad). Data were collected and analyzed using the CFX Manager Software (Bio-Rad). All data were normalized to levels of *rpoA* and analyzed using the comparative cycle threshold (ΔC_T) method (69). The expression levels of the target genes under the various conditions were compared using the relative quantification method (69). Real-time data are expressed as the relative changes in expression levels between cultures in medium with bile salts and those in medium without bile salts. Statistical significance was determined by Student's *t* test, and a *P* value of ≤0.05 was considered significant.

Genetic mutant construction. The Δ*acrB* mutant was constructed using the λ red linear recombination method as previously described (70). PCR was used to amplify a chloramphenicol resistance cassette gene (*cat*) from pKD3 (Table 1) with 5′ and 3′ overhangs identical to the 5′ and 3′ regions of *acrB*. Chloramphenicol-resistant recombinants were identified and selected on chloramphenicol plates and subsequently screened via PCR using confirmation primers (Table S1) that annealed to unique regions up- and downstream of each gene to detect the size difference due to the insertion of the chloramphenicol cassette. For *acrB* complementation analysis, the *acrB* gene was amplified using high-fidelity *Taq* polymerase as previously described (70), digested with HindIII and BamHI, and ligated into digested pUC19. For *galU* complementation analysis, the promoter and *galU* gene were amplified and ligated into pGEMT as previously described (16). Ligation reactions were transformed into *E. coli* DH5α and selected on LB with 100 μg/ml ampicillin. Following sequencing analysis of the inserts, the plasmids were transformed into the appropriate *S. flexneri* 2457T mutants for growth curve analysis of bile salt sensitivity.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/IAI.01067-16.

SUPPLEMENTAL FILE 1, PDF file, 0.4 MB.
SUPPLEMENTAL FILE 2, XLSX file, 0.1 MB.
SUPPLEMENTAL FILE 3, XLSX file, 0.1 MB.

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