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Biofilm Formation and Virulence of *Shigella flexneri* Are Modulated by pH of Gastrointestinal Tract

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**ABSTRACT** *Shigella* infection remains a public health problem in much of the world. Classic models of *Shigella* pathogenesis suggest that microfold epithelial cells in the small intestine are the preferred initial site of invasion. However, recent evidence supports an alternative model in which *Shigella* primarily infects a much wider range of epithelial cells that reside primarily in the colon. Here, we investigated whether the luminal pH difference between the small intestine and the colon could provide evidence in support of either model of *Shigella flexneri* pathogenesis. Because virulence factors culminating in cellular invasion are linked to biofilms in *S. flexneri*, we examined the effect of pH on the ability of *S. flexneri* to form and maintain adherent biofilms induced by deoxycholate. We showed that a basic pH (as expected in the small intestine) inhibited formation of biofilms and dispersed preassembled mature biofilms, while an acidic pH (similar to the colonic environment) did not permit either of these effects. To further elucidate this phenomenon at the molecular level, we probed the transcriptomes of biofilms and *S. flexneri* grown under different pH conditions. We identified specific amino acid (cysteine and arginine) metabolic pathways that were enriched in the bacteria that formed the biofilms but decreased when the pH increased. We then utilized a type III secretion system reporter strain to show that increasing pH reduced deoxycholate-induced virulence of *S. flexneri* in a dose-dependent manner. Taken together, these experiments support a model in which *Shigella* infection is favored in the colon because of the local pH differences in these organs.

**KEYWORDS** *Shigella*, bile acids, biofilm, colon, pH
shortcoming of this model is that in humans M cells are primarily found in ileal Peyer’s patches of the small intestine, whereas the manifestations of Shigella infection are predominantly related to the colon (1, 8). Physiological and pathological studies of Shigella infections in humans and rhesus monkeys, respectively, demonstrate that this genus primarily invades and damages colonic epithelium in areas not necessarily associated with GALT and minimally affects the small intestine (9, 10). In vitro, however, primary gut epithelium infection studies demonstrate that Shigella can invade epithelial cells of small and large intestines (11). Based on these discrepancies between in vivo and in vitro observations, we hypothesize that differences between the luminal environments of small intestine and colon account for the preferential tropism of Shigella for the colon.

Bile is one important luminal factor that enteric pathogens encounter during their colonization and invasion of the gut wall. Bile is a heterogeneous mixture of primary and secondary bile salts, cholesterol, phospholipids, and bilirubin that facilitates the digestion of fat in the small intestine (12). Pathogenic bacteria, including Escherichia coli, Vibrio cholerae, Campylobacter jejuni, and Shigella, have evolved mechanisms to resist the antimicrobial properties of bile, and some even utilize bile as a signal to regulate virulence (13). Relevant here, upon exposure to bile salts, Shigella increases secretion of virulence factors, thus increasing adherence to and invasion of host cells (14, 15). Additionally, long-term bile salt exposure in vitro induces Shigella biofilm formation, a phenomenon that is also observed when V. cholerae encounters bile salts (15, 16). Although a large portion of bile salts are absorbed in the ileum, approximately 400 to 800 mg of bile salts enter the colon daily (17). Thus, despite its importance in increasing pathogenic potency, it is unlikely that bile salts are the sole factor explaining the preferential colonic infection by Shigella.

Concurrent with the inﬂow of bile into the small intestine, however, is the inﬂux of bicarbonate (HCO3−), the physiological base used to buffer acidic contents from the stomach, creating an alkaline luminal environment in the distal small intestine (the mean pH is 7.7, with a range of mostly pH 7.4 to 8.0) (18, 19). In contrast, the colonic lumen is typically more acidic (the average pH is 6.4; the variability is much greater than that in the distal small intestine, with a range of pH 5.0 to 8.0) (18, 19). Interestingly, bicarbonate modulates virulence factors, toxin production, and biofilm formation in V. cholerae (16, 20, 21). However, scant data exist regarding interactions between bicarbonate and Shigella. Given the pH differences between the distal small intestine and the colon, as well as the role of pH in regulating the pathogenic activity of Vibrio cholerae, we hypothesized that pH may play a role in regulating bile salt-dependent virulence of Shigella and its preferential pathogenesis in the colon. We demonstrate that alkalization attenuates Shigella bioﬁlm formation, disperses formed bioﬁlm, does not kill the pathogen, and downregulates transcription of bacterial virulence loci, with a net negative effect on pathogenesis.

In this study, we utilized previously established biofilm formation methods to test whether deoxycylolate-induced bioﬁlms of Shigella ﬂexneri could form under various pH conditions (15). We demonstrated that more basic pH levels, as observed in the small intestine, attenuated bioﬁlm formation without causing bacterial cell death. Additionally, we showed that basic pH conditions dispersed mature bioﬁlms. RNA sequencing of S. ﬂexneri under various pH conditions showed differences in transcriptional proﬁles of bacteria grown in deoxycylolate with or without NaOH. Using a type III secretion system (T3SS) reporter strain of Shigella, we demonstrated that increasing pH could also down-regulate virulence. Collectively, these studies demonstrate that basic conditions, as found in the lumen of the small intestine, are not favorable for S. ﬂexneri pathogenesis, and they provide new mechanistic insights into shigellosis pathogenesis.

RESULTS

Basic conditions attenuate deoxycylolate-induced biofilm formation. Sodium deoxycylolate (NaDC)A, a secondary bile salt found in the small and large intestines, enhances secretion of S. ﬂexneri virulence factors in planktonic culture (14). We initially tested whether this bile acid affected the ability of S. ﬂexneri to form bioﬁlms in vitro.
(Fig. 1A). To optimize conditions to produce biofilms, we grew *S. flexneri* in tryptic soy broth (TSB) supplemented with various concentrations of NaDCA, and then we measured the opacity of biofilms by the optical density at 600 nm (OD\textsubscript{600}). Incubation of *S. flexneri* with 0.05% NaDCA stimulated formation of biofilms that approached 50% of maximum opacity and was thus suitable for testing the effects of external factors on biofilm formation (Fig. 1B).

We hypothesized that alterations of extracellular pH would impact biofilm formation, because the pH of the intestinal lumen varies along its length (18). To simulate the basic conditions found in the small intestine, we adjusted the pH of the TSB containing 0.05% NaDCA with increasing concentrations of NaOH. We characterized the resulting biofilms by OD\textsubscript{600} to measure opacity, by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay to assess microbial viability, and by CFU to quantify the number of planktonic bacteria in the medium that were not incorporated into the biofilm (Fig. 1C and Fig. 2A and B). Increasing NaOH in the TSB decreased biofilm opacity (Fig. 2C) in a dose-dependent manner, with almost complete loss of biofilm at 20 mM (Fig. 2E). Importantly, the pH values that correspond to 5 and 10 mM are in the range of what is found in the human distal small intestine (19). MTT and crystal violet assays corroborated the reduction in biofilm formation with NaOH treatment (Fig. 2D; also see Fig. S1A and B in the supplemental material). Importantly, the attenuation of biofilm formation by increased pH did not appear to be secondary to enhanced bacterial killing by NaOH. In fact, the planktonic phase of *S. flexneri* that was maintained apically of the biofilm was enhanced by increased pH, as measured by OD\textsubscript{600} and CFU per milliliter (Fig. 2F; also see Fig. S1C). Our interpretation of these data is that there was an increased influx of bacteria into the planktonic phase as the biofilm was disassembled under basic conditions.

Taken together, these data support the hypothesis that a basic pH attenuates NaDCA-induced biofilm formation in *S. flexneri* and this effect occurs in a range of pH values that are similar to those in the human small intestinal lumen (Fig. 2B). To further test whether a basic environment, as found in the small intestine, plays a role in decreasing deoxycholate-induced biofilm formation, we repeated our assay with NaHCO\textsubscript{3}, the physiological agent responsible for modulating pH in the small intestine, and we confirmed that a physiologically relevant bicarbonate concentration (0.3%) (22) could prevent biofilm formation (Fig. 2G and H). To control for the possible effects induced by changes in ionic strength, we tested NaCl at the same concentrations and confirmed that NaCl did not affect biofilm formation (Fig. 2I).

**Acidic conditions do not affect biofilm formation but decrease the viability of *S. flexneri*.** To test whether the acidic pH that *S. flexneri* encounters in the large intestine influences biofilm formation, we supplemented TSB with increasing concentrations of HCl (see Fig. S1D). In contrast to the increased biofilm density observed under basic conditions, we found that acidic pH did not significantly change biofilm opacity (see Fig. S1E). Despite the presence of biofilms under all acidic conditions, the MTT and crystal violet assays showed diminished activity, indicating that either the metabolism or the number of bacteria surviving in the biofilms was decreased as the concentration of HCl was increased (see Fig. S1F and G). The number of planktonic bacteria growing in the medium above the biofilm was also decreased with HCl addition (see Fig. S1G and I). Our inability to reduce biofilm assembly and formation under acidic conditions despite decreased viability and/or metabolism for *S. flexneri* prompts consideration of a model in which *S. flexneri* biofilms are better supported in the colon, where the acidic milieu does not prevent bile salt-induced biofilm formation.

**Environmental pH modulates dispersal of mature *S. flexneri* biofilms.** We next asked whether alkalinity affects biofilms that are already assembled and mature. First, we induced biofilm formation with NaDCA, allowed the biofilm to mature for 24 h, and then exchanged the growth medium to TSB without NaDCA over a range of NaOH concentrations (Fig. 3A). We found that the addition of 2.5, 5, or 10 mM NaOH did not significantly affect biofilm opacity, although there was a trend toward reduction. Treatment with 20 mM NaOH fully dispersed the mature biofilm, as indicated by decreased opacity.
The effect of basic pH on biofilm dispersal was less than its effect on biofilm formation. These data were further supported by the MTT assay (Fig. 3C). These data suggest that the disruptive effects of NaOH on *S. flexneri* biofilms were caused not by deprotonation of deoxycholate but rather by direct effects on the pathogen. We found that acidic environments did not significantly disperse mature biofilms or reduce bacterial viability (see Fig. S2A and B).

**RNA sequencing demonstrates that basic conditions attenuate a biofilm-associated transcriptomic program.** To gain additional insight into how pH affects *S. flexneri* biofilm formation, we performed RNA sequencing to determine the transcriptomic alterations in *S. flexneri* caused by pH modulation. We performed this analysis using (i) planktonic cultures in TSB only, (ii) biofilms in TSB plus 0.05% NaDCA, and (iii) planktonic cultures in TSB plus 0.05% NaDCA plus 20 mM NaOH. A global view of the transcriptional profiles of these experimental groups, as shown by principal-component analysis (PCA) plot, indicated that there were indeed transcriptional differences among the experimental groups (see Fig. S3A).
To identify gene signatures that were associated with biofilm formation, we first compared the mRNA transcriptomic profiles between the deoxycholate-induced biofilm and planktonic *S. flexneri* grown in TSB only. Pathway analysis (Database for Annotation, Visualizations and Integrated Discovery [DAVID] v6.7) of genes upregulated in the biofilm showed an enrichment of ABC transporter genes, as well as genes involved in arginine and sulfur metabolism (Fig. 4A). Interestingly, arginine and cysteine metabolic pathways have been reported to be important in biofilm formation in other bacterial species (23, 24). We next compared the biofilm cultures grown with and without NaOH with those grown with and without NaDCA, as outlined in Fig. 2A. The addition of NaOH to the growth medium increased the pH and reduced the opacity of the biofilm, as measured by absorbance at 600 nm (Fig. 2B). The growth of *Shigella* above the biofilm was inhibited by the addition of NaOH, as measured by absorbance at 600 nm (Fig. 2C). The viability of the biofilm, as measured by the MTT assay, was also reduced by the addition of NaOH (Fig. 2D). The addition of NaHCO₃ to the growth medium did not significantly affect the growth or viability of the biofilm (Fig. 2E). We next compared the biofilm cultures grown with and without NaCl, as a control for ionic strength (Fig. 2F). The addition of NaCl did not significantly affect the growth or viability of the biofilm (Fig. 2G). We next compared the biofilm cultures grown with and without NaOH, as a control for pH (Fig. 2H). The addition of NaOH did not significantly affect the growth or viability of the biofilm (Fig. 2I).

**FIG 2** Effects of increased pH on formation of deoxycholate-induced biofilms. (A) Diagram describing the experimental steps for testing the effects of NaOH on biofilm formation induced by NaDCA. (B) Table showing the corresponding pH values when increasing concentrations of NaOH were added to TSB containing 0.05% NaDCA. (C and D) Opacity (measured as OD₆₀₀) (C) and viability (measured with the MTT assay) (D) of biofilms induced by 0.05% NaDCA with increasing concentrations of NaOH, after 24 h at 37°C (n = 3 independent experiments). (E) Representative images of biofilms induced by NaDCA with or without 20 mM NaOH. (F) Growth curves of the planktonic *Shigella* (measured as OD₆₀₀) growing above NaDCA-induced biofilms with increasing NaOH concentrations (n = 3 independent experiments). (G) Biofilm viability with or without 20 mM NaCl (as a control for ionic strength) (n = 2 independent experiments). All bar graphs were plotted as mean and standard deviation. Statistical significance was determined by Student’s t tests with P values of <0.05. **, significant with two-tailed Student’s t test. NS, not significant.
Pathway analysis demonstrated that NaOH repressed deoxycholate-induced biofilm enrichment of ABC transporter, arginine metabolism, and sulfur metabolism genes (Fig. 4B). Heatmap analysis of representative genes from these pathways further supported specific increases in gene transcripts only in the presence of NaDCA (Fig. 4C). Quantitative PCR (qPCR) analysis of candidate genes involved in arginine and cysteine metabolism confirmed the RNA sequencing results (see Fig. S3B).

Taken together, these data demonstrated that basic conditions suppress a biofilm-associated S. flexneri gene program induced by deoxycholate.

**Basic conditions prevent induction of T3SS by deoxycholate.** In addition to stimulating biofilm formation, deoxycholate increases the expression of S. flexneri virulence factors, especially those related to the T3SS, the machinery S. flexneri utilizes to invade host cells (1). Given the attenuation of deoxycholate-induced biofilm formation under basic conditions, we hypothesized that adding NaOH could also reduce the virulence of S. flexneri. To test this, we utilized a Shigella strain that contains a fluorescent reporter for the T3SS (Shigella M90T Sm pTSAR 2.4) (25). In this system, mCherry is constitutively expressed in all bacteria but green fluorescent protein (GFP) is expressed only when the T3SS apparatus is induced. We first demonstrated that addition of 0.2% NaDCA in TSB induced GFP production in this Shigella reporter strain, whereas bacteria growing in TSB alone did not fluoresce green. When the pH of the TSB was adjusted upward with added NaOH, the T3SS activation by deoxycholate was reduced in a dose-dependent manner, with 5 and 10 mM concentrations of NaOH showing significant effects (Fig. 5A and B). This experiment further supports the model in which basic conditions deter Shigella from infection in the small intestine.

**DISCUSSION**

In this study, we utilized a series of in vitro bacterial assays, RNA sequencing, and fluorescence microscopy to demonstrate that alkalization decreases deoxycholate-regulated biofilm formation, which in turn decreases S. flexneri induction of virulence genes. These data call into question the assumption that the initial target of infecting Shigella is the small bowel. We propose, instead, that as S. flexneri transits through the distal small intestine, the basic pH of the luminal environment reduces biofilm formation, virulence, epithelial invasion, and host injury. As S. flexneri reaches the more acidic environment of the proximal colon, it may then revert to a less virulent state, allowing the host to clear the infection.

**FIG 3** Dispersal of biofilms modulated by increased pH. (A) Illustration of the experimental procedures for testing the effects of NaOH on biofilm dispersion and disassembly. (B and C) Opacity (B) and viability (C) of preformed biofilms incubated with increasing concentrations of NaOH for 24 h (n = 3 independent experiments). All bar graphs were plotted as mean and standard deviation. Statistical significance was determined by Student’s t tests with P values of <0.05. **,** significant with two-tailed Student’s t test.
colonic lumen, bacterial biofilm formation is favored, and virulence-related gene expression facilitates epithelial invasion in this distal organ.

*V. cholerae*, a pathogen that preferentially colonizes the small intestine, offers an instructive contrast to *Shigella*. In both bacteria, bile salts induce biofilm formation (16). However, bicarbonate upregulates virulence factors and toxin production in *V. cholerae*, whereas the same condition reduces virulence in *Shigella* (13, 15). The clinical picture reflects the anatomic differences in pathology; cholera is characterized by watery diarrhea, and *Shigella* infections are often characterized by more distal symptoms and signs, such as tenesmus, rectal prolapse, hematochezia, and abundant fecal leukocytes. Thus, our data highlight the importance of biogeographic pathogenesis within the intestinal lumen.

Recent advances in the detection of intestinal pathogens using qPCR revealed that *Shigella* may be more prevalent than previously estimated (26). Antibiotics remain the only treatment for this infection (27), but resistance increasingly challenges case management. Thus, we urgently need to find alternative approaches to treat bacterial diseases like shigellosis (28). We propose a pH-dependent mechanism by which *Shigella* virulence is mediated. These data can form the basis for novel interventions to reduce virulence by targeting biofilm formation under basic pH conditions, to better manage shigellosis.

*Bacterial biofilms play important roles in many biological systems. For example, Vibrio fischeri produces biofilms in the light organ of the Hawaiian squid, Pseudomonas aeruginosa produces biofilms in the lung airways, diminishing respiratory function, and Streptococcus mutans produces polymicrobial biofilms on the enamel surface of teeth,*
**FIG 5** Increased pH inhibits deoxycholate-induced virulence of *Shigella*. (A) Images showing the effects of NaDCA and NaOH on a fluorescent reporter of *S. flexneri* that expresses GFP upon activation of the T3SS (with constitutive expression of mCherry as an internal control). Scale bar = 100 μm. (B) Percentage of GFP-positive cells among mCherry-positive cells under increased NaOH concentrations, plotted as mean and standard deviation (*n* = 2 independent experiments). Statistical significance was determined by Student’s *t* tests. ***, P < 0.01 with two-tailed Student’s *t* test.
causing dental caries (23, 24, 29). Our data demonstrate that in Shigella biofilms, as in P. aeruginosa biofilms and V. fischeri biofilms, arginine and cysteine pathways accompany formation (30, 31). Our work may have implications beyond acute Shigella infections, as gut biofilms have been associated with colorectal cancer and inflammatory bowel disease, and may further the investigation of biofilms related to disorders of the gastrointestinal tract (32, 33).

Although more work is needed to further elucidate the pathways and host factors that control Shigella virulence and biofilm formation, our work expands on our knowledge of Shigella pathogenesis and provides a unique perspective on studying mechanisms of infection. This will prove to be imperative as we are slowly attempting to uncover more and more interactions between the host and pathogen.

MATERIALS AND METHODS

Biofilm formation assays. Shigella flexneri strain 2a 2457T was purchased from ATCC (number 700930) and used within 6 months after receipt. S. flexneri was inoculated into LB medium from the original commercial stock and grown overnight at 37°C with shaking at 250 rpm. After this incubation, the bacteria were pelleted by centrifugation, resuspended in TSB, and measured by OD₆₀₀. The bacterial suspension was then diluted with TSB and adjusted to an OD₆₀₀ of 0.5. From this stock, the bacteria were further diluted 1:800 in TSB that included 0.05% NaDCA. The bacterial suspension was then added to a 96-well plate (180 µl/well). For experiments, 20 µl of H₂O, diluted HCl, or diluted NaOH were added to each well to achieve the desired final concentrations. The plate was incubated under stationary conditions at 37°C for 24 h to allow biofilm formation. The supernatant above the resultant biofilm was removed and set aside for OD₆₀₀ or CFU measurement; the biofilms were washed twice with 100 µl H₂O and measured by OD₆₀₀ before 100 µl of the MTT solution (0.5 mg/ml MTT in TSB) was added to the biofilms. The plate was incubated at 37°C for 10 min before the MTT solution was removed and dissolved with 100 µl dimethyl sulfoxide (DMSO). The OD₅₇₀ of the resulting solution was measured with a Cytation 5 reader. The planktonic cells in the supernatant were sequentially diluted in phosphate-buffered saline (PBS) and plated on LB plates for CFU quantification. A similar procedure was utilized for the testing of NaHCO₃ and NaCl and determination of the dose curve of NaDCA.

S. flexneri growth curve. S. flexneri was grown overnight in LB medium. Bacteria were pelleted by centrifugation, reconstituted to an OD₆₀₀ of 0.5, and then diluted 1:20 in TSB. The bacterial suspension was added to 96-well plates (180 µl/well), to which we then added H₂O, HCl, or NaOH to a final volume of 200 µl. Plates were incubated at 37°C with shaking, and the OD₆₀₀ of each well was measured at 2, 4, and 6 h. Doubling times were calculated from OD₆₀₀ values at 2 and 4 h (see Table S2 in the supplemental material).

Biofilm dispersion assays. An overnight culture of S. flexneri in LB medium was adjusted to an OD₆₀₀ of 0.5 and further diluted 1:800 in TSB with 0.05% NaDCA. The diluted bacterial suspension was added to 96-well plates (200 µl/well) and incubated without shaking at 37°C for 24 h. After 24 h, the supernatant above the biofilm was removed, the biofilms were washed once with H₂O (100 µl), and a new solution of TSB with various concentrations of NaOH or HCl (200 µl/well) was layered on top of the biofilms. After incubation at 37°C without shaking for 24 h, the resulting biofilms were measured by OD₆₀₀ and MTT assay as described above.

RNA sequencing of S. flexneri. The diluted solution of bacteria from an overnight culture (first adjusted to an OD₆₀₀ of 0.5 and then diluted 1:20 in TSB) was further diluted to 1:800 in TSB with or without 0.05% NaDCA. For TSB-only control samples, an aliquot of 200 µl of the aforementioned diluted (without NaDCA) was added to 96-well plates. To assess the effects of NaDCA and/or NaOH, an aliquot of 180 µl of the aforementioned dilutions (with NaDCA) was added with 20 µl of either H₂O or 200 mM NaOH. The plates were incubated under stationary conditions at 37°C for 24 h. The planktonic bacteria in the wells containing TSB only (no NaDCA) or TSB with 0.05% NaDCA and 20 mM NaOH were collected, pelleted by centrifugation at 1300 g (15 min at 4°C), and lysed according to instructions for the Ribopure RNA purification kit for bacteria (Invitrogen). Biofilms from wells containing TSB and 0.05% NaDCA were washed and directly lysed with the same kit. RNA was then purified using the RNeasy mini kit (Qiagen). Genomic DNA removal was performed according to the instructions for the Turbo DNA-free kit (Invitrogen).

Libraries were prepared according to the manufacturer’s protocol and sequenced with the Illumina HiSeq 3000 genome. The Shigella flexneri 2a str. 2457T genome from Ensembl bacteria release 45 was used to align RNA sequencing reads with STAR v2.0.4b. Subread featureCounts v1.4.5 was used to derive gene counts. Gene counts were imported into and analyzed with the R/Bioconductor package EdgeR for normalization and then were imported into the R/Bioconductor package Limma. Differential expression was determined with the Benjamini-Hochberg procedure with false-discovery-rate-adjusted P values of <0.05. The PCA plot was made with the DDSseq2 package, based on all gene counts. Pathway analysis was performed by utilizing DAVID v6.7 (https://david.ncifcrf.gov).

For qPCR verification, 500 ng of RNA was converted to cDNA using the iScript reverse transcription supermix kit (Bio-Rad), and qPCR was performed with the TB Green Advantage qPCR premix. The recA gene was used as a housekeeping gene. The primers used are shown in Table S1 in the supplemental material.
Fluorescence microscopy to probe activation of T3SS. A Congo red-positive colony of Shigella M90T Sm transformed with the pTSAR 2.4 plasmid (a gift from François-Xavier Campbell-Valois) was grown in TSB overnight. The strain was transformed as reported previously (25). The overnight culture was diluted 1:100 into TSB solutions with or without 0.2% NaDCA under various pH conditions (adjusted with H2O, HCl or NaOH) and incubated for 3 h without shaking at 37°C. The activation of the T3SS was assessed with the GFP channel by fluorescence microscopy (25).

Data availability. RNA sequencing data from this study was deposited in the NCBI GEO repository under accession number GSE183515.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 1.1 MB.

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