The synthesis of OspD3 (ShET2) in Shigella flexneri is independent of OspC1

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ABSTRACT
Shigella flexneri is a Gram-negative pathogen that invades the colonic epithelium and causes millions of cases of watery diarrhea or bacillary dysentery predominately in children under the age of 5 years in developing countries. The effector Shigella enterotoxin 2 (ShET2), or OspD3, is encoded by the sen or ospD3 gene on the virulence plasmid. Previous literature has suggested that ospD3 is in an operon downstream of the ospC1 gene, and expression of both genes is controlled by a promoter upstream of ospC1. Since the intergenic region is 328 bases in length and contains several putative promoter regions, we hypothesized the genes are independently expressed. Here we provide data that ospD3 and ospC1 are not co-transcribed and that OspC1 is not required for OspD3/ShET2 function. Most importantly, we identified strong promoter activity in the intergenic region and demonstrate that OspD3/ShET2 can be expressed and secreted independently of OspC1. This work increases our understanding of the synthesis of a unique virulence factor and provides further insights into Shigella pathogenesis.

Introduction
Shigella flexneri is a Gram-negative, facultative intracellular pathogen that causes millions of cases of watery diarrhea or bacillary dysentery annually, predominately in developing countries under the age of 5 years. Symptoms of disease include fever, abdominal cramps, and bloody or mucoid stools. Infection results from the ability of the bacteria to invade the colonic epithelium, a phenotype that requires a number of gene products encoded on the 220 kilobase virulence plasmid. A type-III secretion system (T3SS), the Ipa proteins required for invasion, and other T3SS effector proteins that are important for the pathogen to survive and replicate inside the host are encoded on the virulence plasmid. The mechanism by which S. flexneri induces secretory diarrhea during infection remains to be fully elucidated. Several enterotoxins have been identified in S. flexneri, but it is unclear when each of the enterotoxins are expressed and how each toxin functions in the resulting diarrhea. The current paradigm is that Shigella induces a secretory diarrhea in the jejunum in order to facilitate bacterial transit to the site of invasion in the colon. One of the enterotoxins, ShET2, is encoded by a gene called sen or ospD3 on the virulence plasmid of all Shigella strains and serotypes.

Another important aspect of Shigella infection is polymorphonuclear cell (PMN) infiltration, which occurs after the bacteria begin to invade the colonic epithelium. It is well established that S. flexneri infection induces a massive PMN infiltration during infection to enhance bacterial invasion at the basolateral surface of epithelial cells, and this infiltration is responsible for the destruction of the colonic epithelium. PMN infiltration results from interleukin-8 (IL-8) secretion from infected epithelial cells, which is induced through nuclear factor κB (NF-κB) activation during intracellular replication of Shigella. The S. flexneri ospC1 and ospD3 gene products have been shown to target PMN infiltration and IL-8 secretion, respectively. First, a ΔospC1 mutant has a significant
Results

Transcription initiation sites are detected in the \( \text{ospC1-ospD3 intergenic region} \)

Results from high-throughput sequencing of RNA transcripts (RNA-seq) isolated from \( S. \text{flexneri} \) 2a strain 2457T grown in broth cultures identified two potential transcriptional initiation sites for the \( \text{ospD3} \) gene within the 328 base pair intergenic region (Fig. 1). Based on the increases in transcript reads in conjunction with the proximity of promoters and ribosome binding sites (RBS), the first site is located 43 nucleotides downstream of the \( \text{ospC1} \) stop codon while the second site is located 81 nucleotides upstream of the annotated \( \text{ospD3} \) translational start codon. The BPROM promoter prediction program identified a total of 4 putative promoter regions within the intergenic region (sequences in bold), and two consensus AAGGAA RBS were also identified (Fig. 1). There was no difference in transcription initiation sites from RNA isolated from intracellular bacteria (data not shown). Based on these observations, we pursued the possibility that \( \text{ospD3} \) was transcribed independently from \( \text{ospC1} \).

\( \text{ospC1 and ospD3 are not co-transcribed} \)

In order to confirm the RNA-seq data and demonstrate that the \( \text{ospC1} \) and \( \text{ospD3} \) genes are not co-transcribed, we performed non-quantitative RT-PCR analysis of the \( \text{ospC1} \) to \( \text{ospD3} \) coding region. We hypothesized that if the genomic region was truly an operon, we should be able to amplify the entire 3.4 kb region encompassing \( \text{ospC1} \) and \( \text{ospD3} \) with a single set of flanking primers. By utilizing a series of primer sets that anneal along the coding region, or in the \( 5' \) or \( 3' \) end of \( \text{ospC1} \) and \( \text{ospD3} \), we could not amplify a 3.4 kB polycistronic product representing both genes, even when we heat denatured the RNA template to remove possible secondary structures according to the manufacturer’s instructions for the cDNA synthesis kit. RNA isolated from the
Figure 1. RNA-sequencing identifies transcription initiation sites in the ospC1 and ospD3 intergenic region. The analysis of the ospC1 to ospD3 coding region on the virulence plasmid is presented from RNA isolated from broth cultures of Z457T. RNA-sequencing analysis identified two transcriptional start sites (white box), while the BPROM promoter prediction software predicted 4 putative promoter regions highlighted in bold, each with $-35$ and $-10$ sequences. There are two AAGGAA consensus ribosome binding sites (gray boxes) and two putative translational start sites for ospD3 (circled "m" for methionine).
**Figure 2.** Non-quantitative RT-PCR analysis demonstrates that the *ospC1* and *ospD3* genes are not co-transcribed. A) Using RNA isolated from broth cultures and several different primer sets, we were able to amplify the *ospC1* and *ospD3* genes along with a 1.62 kb fragment from the intergenic region to the 3’ end of *ospD3*. However, we were never able to amplify the entire 3.4 kb region, even after heating the RNA template prior to cDNA synthesis to remove any possible secondary RNA structures that may be present as recommended by the manufacturer (data not shown). RNA was isolated from both wildtype 2457T and the Δ*ospC1* Δ*ospD3* double mutant (negative control). All primer sets were validated using DNA as a template, which generated the expected sized products (Fig. S1). For both strains, the house-keeping gene *rpoA* was amplified (see Fig. 3). The *ipa* analysis served as a positive control to amplify 3.1 kb from a known operon, with the forward primer annealing within *ipaB* and the reverse primer annealing within *ipaD*. As expected, amplification occurred for both 2457T and the double mutant. B) Summary of the RT-PCR experiments in which 15 primer sets were used in total. The positive results in black represent the products that were obtained with 8 primer sets. Lines marked with (a) are the amplified products present in Figure 2A while lines marked with (‘) represent amplicons that included regions up or downstream of the individual genes. The gray dotted lines represent faint products that were detected but were not consistently detected upon repeated experiments with independent samples of RNA. Faint detection was not due to improper extension time during the PCR reactions. The gray solid lines represent experiments in which we attempted to amplify the entire 3.4 kb coding region using 3 separate primer sets. Not all amplifications are present in Figure 2A. We consistently were unable to detect any products despite successful amplification of a 3.1 kb fragment from the *ipa* operon coding region. Based on our results, we predict that the genes are not co-transcribed and that two transcripts result (represented by #1 and #2 at the bottom of the figure).

The double Δ*ospC1* Δ*ospD3* mutant served as the negative control in all experiments. All primers were validated using genomic DNA as a template (Fig. S1), and additional controls lacking the reverse transcriptase were performed (see Fig. 3). A positive control for amplification of a 3.1 kb product via RT-PCR was from the *ipa* operon encoded on the virulence plasmid (Fig. 2A). In addition, we utilized RT-PCR to determine if *ospD3* can be expressed in a Δ*ospC1* mutant. As shown in Figure 3, both a large and a small transcript specific to *ospD3* can be amplified from the Δ*ospC1* mutant. For these experiments, the same *ospD3* reverse
primer was used in conjunction with two different forward primers. Finally, *ospD3* transcription was detected in bacterial strains in which the *ospC1* promoter was deleted (Fig. 4). The data suggested that *ospD3* transcription is initiated from a separate promoter; and based on the summary of results, we predict that *ospC1* and *ospD3* have distinct transcripts (Fig. 2B).

Three distinct promoter regions drive expression of the *ospD3* coding region

We utilized β-galactosidase (β-gal) assays \(^{21,22}\) to verify putative functional promoter regions for the *ospD3* transcripts. The β-gal assays were performed on four episomal transcriptional fusions of intergenic region fragments that included putative promoter and RBS sequences (see Materials and Methods). As shown in Figure 5, promoter activity was detected in the intergenic region for three out of the four constructs tested, each with decreasing lengths of the intergenic region. The empty vector (EV) control verified there was no transcription initiation from the vector sequence. Sequence 3 represented the minimum length that retained promoter activity, since activity was completely lost with sequence 4. These results confirmed that *ospD3* can be expressed from an independent promoter relative to *ospC1*.

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**Figure 3.** Detection of *ospD3* transcripts in the Δ*ospC1* mutant via non-quantitative RT-PCR. A) The products amplified for the *ospC1* and *ospD3* analysis are depicted in the diagram. For *ospD3*, two primer sets were used to amplify the full-length gene and a smaller fragment. For both reactions, the same reverse primer was used with different forward primers. B) Results from RNA isolated from broth cultures demonstrated that both products of *ospD3* transcript were detected in the Δ*ospC1* mutant, indicating that *ospD3* expression does not require the *ospC1* promoter. We also verified that the *ospC1* transcript was detected in the Δ*ospD3* mutant. Wildtype 2457T served as a positive control, and the virulence plasmid-cured strain BS103 and the double Δ*ospC1 + ΔospD3* mutant served as negative controls. C) Control experiments were performed to ensure that the house-keeping gene *rpoA* was detected in all samples, and that no amplification of product occurred when reverse transcriptase (RT) was removed from the reaction to ensure there was no DNA contamination in the RNA samples.
Protein expression and secretion analyses verify independent expression of OspD3/ShET2

In order to confirm the previous findings and analyze the protein that is expressed, we constructed C-terminal hemagglutinin (HA) tagged fusions on a low-copy plasmid to OspC1, to OspD3 following the entire OspC1 to OspD3 coding region, and to OspD3 following the upstream intergenic region (Fig. 6A). For the OspC1-HA construct, expression was driven by the native ospC1 promoter and included the upstream MxiE box (5'-GTATCGTTTTTTTATAG-3') sequence. For the OspC1-OspD3-HA construct, the entire coding region was included from the MxiE box to the end of the ospD3 gene. For the OspD3-HA single construct, 215 bases of the intergenic region, upstream of the annotated start site, were added to include the putative promoter region. The plasmids were transformed into S. flexneri strain 2457T, and expression and Congo Red (CR) secretion analyses were performed as previously described. For the OspC1-OspD3-HA construct, the entire coding region was included from the MxiE box to the end of the ospD3 gene. For the OspD3-HA single construct, 215 bases of the intergenic region, upstream of the annotated start site, were added to include the putative promoter region. The plasmids were transformed into S. flexneri strain 2457T, and expression and Congo Red (CR) secretion analyses were performed as previously described. For the OspC1-OspD3-HA construct, the entire coding region was included from the MxiE box to the end of the ospD3 gene. For the OspD3-HA single construct, 215 bases of the intergenic region, upstream of the annotated start site, were added to include the putative promoter region. The plasmids were transformed into S. flexneri strain 2457T, and expression and Congo Red (CR) secretion analyses were performed as previously described.

Additionally, OspD3 was detected via the T3SS as shown in supernatants from the CR secretion assay (Fig. 6B). Equal loading for all analyses was confirmed with Coomassie blue staining of total protein (data not shown).

Given the functional role of OspD3/ShET2 as an enterotoxin, we utilized the OspD3-HA single construct to prepare a protein sample similar to the Ussing chamber samples utilized in the enterotoxin assay. The amount of concentrated supernatants normally used for each Ussing analysis (400 μl) was TCA precipitated and analyzed by Western blot using the primary HA antibody. As shown in Figure 6C, OspD3 is present in the sample, confirming the CR secretion assay and demonstrating that the protein can be secreted at a robust level from the single gene construct.

Finally, to identify the translational start site of OspD3/ShET2, the HA-tagged single construct protein was immunoprecipitated using the HA tag, and the purified protein was analyzed for N-terminal sequencing. The purified protein was confirmed to have a translational initiation site that corresponded to the annotated start site at position 79,610 (GenBank NC_004851) or the first circled methionine in Figure 1.
Figure 5. The β-galactosidase assay identifies promoter regions for ospD3. A) Four sequences spanning the intergenic region were tested for β-galactosidase activity, each with a decreasing size in which the base-pair length of the sequence is provided in the parentheses after the sequence name. All activity was significant above the empty vector negative control, except for intergenic region 4. Plotted is the average Miller Units calculated +/− the SEM. Three independent assays were performed. Statistical analyses were performed with a Student’s t-test between the indicated comparisons. The p-value for intergenic 1 sequence versus intergenic 2 or intergenic 3 sequence is <0.001 while the p-value for intergenic 2 vs. intergenic 3 is <0.03. B) Schematic representation of the intergenic region for analysis in the β-galactosidase assay. The intergenic region is separated by 328 bases in S. flexneri, which has several features for the initiation of transcription and translation. First, there were two transcriptional start sites predicted by RNA-seq, which are represented by the black diamonds. Second, the bacterial promoter prediction program BPROM (see text) predicted 4 putative promoters (each with a -35 and -10 sequences) in this region. The first is immediately after the end of ospC1, followed by the second in close proximity. The start of the single OspD3 protein expression construct (see Fig. 5) is immediately downstream of the second putative promoter region. Therefore, the construct includes putative promoters three and four. The third putative promoter is near the annotated translational start site (+1) of ospD3, and the fourth is just downstream of the third (promoter). Third, two conserved Shine-Dalgarno (SD) sequences of AAGGAA for ribosome binding are detected immediately upstream and downstream of the +1 site. An alternative start site (+48) is in-frame with the second SD sequence. For the cloning of the intergenic region, 4 sets of sequence were amplified by PCR to be analyzed in the β-galactosidase assay, represented by the 4 lines at the bottom of the figure.
Virulence assays demonstrate that OspC1 is not required for OspD3/ShET2 function

The OspD3/ShET2 protein has been associated with two distinct pathogenic roles: enterotoxin activity5,6 and regulating IL-8 secretion in infected epithelial cells.13 We utilized two virulence assays to demonstrate that a ΔospC1 mutation does not affect OspD3/ShET2 activity. The first assay measures the enterotoxin activity associated with OspD3/ShET2 in Ussing chamber experiments. The ΔospD3 mutant as previously demonstrated5 had an approximate 40% (+/- 14%) reduction in enterotoxin activity relative to wildtype 2457T (p < 0.01) as measured by the change in short circuit current (ΔIsc) across mouse jejunal tissue upon addition of concentrated culture supernatants (Fig. 7). However, the ΔospC1 mutation had no significant effect since the mutant induced the ΔIsc similarly as 2457T. The double mutant had a 35% (+/-17%) reduction in toxin activity, indicating that the ΔospC1 mutation did not contribute to the decrease in activity. The data demonstrated that neither the ospC1 transcript nor the OspC1 protein is required for enterotoxin activity.

The second assay analyzes the amount of interleukin-8 (IL-8) secreted from epithelial cells in response...
to Shigella infection. In human colonic HCT-8 cells (Fig. 8), HT-29 cells (Fig. S2A), and T84 cells (Fig. S2B), the \( \Delta ospD3 \) mutant consistently induced significantly greater IL-8 secretion \((p < 0.01)\) compared to wildtype bacteria. The double \( \Delta ospC1 + \Delta ospD3 \) mutant also induced an increased secretion level similar to the \( \Delta ospD3 \) mutant. However, the \( \Delta ospC1 \) mutation had no effect on IL-8 secretion compared to wildtype. Control experiments verified that the increased amount of IL-8 secretion from cells infected with the \( \Delta ospD3 \) mutant was not due to an increase in invasion or intracellular replication relative to wildtype bacteria (data not shown). Finally, analysis of the strain expressing the OspD3-HA single gene construct resulted in less IL-8 secretion relative to wildtype bacteria (Fig. 8), which indicates reduced IL-8 secretion due to the presence of more OspD3 protein. The data not only confirm a role for OspD3 in IL-8 secretion from infected cells, but demonstrate that the \( \Delta ospC1 \) mutation has no effect on OspD3 activity.

**Discussion**

Several bacterial virulence proteins have dual functions, particularly in *S. flexneri*. OspD3/ShET2 is a unique *Shigella* virulence factor that has been shown to function as both an enterotoxin\(^5,6\) and have an effect on IL-8 secretion during intracellular replication of the bacteria.\(^13\) Given the importance of these functions in infection, we sought to further characterize the gene and protein at both the transcriptional and translational levels to appreciate the expression of this important virulence factor. Previous literature suggested that *ospD3* transcription requires the promoter upstream of *ospC1* and that the two genes are co-transcribed;\(^13,16,17\) however, in-depth analysis to verify these findings was lacking. Understanding how bacterial pathogens regulate the expression of virulence factors is important to our appreciation of pathogenesis and our ability to combat infectious diseases.

The combination of the RNA-seq, non-quantitative RT-PCR, the \( \beta \)-gal assays, the protein expression and secretion, and the mutation analyses all demonstrate that *ospD3* can be expressed independently of *ospC1*. In the RT-PCR analysis (Fig. 2), we were unable to consistently amplify products above 2.0 kB despite some amplification of the products beyond the coding regions. Most importantly, we were unable to amplify products ranging from 3.1 to 3.4 kB that would encompass both genes, unlike our positive control in the *ipa* operon. This analysis strongly indicates that
ospC1 and ospD3 are not co-transcribed. Additional evidence to support the independent expression of ospD3 came in the detection of ospD3 transcript in the DospC1 mutant (Fig. 3). The DospC1 mutation was constructed by inserting a chloramphenicol resistance cassette in the place of ospC1. The 30 end of ospC1 and entire intergenic region remain present in this mutation;11 and therefore, the appropriate spacing between the end of the chloramphenicol cassette and the start of ospD3 remains intact. While the ospC1 promoter is still present in this construct, the ¡10 promoter sequence has been mapped to 237 bases upstream of the ospC1 translational start codon.33 The ospC1 promoter region is therefore 1.98 kB upstream of the ospD3 translational start codon. Using this sequence information, we deleted the ospC1 promoter in both the wild-type and ΔospC1 mutant to verify that ospD3 transcription was still present (Fig. 4). This data, in conjunction with the ospD3 promoters identified in the intergenic region (Fig. 5), clearly demonstrate that ospD3 transcription is initiated from an independent promoter in either wild-type or the ΔospC1 mutant. Additionally, the HA-tagged single gene construct (Fig. 5) confirmed that OspD3/ShET2 can be expressed from the transcription initiation sites within the intergenic region. Therefore, the data verify that transcription initiation of ospD3 is not dependent on the ospC1 promoter. Finally, to provide further evidence that the genes are regulated separately and not co-transcribed, several bioinformatic studies in the literature have identified differential expression patterns or levels for ospC1 and ospD3 under various conditions in the supplemental tables provided.19,34-36 Genes in known operons, such as the ipaADCB operon, are expressed together at approximately the same levels.35 In all, our experimental analyses and expression data provided by the literature confirm that ospD3 is expressed separately and at distinct levels relative to ospC1.

With regard to the ospD3 intergenic region, two transcription initiation points were predicted, as well as several putative promoters elements identified by the BPROM software,20,37 and two putative Shine-Dalgarno (SD) or RBS of AAGGAA are in frame with a two distinct ATG translational start codons (Fig. 1). A recent publication by Hensley, et al reported the consensus SD sequence utilized by Shigella to be AAG-GAA.37 Therefore, the different intergenic sequences were analyzed using β-gal assays. When all promoter sites and RBS sites are included, β-gal activity is very

Figure 8. IL-8 secretion analysis from infected epithelial cells. HCT-8 cells were infected with wildtype 2457T and various S. flexneri mutants. Afterwards, the culture media was processed for IL-8 ELISAs to determine the amount of IL-8 secreted from the epithelial cells over the course of the infection. Cells infected with the ΔospC1 mutant had the same amount of IL-8 secretion compared to 2457T; however, cells infected with the ΔospD3 mutant had a significant increase in IL-8 secretion relative to 2457T (p < 0.001). The double ΔospC1 + ΔospD3 also had a significant increase in IL-8 secretion (p = 0.01). The wildtype strain expressing the HA-tagged OspD3 (pOspD3) had significantly lower IL-8 expression relative to 2457T (p < 0.05). The virulence plasmid-cured strain BS103 served as a noninvasive infection control while media alone served as a negative control for IL-8 secretion. The average IL-8 secretion (pg/ml) relative to 2457T is plotted +/- the SEM. Statistical analyses were performed with a student’s t-test between 2457T and the mutants or the strain expressing the HA-tagged OspD3 (pOspD3). Asterisks indicate significantly altered IL-8 secretion relative to wildtype 2457T.
strong (Fig. 5). Intergenic sequence 2 and 3 were tested in an attempt to reduce the number of promoter sites relative to the two possible translational start sites. Both sequences are functional and resulted in significant LacZ production, indicating that each promoter region and translational start site could be utilized for ospD3 expression (Fig. 5B). The data are confirmed by the lack of β-gal activity with intergenic sequence 4 since the second SD site is eliminated from this construct. N-terminal sequencing of the HA-tagged single gene construct demonstrated that the first ATG codon is the predicted translational initiation point; however, we cannot rule out that the second translational start site is used for the protein due to a lack of high level sensitivity with this analysis. The Shigella proteins IcsP37 and Spa3338 have both been demonstrated to use alternate start codons to produce different lengths of each protein. It is interesting to note that more OspD3/ShET2 protein was produced from the single construct compared to the OspC1-OspD3 construct. Again, this difference may be due to the different translational start sites utilized in different environmental conditions, or due to the possibility that transcriptional activators or repressors important for regulating OspD3/ShET2 expression bind to the intergenic region. Future analysis of regulatory elements will help to clarify the regulation of OspD3/ShET2 expression.

The virulence assays have verified that OspD3/ShET2 activity is not dependent upon OspC1 expression in that the ΔospC1 mutation does not affect toxin activity or IL-8 secretion from infected cells, which is supported by observations in previous studies performed in polarized epithelial cells.12 We recognize that our findings in the IL-8 secretion assay contrast with the conclusions of Farfán, et al that epithelial cells infected with a ΔospD3 mutant secreted less IL-8 than wildtype bacteria from a monolayer of T84 cells.13 Therefore, we verified our results in three separate cell lines to confirm that the ΔospD3 mutation leads to greater IL-8 secretion from infected cells. The interplay between the various Shigella type-III effectors, IL-8 secretion, and subsequent PMN infiltration is quite complex. While invasion at the basolateral pole of intestinal epithelial cells induces IL-8 secretion and PMN infiltration to enhance bacterial access to the submucosa early in infection,11,39 other type-III effectors have been shown to down-regulate IL-8 secretion and PMN infiltration during later stages of infection in an attempt to mitigate the immune response.12,40,41 Since infection of epithelial cells with the ΔospD3 mutant induced a greater amount of IL-8 secretion relative to wildtype bacteria, we hypothesize that OspD3 targets a eukaryotic protein to down-regulate IL-8 expression possibly in an attempt to regulate PMN infiltration.42 As mentioned above, one of the consequences of PMN infiltration in the gut is diarrhea.14 Future studies identifying the eukaryotic target(s) of OspD3/ShET2 will shed light into the role this bacterial effector plays in regulating IL-8 secretion, and will allow us to determine how or if this role relates to the enterotoxin activity of the protein.

In conclusion, our study is the first to provide extensive experimental data that ospD3 is expressed independently of ospC1. Our analysis verifies that OspD3/ShET2 functions as both an enterotoxin and a regulator of IL-8 secretion from infected epithelial cells, and that OspC1 does not contribute to enterotoxin activity. This work serves as the basis for future studies to identify the eukaryotic targets of OspD3/ShET2 to understand how this unique bacterial virulence factor affects two seemingly diverse events.

Materials and methods

Bacterial strains and growth conditions

Table 1 lists the bacterial strains and plasmids used in this study. Bacteria were routinely cultured at 37°C either in tryptic soy broth or Luria-Bertani broth with aeration, or on tryptic soy broth plates with 1.5% agar and 0.025% Congo Red (CR; Sigma). Antibiotics were used at the following concentrations: chloramphenicol, 5 μg/ml; kanamycin, 50 μg/ml; ampicillin, 100 μg/ml; and tetracycline, 10 μg/ml.

Animals used in the study

Female C57BL/6 mice were purchased from the National Cancer Institute (Frederick, MD) and were used for Ussing experiments between 6 and 14 weeks of age. This study was carried out in strict accordance with the recommendations of the Institutional Animal Care and Use Committee (IACUC) at the University of Maryland at Baltimore (approved protocol #0111044). All efforts were made to minimize suffering of mice during euthanasia.
**Table 1** Strains and plasmids used in this study.

| Name | Description | Source or Reference |
|------|-------------|---------------------|
| **Strains** | | |
| 2457T | Wildtype *S. floueri* 2a | 47 |
| BS103 | Plasmid-cured 2457T | 48 |
| BS766 | 2457T transformed with pKM208 | 25 |
| BS772 | 2457T/ΔospC1:cat | 11 |
| BS796 | 2457T/ΔospD3:cat | 5 |
| ΔospC1 + ΔospD3 | 2457T/ΔospC1 + ΔospD3, cmR | This study |
| 2457T/Δpc1 | 2457T/ΔospC1 promoter | This study |
| BS772/Δpc1 | BS772/ΔospC1 promoter | This study |
| 2457T (pOsPc1) | 2457T transformed with pOsPC1 | This study |
| 2457T (pOsPC1-OspD3) | 2457T transformed with pOsPC1-OspD3 | This study |
| 2457T (pOspD3) | 2457T transformed with pOspD3 | This study |
| 2457T (pRW50) | 2457T transformed with pRW50 empty vector | This study |
| 2457T (pRW50_1) | 2457T transformed with pRW50_1 for the β-galactosidase assay | This study |
| 2457T (pRW50_2) | 2457T transformed with pRW50_2 for the β-galactosidase assay | This study |
| 2457T (pRW50_3) | 2457T transformed with pRW50_3 for the β-galactosidase assay | This study |
| 2457T (pRW50_4) | 2457T transformed with pRW50_4 for the β-galactosidase assay | This study |
| **Plasmids** | | |
| pKD3 | oriR6K, bla, cat | 49 |
| pKD4 | oriR6K, bla, aphA-3 | 49 |
| pKM208 | Temperature-sensitive red-, gam-, lacI-expressing plasmid driven by P_red promoter, bla | 49 |
| pDZ1 | Cloning intermediate for 2HA fusions, cat | 11 |
| pOsPC1 | ospC1 cloned into pDZ1, cat | This study |
| pOsPC1-OspD3 | ospC1 and ospD3 cloned into pDZ1, cat | This study |
| pOspD3 | ospD3 cloned into pDZ1, cat | This study |
| pRW50 | Low-copy-number promoterless lacZ expression vector, tet | 21 |
| pRW50_1 | Promoter sequence intergenic #1 cloned into pRW50 for the β-galactosidase assay | This study |
| pRW50_2 | Promoter sequence intergenic #2 cloned into pRW50 for the β-galactosidase assay | This study |
| pRW50_3 | Promoter sequence intergenic #3 cloned into pRW50 for the β-galactosidase assay | This study |
| pRW50_4 | Promoter sequence intergenic #4 cloned into pRW50 for the β-galactosidase assay | This study |

**Bacterial strain construction**

The ΔospC1 + ΔospD3 double mutant was constructed using the λ red linear recombination method as previously described.25 Given the size of the ospC1 to ospD3 genomic region (3.4 kb), the chloramphenicol cassette was designed to recombine into and span the middle 2.57 kb of the ospC1 to ospD3 genomic region. As a result, approximately 422 bp of the 5’ end of ospC1 and 446 bp of the 3’ end of ospD3 remain present in the mutant strain. For the ospC1 promoter deletions in strains 2457T and BS772, the same protocol was followed using the kanamycin resistance cassette. Please refer to Table S1 for the primer sequences used to make the deletion mutants. Primers that annealed outside of the coding regions were used to verify deletion of the genes via a reduction in PCR product size relative to wildtype bacteria.

The primers used to construct the C-terminal hemagglutinin (HA) tags are listed in Table S1. The genes, with upstream promoter regions, were amplified by PCR with primers harboring 5’ restriction enzyme sites (Table S1) using high fidelity Taq polymerase (Invitrogen). The PCR products were digested with the appropriate restriction enzyme (pDZ1, Acc65I and BgII) and ligated into digested pDZ1 (promoterless) to generate each of the plasmids. The plasmids were subsequently transformed into wildtype 2457T, and selection for positive transformants occurred on tryptic soy broth plates with 1.5% agar, 0.025% CR, and 5 μg/ml chloramphenicol. Sequencing of the plasmids was performed to ensure no mutations were introduced during the cloning process. The primers used for the sequencing annealed outside of the MCS for the plasmids and are listed in Table S1.

The primers used to create the transcriptional lacZ fusions for the β-galactosidase assays are listed in Table S1. The putative promoter sequences were amplified by PCR using high fidelity Taq polymerase and subsequently annealed into the pGEMT vector (Promega). The resulting plasmids were then digested with EcoRI and HindIII, the promoter fragments were gel purified and ligated into the lacZ promoterless plasmid pRW50.21,43 The lacZ gene in plasmid pRW50 also does not contain a ribosome binding site; and therefore, the ospD3 putative promoter and RBS sequences were tested to drive expression of lacZ. The plasmids were transformed into *E. coli* DH10B, sequenced, and then transformed into 2457T. Selection of positive transformants occurred on tryptic soy...
broth plates with 1.5% agar, 0.025% CR, and 10 μg/ml tetracycline.

**Intestinal epithelial cell maintenance and infections**

Epithelial cells were routinely maintained in tissue culture media (1X DMEM for HT-29 cells, RPMI for HCT-8 cells, and DMEM-F12 for T84 cells) with 10% fetal bovine serum at 37°C with 5% CO₂. For infections, HT-29, HCT-8, or T84 epithelial cells were seeded into 12-well tissue culture plates to establish a semi-confluent monolayer (approximately 75% confluency was routinely reached). Bacterial strains were subcultured in broth until the OD₆₀₀ reached mid-log phase (approximately 0.7). After standardization to an OD₆₀₀ of 0.35 (which corresponds to approximately 1.0 × 10⁸ CFU/ml), the bacteria were washed with 1X PBS, resuspended in 1 ml tissue culture media, and added to the epithelial cells. The tissue culture plates were spun at 3,000 rpm for 10 minutes to facilitate bacterial contact for invasion. The plates were incubated at 37°C with 5% CO₂ for 45 minutes to ensure that a majority of the monolayer was infected. Afterwards, the plates were washed with 1X PBS and fresh media with 50 μg/ml gentamicin was added to the monolayers to kill extracellular bacteria for 45 minutes. After washing the monolayers and adding fresh media with 50 μg/ml gentamicin, plates were incubated at 37°C with 5% CO₂ for 18 hours to allow IL-8 secretion to accumulate into the culture media. On the following day, the 1 ml of culture media from each well was collected and centrifuged at 10,000 × g for 10 minutes to remove any cell debris or bacteria. The supernatants were collected and stored at −80°C for subsequent RNA isolation.

**RNA isolation**

RNA was isolated from bacterial cultures or infected epithelial cells using either Qiagen’s RNA protect and RNeasy kits or the Trizol method (Invitrogen) according to manufacturer’s protocols. DNA was digested with Turbo DNase (Invitrogen), and concentrations of total RNA were determined using a NanoDrop ND-1000 spectrophotometer. The cDNA was synthesized from total RNA using Superscript III First Strand Synthesis kit (Invitrogen) or RevertAid cDNA first strand synthesis kit (Thermo Scientific) according to manufacturer’s protocol. All RNA was first confirmed to be free of DNA contamination by performing separate cDNA synthesis reactions with and without reverse transcriptase, followed by PCR amplification of the house-keeping gene rpoA (Table S1).

**RNA sequencing**

The data generated from the RNA-seq analysis of 2457T RNA from broth cultures and intracellular infections were performed in the Morris, et al study.¹⁹ The RNA-seq figures were generated using Mochiview.⁴⁴

**Non-quantitative reverse-transcription PCR analysis**

cDNA was synthesized from total RNA isolated from broth cultures using the RevertAid cDNA first strand synthesis kit (Thermo Scientific) according to manufacturer’s protocol. All RNA was first confirmed to be free of DNA contamination by performing separate cDNA synthesis reactions with and without reverse transcriptase, followed by PCR amplification of the house-keeping gene rpoA. The various PCR reactions were performed with the primer sets outlined in Table S1 using the 2X Taq-Pro Complete PCR mix (Denville Scientific). All primer sets were validated and tested for proper DNA amplification prior to the experiment (Fig. S1). The annealing temperatures were adjusted accordingly for each primer set, and the extension time was adjusted for the size of each product. The products of the reactions were visualized by gel electrophoresis on 1% agarose gels stained with ethidium bromide on a Bio-Rad GelDoc system.

**β-galactosidase assay**

The β-gal assay was performed as previously described.²¹²² β-gal activity was measured through the enzymatic hydrolysis of o-nitrophenyl-β-D-galactopyranoside in bacteria from overnight cultures resuspended in Z buffer, and β-gal activity in Miller units was calculated as previously described.²² The average of at least three independent experiments
is presented with the average Miller unit obtained for each experiment +/− the standard error of the mean (SEM). Statistical analyses were performed with a student’s t-test between the indicated comparisons.

**Protein expression and secretion**

For protein expression analysis, bacteria harboring the various plasmids were subcultured in LB or TSB at 37°C with the appropriate antibiotic selection to mid-log phase (OD₆₀₀ of 0.7). Afterwards, bacteria were pelleted and resuspended in 1 ml of SDS loading buffer. Samples were stored at −20°C. The Congo red (CR) secretion assay was used to identify proteins secreted by the bacteria through the T3SS, and was performed as previously described.²⁴ Briefly, bacteria were grown to mid-log phase, resuspended in 1X PBS, and CR was added to a final concentration of 30 μg/ml. The bacteria were incubated at 37°C for 1 hour. After incubation, the bacteria were pelleted by centrifugation, and the supernatant was collected and filtered through a 0.22-μm pore filter and then stored at −20°C. The proteins in the supernatant represent the secreted proteins and were concentrated by trichloroacetic acid (TCA) precipitation. TCA pellets were resuspended in 50 μl sodium dodecyl sulfate (SDS) loading buffer for protein analysis and stored at −20°C. The bacterial pellets, representing the non-secreted proteins, were resuspended in 500 μl of SDS loading buffer and stored at −20°C.

To analyze OspD3 protein present in the Ussing chamber samples, strain 2457T (pOspD3) was subcultured in 600 ml LB with 5 μg/ml chloramphenicol at 37°C and grown to an OD₆₀₀ of 1.0. The bacteria were subsequently pelleted, and the supernatants were filtered and concentrated to 2 ml as previously described.⁵ A 400 μl aliquot, representing the amount of the sample analyzed on each Ussing chamber, was TCA precipitated, resuspended in 20 μl of SDS loading buffer for subsequent Western blot analysis, and stored at −20°C.

**Protein and western blot analysis**

For total protein analysis each sample was resolved by SDS-polyacrylamide gel electrophoresis (PAGE), and Coomassie blue stain was used to visualize total protein. For Western blot analysis proteins were transferred to a PVDF membrane and blocked with 10% dry milk in 1X PBS. HA-tagged proteins were detected with anti-HA antibody (mouse mono HA.11 (16B12); Covance) at a concentration of 1:3,000 in PBS-Tween (PBST) with 10% dry milk overnight at 4°C. Histagged proteins were detected with anti-his antibody (mouse tetra-his; Qiagen) at a concentration of 1:2,000. IpaB was detected with mouse monoclonal anti-IpaB (1:20,000 dilution) antibody as previously described.⁴⁵ After washing, Alexa Fluor 700 goat anti-mouse immunoglobulin G (H + L) antibody (Molecular Probes) was added at a 1:3,000 concentration in PBST with 10% dry milk for 1 h. All washes of the PVDF membrane were performed with 1X PBST for 5 minutes at room temperature. Western blots were scanned using the Odyssey infrared detection system (Li-Cor).

**Protein purification for N-terminal sequencing**

The HA-tagged OspD3 single construct was immuno-precipitated with the Pierce magnetic HA-Tag IP/Co-IP kit (Thermo) according to manufacturer’s instructions. For analysis 60 ml cultures were used, with elutions from multiple columns combined and TCA precipitated into a single protein sample. TCA pellets were resuspended in 25 μl of SDS loading buffer. 20 μl of the sample was resolved with SDS-PAGE while the remaining 5μl of the sample was analyzed by Western blot analysis with the anti-HA antibody. The protein band corresponding to the OspD3 full-length protein was sent to Taplin Mass Spectrometry Facility at Harvard Medical School. The HA-tagged OspD3 immunoprecipitations were compared to 2457T negative controls to ensure the proper, Western blot-confirmed protein band was sent for analysis.

**Ussing chamber analysis**

Ussing chamber experiments to measure enterotoxin activity from wildtype or mutant strains of *S. flexneri* across mouse jejunal tissue were performed as previously described.⁵ Briefly, samples were prepared by subculturing overnight cultures into 600 ml Luria-Bertani Broth (LB) 37°C with shaking until the optical density (OD₆₀₀) reached 1.0. Bacteria were pelleted, and culture supernatants were filter-sterilized and subsequently concentrated to a final volume of 2 ml. Assessment of secretory activity in response to secreted bacterial proteins (400 μl aliquots of the 2 ml concentrated supernatants) was performed using jejunal mucosae stripped of muscle and mounted in
Ussing chambers. The short circuit current (Isc) was monitored continuously for at least 30 minutes post-toxin addition; the change in Isc (ΔIsc) reflects the maximum change during this time interval. Responses from all tissue segments exposed to secreted proteins from one mouse were averaged to yield a mean response per animal. Each sample was tested on a minimum of eight animals with at least 3 biologically independent concentrated culture supernatants. For every experiment, secreted proteins from wildtype S. flexneri 2457T served as positive controls. Values are expressed as the average of the independent experiments +/- SEM. Statistical analyses were performed with a student’s t-test between 2457T and the mutants. The experimental protocol was approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Maryland at Baltimore (protocol #0111044).

IL-8 ELISA assay
Enzyme-linked immunosorbent assays were used to analyze the amount of IL-8 secreted into the culture media of infected epithelial cells using the DuoSet ELISA Development System for human CXCL8/IL-8 (R&D Systems). The OD450 was determined using an ELISA microplate reader (Multiscan Ascent; Thermo Lab systems). Analysis was performed in HCT-8, HT-29, and T84 cells. All samples had technical triplicates for each analysis, and all samples were analyzed for at least two independent experiments. Values are expressed as the average of the independent experiments +/- SEM. Statistical analyses were performed with a student’s t-test between 2457T and the mutants. The experimental protocol was approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Maryland at Baltimore (protocol #0111044).

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Abbreviations
HA hemagglutinin
IL-8 interleukin-8
PMN polymorphonuclear cell
RBS Ribosome binding site
RNA-seq RNA sequencing
SD Shine-Dalgarno
ShET2 Shigella enterotoxin 2
T3SS Type-III secretion system
β-gal β-galactosidase

Disclosure of potential conflicts of interest
No potential conflicts of interest were disclosed.
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