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Characterization of the *ospZ* Promoter in *Shigella flexneri* and Its Regulation by VirB and H-NS

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OspZ is an effector protein of the type III secretion system in *Shigella* spp. that downregulates the human inflammatory response during bacterial infection. The *ospZ* gene is located on the large virulence plasmid of *Shigella*. Many genes on this plasmid are transcriptionally repressed by the nucleoid structuring protein H-NS and derepressed by VirB, a DNA-binding protein that displays homology to the plasmid partitioning proteins ParB and SopB. In this study, we characterized the *ospZ* promoter and investigated its regulation by H-NS and VirB in *Shigella flexneri*. We show that H-NS represses and VirB partially derepresses the *ospZ* promoter. H-NS-mediated repression requires sequences located between −731 and −412 relative to the beginning of the *ospZ* gene. Notably, the VirB-dependent derepression of *ospZ* requires the same VirB binding sites as are required for the VirB-dependent derepression of the divergent *icsP* gene. These sites are centered 425 bp upstream of the *ospZ* gene but over 1 kb upstream of the *icsP* transcription start site. Although these VirB binding sites lie closer to *ospZ* than *icsP*, the VirB-dependent increase in *ospZ* promoter activity is lower than that observed at the *icsP* promoter. This indicates that the proximity of VirB binding sites to *Shigella* promoters does not necessarily correlate with the level of VirB-dependent derepression. These findings have implications for virulence gene regulation in *Shigella* and other pathogens that control gene expression using mechanisms of transcriptional repression and derepression.

Shigella species are Gram-negative, nonmotile rods that are causative agents of bacillary dysentery. The pathogenesis of *Shigella* spp. is primarily attributed to a large virulence plasmid (~230 kb) that carries many of the genes required for colonization of humans and primates, including invasion of the colonic epithelium and actin-based motility within the host cell cytoplasm. The precise regulation of virulence genes is central to *Shigella* pathogenicity. This regulation relies heavily upon the antagonistic relationship between the nucleoid structuring protein H-NS, which functions as a transcriptional repressor, and one of two transcription factors encoded by the large virulence plasmid, VirF and VirB (1), which function to counteract H-NS-mediated repression (2, 3) in a process called derepression (reviewed in reference 4).

OspZ is encoded by the large virulence plasmid, is secreted by the type III secretion system (5, 6), and is homologous to NleE of enteropathogenic *Escherichia coli* (7). NleE and full-length OspZ block nuclear translocation of the p65 subunit of NF-κB, thereby downregulating the interleukin-8 (IL-8) response during bacterial infection (8). Full-length OspZ is therefore a member of a growing family of bacterial proteins that cause the suppression of NF-κB-regulated genes, thereby subverting innate immune signaling within the host (9–11). *ospZ* of *Shigella flexneri* serotype 6 encodes full-length OspZ, but the protein in *S. flexneri* serotype 2a has a 42-amino-acid truncation at the C terminus. The truncated form of OspZ in *Shigella flexneri* 2a is unable to inhibit p65 nuclear translocation (8). Instead of reducing polymorphonuclear leukocyte (PMN) migration, an activity predicted for the full-length OspZ protein, the truncated form of OspZ has been shown to stimulate PMN migration across polarized T84 intestinal epithelial cells (7). Although the activity of both forms of the OspZ protein has been characterized and implicated in the virulence of *Shigella* species (7, 8), the promoter region of *ospZ* in *Shigella* species remains largely uncharacterized.

The *ospZ* promoter region lies within an unusually long intergenic region on the large *Shigella* virulence plasmid (depicted in Fig. 1A). This intergenic region also contains the promoter of the divergent gene *icsP*. IcsP is an outer membrane protease that cleaves IcsA, the actin tail assembly protein, from the surface of *Shigella* and functions to regulate the actin-based motility of *Shigella* within epithelial cells (12, 13). Like many virulence genes in *Shigella*, *icsP* is transcriptionally repressed by H-NS and derepressed by VirB (14). This mechanism of gene regulation has been termed "silencing and anti-silencing" (4). Two VirB binding sites, located over 1 kb upstream of the *icsP* transcription start site (TSS), are essential for the VirB-dependent regulation of the *icsP* promoter (15). These VirB binding sites are centered only 425 bp upstream of the *ospZ* gene (Fig. 1A).

As a starting point for this work, we chose to characterize the *ospZ* TSS and its associated promoter elements. Since *ospZ* lies divergent to *icsP* on the *Shigella* virulence plasmid, we tested our hypothesis that the *ospZ* promoter, like that of *icsP*, is regulated by VirB and H-NS. Having established the involvement of H-NS and VirB as regulatory proteins, we identified DNA sequences necessary for the regulation of the *ospZ* promoter (P*ospZ*). The two

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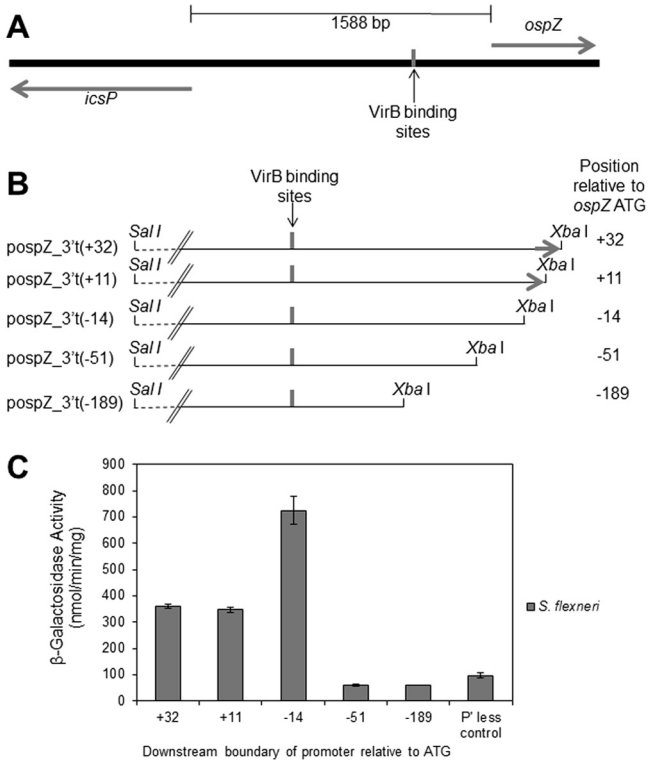


FIG 1 Analysis and characterization of the *ospZ* promoter. (A) Overview of the intergenic region between *icsP* and *ospZ*. VirB binding sites identified by Castellanos et al. (15) are indicated and centered 425 bp and 1,160 bp upstream of the *ospZ* and *icsP* translation start sites, respectively. (B) Schematic of the 3′-truncated *ospZ* promoter fragments found in the pospZ_3′t series. For each construct, the position of the 3′ truncation relative to the *ospZ* ATG is given in parentheses. The 5′ end of the *ospZ* gene (denoted by the arrow) is found only in pospZ_3′t(+32) and pospZ_3′t(+11). (C) Activity of the pospZ_3′t series in wild-type *S. flexneri*. pMIC21 serves as the promoterless (P′ less) control. Assays were run in triplicate, and the means and standard deviations are shown.

major goals of this study were to improve our understanding of the transcriptional regulation of *ospZ*, a gene implicated in the pathogenicity of *Shigella* species, and to further examine how H-NS-mediated repression and VirB-dependent derepression of virulence genes are orchestrated on the *Shigella* virulence plasmid.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* was grown on LB agar (LB broth containing 1.5% [wt/vol] agar), and *S. flexneri* strains were grown on Trypticase soy agar (TSA; Trypticase soy broth [TSB] containing 1.5% [wt/vol] agar). To ensure that *Shigella* strains maintained the virulence plasmid, Congo red binding was tested on TSA plates containing 0.01% (wt/vol) Congo red. *E. coli* and *S. flexneri* strains were grown at 37°C with aeration in Luria-Bertani (LB) broth for all assays. Where appropriate, antibiotics were added at the following final concentrations: ampicillin, 100 μg/ml, and chloramphenicol, 25 μg/ml.

Plasmid construction. The *ospZ* promoter region was PCR amplified from the large virulence plasmid of *S. flexneri* serotype 2a strain 2457T using primers W152 and W153. (Primers used in this study are listed in Table 2.) The resulting fragment was cut with the restriction endonucleases SalI and XbaI and cloned into pHJW20 (15) in order to replace the *icsP* promoter region. The resulting plasmid, pDB05, carries the entire *ospZ* promoter (1,613 bp upstream of the *ospZ* translation start site) and the first 32 bp of the *ospZ* coding region, cloned upstream of a translation stop site in each reading frame and a promoterless *lacZ* gene, so that expression of *lacZ* is directly regulated by the *ospZ* promoter.

The 3′ promoter truncations of *ospZ* were PCR amplified from pDB05 using forward primer W152 in combination with reverse primer W203, W202, W201, or W154. PCR products were digested with SalI and XbaI and then ligated to pDB05 also digested with SalI and XbaI. This created a series of P*ospZ-lacZ* fusion plasmids with the same 5′ end but different 3′ ends: +11, −14, −51, and −189 relative to the translation start site of the *ospZ* gene, respectively. These plasmids and pDB05 are described as the pospZ_3′t series in this work.

The 5′ promoter truncations of *ospZ* were isolated or PCR amplified from pDB05 using forward primers W152, W180, W181, W291, and W336 in combination with reverse primer W153. PCR products were digested with SalI and XbaI and were ligated to pAFW04, which had also been digested with SalI and XbaI. This created a series of P*ospZ-lacZ* fusion plasmids with 5′ boundaries of −1613, −1203, −731, −495, and −412 relative to the *ospZ* translation start site. The 3′ end of each promoter fragment was the same, +32 relative to the *ospZ* translation start site. These plasmids are described as the pospZ_5′t series in this work.

To create pDB15, the plasmid pMIC16 (15) was digested with restriction enzymes BglII and PstI to remove a 197-bp fragment containing the mutated VirB binding sites. The resulting fragment was subsequently cloned into pospZ_5′t(−1613) to replace the 197-bp BglII PstI restriction fragment containing the wild-type VirB binding sites.

To create pospZ_Δ45, a three-step PCR was used. In step one, two DNA fragments upstream and downstream of the region to be deleted were amplified from pospZ_5′t(−1613) DNA using the following primer pairs: W73-W357 and W356-W160. In step two, the upstream fragment was further amplified using W73 and W355 (W355 is identical to W357 except that its 5′ end is complementary to sequences downstream of the region to be deleted). In step three, the fragments generated in steps one and two were mixed and amplified using W73 and W160. The resulting DNA was then cut with BglII and XbaI and used to replace the existing BglII and XbaI fragment present in pospZ_5′(−1613).

All constructs generated in this study were confirmed by DNA sequencing.

β-Galactosidase assays. β-Galactosidase activity was determined using the Miller protocol (16). Overnight cell cultures were back-diluted 1:100 and grown for 5 h at 37°C before being assayed, because prior experiments have shown that both *icsP* and *ospZ* promoter activities are maximal after 5 h (14). In experiments in which *virB* was induced from the pBAD18 derivative, *Shigella* and *E. coli* strains were grown for 3 h and 5 h, respectively, at either 30 or 37°C prior to induction with 0.2% (wt/vol) L-arabinose. Cells were then grown at either 30 or 37°C for an additional 2 h before being assayed.

Primer extension analysis. The *ospZ* TSS was identified through RNA extraction and primer extension analysis as described recently (17) using a protocol adapted from that of Aiba et al. (18). Total cellular RNA was extracted using the hot-phenol method from 10^9 cells harvested from early-stationary-phase cultures. Samples were digested with DNase I (Invitrogen) for 1 h at 37°C in DNase I buffer (Ambion), and total RNA integrity was verified by formaldehyde gel electrophoresis and ethidium bromide staining. Primers W153 and W160 were 5′ end labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase (Promega). One picomole of ^{32}P-labeled primer and 5 μg of total RNA were dissolved in 30 μl of hybridization buffer (18). The annealing reaction mixture was heated at 50°C for 5 min, incubated at 75°C for 15 min, and maintained at 45°C for a total of 3 h. Samples were ethanol precipitated and cDNA was generated using Superscript II reverse transcriptase (Invitrogen) at 37°C for 50 min. Reactions were aborted by heating samples to 70°C for 10 min, and RNA

TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
Strains		
S. flexneri		
2457T	*S. flexneri* serotype 2a	41
AWY3	2457T *virB*::Tn*5*	14
E. coli		
MC4100	*E. coli* strain K-12 with *araD* and *lacZ* deletion	42
MC4100 *hns*	MC4100 *hns*::Kn. The first 37 amino acids of H-NS are expressed, giving rise to a *trans*-dominant negative effect over other H-NS-like proteins in the cell.	43
Plasmids		
pHJW20	P*icsP-lacZ* reporter plasmid derived from pACYC184. Carries 1,259 bp upstream and 48 bp downstream of the *icsP* gene on a PstI-XbaI DNA fragment. Restriction with SalI and XbaI completely removes *icsP* promoter and gene sequences.	15
pDB05	pHJW20 carrying 1,613 bp of wild-type sequence upstream and 32 bp downstream of the *ospZ* translation start site in the SalI and XbaI sites. Also known as pospZ_3′t(+32).	This work
pospZ_3′t(+11)	pHJW20 carrying 1,613 bp of wild-type sequence upstream and 11 bp downstream of the *ospZ* translation start site in the SalI and XbaI sites	This work
pospZ_3′t(−14)	pHJW20 carrying wild-type sequences located between 1613 bp and 14 bp upstream of the *ospZ* translation start site in the SalI and XbaI sites	This work
pospZ_3′t(−51)	pHJW20 carrying wild-type sequences located between 1613 bp and 51 bp upstream of the *ospZ* translation start site in the SalI and XbaI sites	This work
pospZ_3′t(−189)	pHJW20 carrying wild-type sequences located between 1613 bp and 189 bp upstream of the *ospZ* translation start site in the SalI and XbaI sites	This work
pAFW04	Identical to pHJW20, but the lambda *oop* terminator is cloned immediately upstream of the *icsP* promoter fragment to prevent possible transcriptional read-through into the promoter region. Restriction with SalI and XbaI completely removes *icsP* promoter sequences but leaves the lambda *oop* terminator.	This work
pospZ_5′t(−1613)	pAFW04 carrying 1,613 bp of wild-type sequence upstream and 32 bp downstream of the *ospZ* translation start site on a SalI-XbaI DNA fragment. Identical to pDB05, but the lambda *oop* terminator is cloned immediately upstream of the *ospZ* promoter fragment to prevent possible transcriptional read-through into the promoter region.	This work
pospZ_5′t(−1203)	pAFW04 carrying 1,203 bp of wild-type sequence upstream and 32 bp downstream of the *ospZ* translation start site on a SalI-XbaI DNA fragment	This work
pospZ_5′t(−731)	pAFW04 carrying 731 bp of wild-type sequence upstream and 32 bp downstream of the *ospZ* translation start site on a SalI-XbaI DNA fragment	This work
pospZ_5′t(−495)	pAFW04 carrying 495 bp of wild-type sequence upstream and 32 bp downstream of the *ospZ* translation start site on a SalI-XbaI DNA fragment	This work
pospZ_5′t(−412)	pAFW04 carrying 412 bp of wild-type sequence upstream and 32 bp downstream of the *ospZ* translation start site on a SalI-XbaI DNA fragment	This work
pospZ_Δ45	pAFW04 with a 45-bp deletion between −270 and −225 relative to the *ospZ* translation start site	This work
pMAP07	pAFW04 lacking all promoter sequences	This work
pMIC16	pBluescript II KS(+) carrying 809 bp upstream and 331 bp downstream of the *ospZ* translation start site with 14-bp substitutions in the VirB binding sites	15
pDB15	pospZ_5′t(−1613) carrying 14-bp substitutions in the VirB binding sites, so that the original sequence ATTTC AGtATGAAAT was altered to GCCCAGCtCGACCCG[a]	This work
pMIC21	pHJW20 lacking all promoter sequences	15
pBAD-virB	pATM324 derived from pBAD18; Amp^r	44
pBAD18	Arabinose-inducible pBAD expression vector, pBR *ori*; Amp^r	45

[a] Uppercase letters represent sites found to be essential for VirB-dependent regulation of the *icsP* promoter (16) or the corresponding mutated sites. Note that the original sites are organized as a near-perfect inverted repeat, separated by a single nucleotide (lowercase).

was removed by digestion with 10 mg/ml of RNase A (Sigma) for 30 min at 37°C. Samples were ethanol precipitated and dissolved in 5 μl of loading dye (95% [vol/vol] formamide, 20 mM EDTA, 0.05% [wt/vol] bromophenol blue, 0.05% [wt/vol] xylene cyanol; Sequenase, version 2.0, DNA sequencing kit [Affymetrix]) prior to separation by electrophoresis on a 6% (vol/vol) glycerol-tolerant polyacrylamide gel (PAGE) containing 7 M urea. PAGE gels were transferred to Whatman paper and vacuum dried. Dried gels were exposed to a phosphor screen overnight and visualized using a Typhoon 9410 variable-mode imager (Amersham). A sequencing ladder generated from pBluescript II KS(+) (Stratagene) and an M13 reverse primer with the Sequenase, version 2.0, DNA sequencing kit (Affymetrix) was routinely used to determine the sizes of primer extension products.

RESULTS

Analysis of the *ospZ* promoter region. To identify the location of associated *ospZ* promoter sequences, a series of 3′ truncations of putative promoter sequences were created. Promoter fragments were generated with the same upstream boundary (−1613 relative to the beginning of the *ospZ* gene) but different downstream boundaries ranging from + 32 to −189 relative to the beginning

TABLE 2 Oligonucleotide primers used in this study

Primer	DNA sequence[a]
W73	CAATAAAATGGTTGGTTGAAGGTCGTG
W152	CCGAGTCGACCAAGTACAAAGAATTTTAATTTCATCG
W153	CCGATCTAGAACGTTCTTAATATTCTTGATGGGAC
W154	CCGATCTAGAAAACCAGAACCTCGCTTAGGCC
W160	TTTGCGCTCCTTCAACTGGGCA
W180	CCGAGTCGACCCTCCTGTATTTGACGTAACGTG
W181	CCGAGTCGACTAATGTGGTTGTCCGATTAAGGAC
W201	TTACTTCTAGACCTAAGTGGAATGTCTCCACGG
W202	TTACTTCTAGACTCAAATATAAACATTACCATGAAC
W203	TTACTTCTAGAGGACTAATCATTTTAATCTCTATACTC
W291	CCGAGTCGACGGATAAGCTATTGTCCTTATTCCAC
W336	ATTTTGTCGACGAAGTATTCAATCAAACAATTACACC
W355	GTGGGTAGGATCAATAAAGACGTTTTATATGTGTCTGC
W356	GTAATATGGACACAGGCCTAAGCG
W357	CCTCGCTTAGGCCTGTGTCCATATTACGTGGGTAGGAT CAATAAAGACGTTTTATATGTGTCTGC
M13	GAGCGGATAACAATTTCACACAGG

[a] Underlined sequences represent unique restriction sites introduced for cloning purposes.

of the *ospZ* gene (Fig. 1B). Each promoter fragment was then cloned upstream of a promoterless *lacZ* gene in a reporter plasmid, and β-galactosidase activity was measured in a wild-type *S. flexneri* 2a strain. Our data show that the three longest constructs display β-galactosidase activity above baseline, but the two shortest constructs do not (Fig. 1C). This indicates that under the conditions tested, the only active promoter(s) lie(s) within 50 nucleotides (nt) of the 5′ end of the *ospZ* gene. The β-galactosidase activity generated by cells carrying pospZ_3′t(−14) was routinely 2-fold higher than that generated by cells carrying plasmids with longer promoter fragments. Although the underlying reason for this remains unknown, it is possible that the unique 3′ junction between the *ospZ* sequence and the *lacZ* gene increases mRNA stability or translation efficiency of *lacZ* in this construct.

Transcription start site mapping. To precisely map the TSS of the *ospZ* promoter, we next performed primer extension analysis on mRNA isolated from the wild-type *S. flexneri* 2a strain or the same strain carrying the P*ospZ-lacZ* fusion plasmid, pDB05. Two primers were used for our analyses: W153 is complementary to the beginning of the *ospZ* transcript, a sequence that is present in both the *ospZ* gene and the P*ospZ-lacZ* fusion plasmid, and W160 is complementary to the beginning of the *lacZ* transcript, a sequence present only in the P*ospZ-lacZ* fusion plasmid. Regardless of whether the *ospZ* promoter was carried by the large virulence plasmid or the low-copy-number P*ospZ-lacZ* reporter, a single primer extension product was observed when primer W153 was used (Fig. 2A). This indicates that a single TSS is associated with the *ospZ* gene and that this TSS is used regardless of whether the transcript is generated from the native *ospZ* gene or the P*ospZ-lacZ* fusion plasmid. When primer W160 was used for primer extension analysis of mRNA isolated from cells carrying the P*ospZ-lacZ* fusion plasmid, a larger product was detected. This was expected because primer W160 binds downstream of W153. Even so, the size of this product mapped exactly to the position of the TSS identified by primer W153. The identification of a single transcription start site 28 nt upstream of the *ospZ* translation start site by primer extension analyses is consistent with our previous findings (Fig. 1C), which predicted that the *ospZ* TSS lies within 50 nucleotides of the start of the *ospZ* gene. Using this

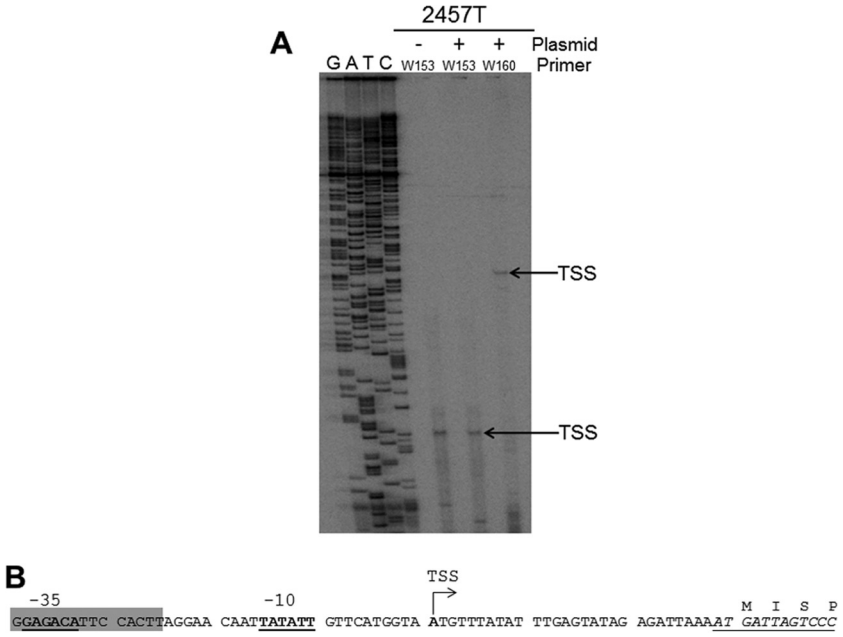
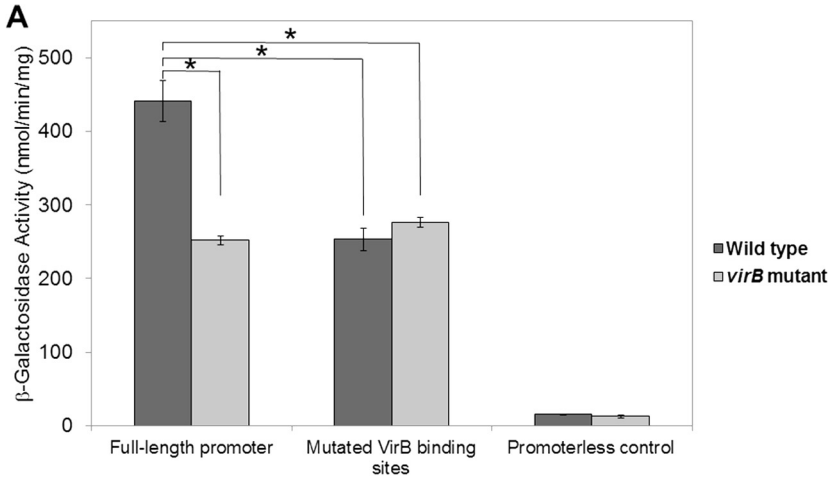


FIG 2 Identification of the transcription start site of the *ospZ* gene. (A) Primer extension analysis of *ospZ* transcripts generated by the wild type (2457T) or the wild type carrying the plasmid pDB05. The primer W153 binds within *ospZ*, and the primer W160 binds within *lacZ*. Arrows indicate identified transcription start sites, which map to the same nucleotide position. (B) Sequence of the *ospZ* promoter region and beginning of the gene. The *ospZ* transcription start site identified by this work is indicated (bold type and designated TSS), and associated potential −10 and −35 elements are shown (bold and underlined). The gene sequence is italicized, and encoded amino acids are given. The sequence bearing strong sequence similarity to the 3′ end of IS*3* (92% identity over 185 nt) is highlighted in gray (GenBank number AL391753.1 [32]).



B ***S. flexneri***

Strain	Fold Increase in *ospZ* promoter activity (mean ± S.D.) at:			
	30°C		37°C	
	pBAD-virB	pBAD18	pBAD-virB	pBAD18
AWY3 pospZ_5′(-1613)	1.54 ± 0.11	0.83 ± 0.01	1.93 ± 0.11	0.84 ± 0.01
AWY3 pDB15	0.84 ± 0.02	1.0 ± 0.04	0.75 ± 0.04	1.0 ± 0.01
2457T pospZ_5′(-1613)	1.74 ± 0.04	0.86 ± 0.03	2.32 ± 0.04	1.35 ± 0.06
2457T pDB15	0.93 ± 0.12	0.91 ± 0.02	0.81 ± 0.05	0.89 ± 0.03

FIG 3 Activity of the P*ospZ-lacZ* fusions in wild-type *Shigella* and a *virB* mutant. (A) β-Galactosidase activity resulting from cells carrying either the wild-type full-length promoter (pospZ-5′t(−1613), the full-length promoter containing mutated VirB binding sites (pDB15), or a promoterless control (pMAP07) grown at 37°C. Assays were run in triplicate, and the means and standard deviations are shown.*, $P < 0.01$. (B) Fold increase in *ospZ* promoter activity in cells grown with (pBAD-virB) or without (pBAD18) induction of *virB*. In each case, β-galactosidase activity from the promoterless control in each strain background was subtracted from those generated by cells carrying P*ospZ-lacZ* fusions. The resulting data were normalized to activities obtained in the *virB* mutant strain carrying pDB15 (construct with the mutated VirB binding sites) when these cells were grown at either 30 or 37°C. Assays were run in triplicate, and the means and standard deviations are shown.

information, the potential −10 and −35 promoter elements of the *ospZ* promoter were identified (Fig. 2B) and were found to display a 5/6 and 4/6 match to each consensus sequence, respectively.

VirB upregulates the *ospZ* promoter. Our previous studies of the genetic locus containing *icsP* and *ospZ* have demonstrated that two VirB binding sites, organized as an inverted repeat and located over 1 kb upstream of the *icsP* TSS, are required for the VirB-dependent regulation of the *icsP* promoter (15). These VirB binding sites are located closer to the beginning of the divergent *ospZ* gene than the *icsP* gene (Fig. 1A). Therefore, we next chose to determine whether or not these VirB binding sites influence the activity of the *ospZ* promoter. To do this, β-galactosidase assays were performed in wild-type *Shigella* and a *virB* mutant background carrying either the full-length *ospZ* promoter [pospZ_5′t(−1613)] or a derivative that contained base pair substitutions throughout the VirB binding sites (pDB15). Our data show that the activity of the *ospZ* promoter carrying wild-type VirB binding sites is significantly higher in wild-type *Shigella* than in the *virB* mutant and that this increase is completely dependent on the previously identified VirB binding sites (15) (Fig. 3). Notably, complementation of the *virB* mutant with a plasmid carrying an inducible copy of *virB* rescued the VirB-dependent regulation of the *ospZ* promoter (Fig. 3B) at both 30 and 37°C, but this effect was observed only in *virB* mutants carrying constructs bearing wild-type VirB binding sites (and not in those cells carrying pDB15). Taken together, these results indicate that *ospZ* promoter activity is dependent upon VirB for its activity and that this dependency requires the two VirB binding sites, which were previously characterized as regulating the *icsP* promoter (15).

H-NS represses the *ospZ* promoter at 37°C. The nucleoid structuring protein H-NS plays a major role in silencing the transcription of virulence genes on the *Shigella* virulence plasmid (19–22). At the nonpermissive temperature of 30°C, many genes on the virulence plasmid are repressed by H-NS (23). Upon a switch to 37°C, a complex regulatory cascade is triggered, which results in the production of VirF and subsequently VirB (3, 24, 25). Although some virulence genes are derepressed solely by the increase in temperature (23), others are derepressed at 37°C by either VirF or VirB, which function to relieve the H-NS-mediated repression of these virulence gene promoters on the large virulence plasmid (3, 14, 21, 26).

Our previous studies at the *icsP* and *ospZ* locus have revealed that H-NS is able to repress the *icsP* promoter at 37°C (14). Therefore, we hypothesized that H-NS would also repress transcription of the divergent gene *ospZ* at 37°C. To test this hypothesis and to identify regions of DNA sequences required for H-NS-mediated regulation, a series of 5′-truncated P*ospZ-lacZ* fusion plasmids were constructed (named the pospZ_5′t series) and introduced into the wild-type *E. coli* strain MC4100 and an *hns* mutant derivative. β-Galactosidase levels were then measured after the transformed cells were grown at 37°C. These *E. coli* strains were used to avoid interference arising from the H-NS-dependent regulation of *virB* in *Shigella* (27) and the notorious genetic instability of *Shigella hns* mutants (A. T. Maurelli, personal communication). A

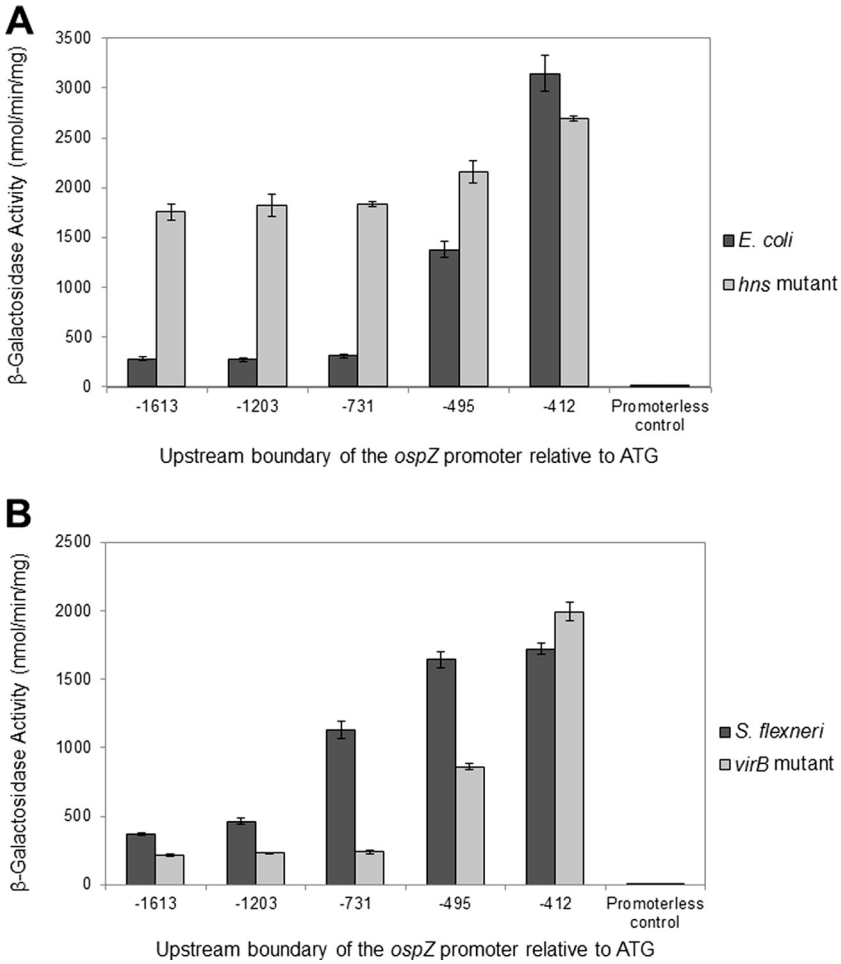


FIG 4 Activity of the *ospZ* promoter truncation series (pospZ_5′t series) in wild-type *E. coli* and an *hns* mutant (A) and wild-type *S. flexneri* 2457T and a *virB* mutant derivative (B). pMAP07 serves as the promoterless control. Assays were run in triplicate, and each assay was repeated three times. Means and standard deviations of representative data are shown. Note that the activities of pDB05 and pospZ_5′t(−1613) were found to be identical to each other (data not shown).

similar strategy has been used by us (14) and others to study H-NS-dependent regulation of *Shigella* promoters (21).

Consistent with our hypothesis, the activity of the longest construct (−1613) was 6-fold lower in the wild-type background than in the *hns* mutant background (Fig. 4A). Similar expression levels were observed in wild-type cells carrying the promoters truncated to −1203 and −731 relative to the *ospZ* gene, indicating that these promoters were also repressed by H-NS. As we successively removed sequences from the 5′ end of the *ospZ* promoter starting at −731, promoter activity was seen to significantly increase in the wild-type background in a stepwise manner, until it was equivalent to that observed in the *hns* mutant background. These observations are consistent with the removal of sequences required for H-NS-mediated repression. Based on these analyses, sequences between −495 and −412 allow partial repression of the *ospZ* promoter by H-NS, but sequences between −731 and −495 are needed for full repression of the *ospZ* promoter by H-NS.

VirB production in a wild-type *Shigella* background at 30°C or in an *E. coli* background at 37°C is sufficient to partially derepress the *ospZ* promoter. The *virB* gene is maximally transcribed at 37°C (28). While VirB production is normally very low at 30°C (29), it has been shown that VirB-regulated genes can overcome H-NS-mediated repression if *virB* expression is induced from an inducible promoter at this temperature (28, 30). To test if VirB production at 30°C can overcome the H-NS-mediated repression of *ospZ* in a wild-type *Shigella* background, wild-type *Shigella* strains carrying a P*ospZ-lacZ* reporter and either a *virB* inducible plasmid or an empty plasmid control (pBAD18) were grown under inducing conditions. Our data show that VirB production in a wild-type *Shigella* background at 30°C leads to a 1.74-fold increase in *ospZ* promoter activity, but this increase is not observed when the identified VirB binding sites are mutated in the reporter constructs (Fig. 3B). Interestingly, when *virB* was expressed from an inducible plasmid in wild-type cells grown at 37°C, *ospZ* promoter activity was seen to increase further (2.32-fold versus 1.35-fold), even though VirB is naturally produced at 37°C in wild-type cells. Because additional VirB protein can further elevate *ospZ* promoter activity, the VirB-dependent effect on the *ospZ* promoter observed in wild-type cells does not appear to be saturating under the conditions used in our assay.

At the *icsP* promoter the VirB protein has no effect in the absence of H-NS, indicating that VirB functions solely to antagonize H-NS-mediated repression (14). Our data show that VirB increases *ospZ* promoter activity (Fig. 3) and that H-NS is required

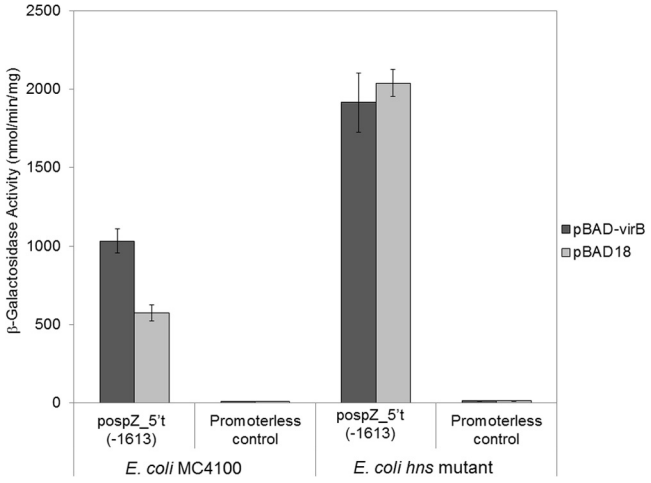


FIG 5 Activity of the *ospZ* promoter (pospZ_5′t(−1613) in a wild-type *E. coli* strain and an *hns* mutant derivative after induction of *virB* from an L-arabinose-inducible plasmid. pMAP07 serves as the promoterless control. Assays were run in triplicate, and the means and standard deviations are shown. Note that the activities of pDB05 and pospZ_5′t(−1613) were found to be identical to each other (data not shown).

for full repression of the *ospZ* promoter (Fig. 4A). We next wanted to test whether VirB functions to relieve H-NS-mediated repression or whether it functions similarly to an activator, increasing the activity of the *ospZ* promoter even in the absence of H-NS. To test this, a pBAD expression vector carrying *virB* or an empty plasmid control (pBAD18) was introduced into the *E. coli* strain MC4100 or an *hns* mutant derivative carrying either the full-length *ospZ* promoter fragment [pospZ_5′t(−1613)] or a promoterless *lacZ* reporter, and β-galactosidase activities were measured 2 h postinduction. Our data show that in wild-type *E. coli*, *ospZ* promoter activity increased 2-fold after *virB* expression (Fig. 5), consistent with our previous results obtained in a *Shigella* background (Fig. 3). In contrast, in the MC4100 *hns* mutant, promoter activity was not affected by expression of *virB* (Fig. 5). These data demonstrate that VirB functions to partially overcome H-NS-mediated repression of the *ospZ* promoter. Furthermore, since levels of derepression of the *ospZ* promoter following exogenous expression of *virB* in an *E. coli* background (Fig. 5) were similar to those observed in a wild-type *Shigella* background, this strongly suggests that VirB is the only *Shigella*-specific protein required for derepression of the full-length *ospZ* promoter in our assays.

Activity of the P*ospZ* truncation series in an *S. flexneri* background. To further characterize the VirB-dependent regulation of the *ospZ* promoter, we next measured the activity of our 5′ promoter truncation series in a wild-type *Shigella* background or a *virB* mutant derivative (Fig. 4B). The pattern of promoter activity observed in the *virB* mutant was very similar to that seen in the wild-type *E. coli* strain (Fig. 4A). Both of these strains express H-NS but do not express *virB*. These data therefore validate our use of *E. coli* strains in experiments to test the involvement of H-NS in the regulation of the *ospZ* promoter (Fig. 4A). As expected, all promoter truncations, except −412, which lacks the required VirB binding sites, displayed a VirB-dependent increase in promoter activity. This supports our finding that the VirB binding sites located at position −425 relative to the beginning of the *ospZ* gene are required for the VirB-dependent regulation of the *ospZ* promoter. Surprisingly, the promoter truncated to −731 displayed a 4.5-fold increase in VirB-dependent activity, whereas all other VirB-regulated promoters in this series displayed a 2-fold increase. The reason for this remains unclear, but this observation appears to be an artifact inherent to the pospZ_5′t(−731) construct because first, only a 2-fold increase is seen in promoter activity when measured in the context of the entire intergenic region, and second, *ospZ* expression was previously reported to increase 2- to 3-fold in response to temperature (5).

Comparison of the *ospZ* and *icsP* genetic locus in other *Shigella* strains and species. Finally, to assess whether our characterization of the *ospZ* promoter was relevant to other *Shigella* strains and species, DNA sequences of *ospZ* promoter regions were collected and compared to the entire *icsP* and *ospZ* intergenic region taken from *Shigella flexneri* serotype 2a, type strain 2457T (Table 3). Our analysis revealed that most sequenced strains show complete sequence identity to the VirB binding sites, promoter elements, and most of the intergenic region in 2457T, despite the occasional point mutation (no more than 4 in any sequence). These findings are consistent with our hypothesis that VirB regulates *ospZ* genes and *icsP* genes in these *Shigella* strains and species as well. The only sequences displaying a significant deviation from those found in 2457T were found in *S. dysenteriae* serotype 2 [(10)4279] and *S. boydii* serotype 4 (Sb227, YP_406371). The available sequences of these strains indicate that a 45-bp deletion exists between 270 and 225 bp upstream of the *ospZ* gene (Table 3). To test the impact that this 45-bp deletion has on *ospZ* promoter activity, a 45-bp deletion identical to that found in *S. dysenteriae* serotype 2 and *S. boydii* serotype 4 was created in our P*ospZ-lacZ* reporter plasmid (pospZ_Δ45), and promoter activity was measured in *S. flexneri* serotype 2a strain 2457T and a *virB* mutant derivative. Although the deletion slightly lowered overall promoter activity, *ospZ* promoter activity was still 2.5-fold higher in the wild-type *S. flexneri* strain than in the *virB* mutant (Table 4). These data strongly suggest that the 45-bp deletion found in *S. dysenteriae* serotype 2 and *S. boydii* serotype 4 does not interfere with VirB-dependent regulation of the *ospZ* promoter.

DISCUSSION

The *ospZ* gene, formerly called *orf212* (5, 7), is carried by the large virulence plasmid in all four *Shigella* species. This gene is located upstream of and divergent from the *icsP* gene in all *Shigella* strains and species analyzed (coordinates provided in Table 3), even though this gene is not annotated on the published sequences of the large virulence plasmids found in *Shigella flexneri* serotypes 2a and 5a (pCP301 [AF386526.1] and pWR501 [AF348706.1], respectively).

The OspZ protein is homologous to NleE, which is encoded by attaching and effacing pathogens *Citrobacter*, enterohemorrhagic *E. coli* (EHEC), and enteropathogenic *E. coli* (EPEC) (7, 8), in which it has been shown to block translocation of the p65 subunit of the transcription factor NF-κB to the host cell nucleus, resulting in NF-κB inhibition, and decreased IL-8 expression in EPEC-infected intestinal epithelial cells (8). The OspZ/NleE protein encoded by these bacteria is 224 to 230 amino acids long. In contrast, most *S. flexneri* strains produce a C-terminally truncated protein that is only 188 amino acids long (the only exception is *S. flexneri* serotype 6 [8] [Table 3]). The truncated form of OspZ has also been implicated in *Shigella* virulence because it is required for

TABLE 3 Analysis of DNA sequences found at the *ospZ* and *icsP* locus in *Shigella* spp.[a]

Species, serotype, and strain name	GenBank accession no., NCBI reference sequence no., or source of strain	OspZ product (full-length, 230 aa) and coordinates of gene on published sequence	Identity to *ospZ* and *icsP* intergenic region taken from 2457T (%)	Description of sequence change in intergenic region
S. boydii, Sb227	CP000037	Full length (8); complement (123143–123835)	97.0	45-bp deletion between −270 and −225 relative to *ospZ* gene, and 3 single point substitutions
S. boydii CDC3083-94	NC_010660.1	Full length; complement (206053–206745)	99.7	2 single point substitutions
S. dysenteriae serotype 1, Sd197	CP000035.1	Full length (7); complement (179127–179819)	99.7	4 single point substitutions
S. dysenteriae serotype 2, (10)4279	This work	Full length	97	45-bp deletion between −270 and −225 relative to *ospZ* gene, and 3 single point substitutions
S. flexneri serotype 2a, 2104	RRB strain	42-aa deletion, truncated to amino acid 188 (8)	100	NA
S. flexneri serotype 6, 0106164	RRB strain	Full length (8)	99.7	Single point substitution
S. flexneri serotype 2a, 2457T	This work	42-aa deletion, truncated to amino acid 188	Reference sequence	NA
S. flexneri serotype 2a, pCP301	AF386526.1	42-aa deletion, truncated to amino acid 188; complement (218144–218711)	100	NA
S. flexneri serotype 5a, M90T pWR501	AF348706.1	42-aa deletion, truncated to amino acid 188; complement (218378–218941)	99.9	2 single point substitutions
S. flexneri serotype 5a, M90T pWR100	AL391753.1	42-aa deletion, truncated to amino acid 188; complement (212330–212896)	99.9	2 single point substitutions
S. sonnei, Ss046	NC_007385.1	Full length (8); complement (210796–211488)	99.7	2 single point substitutions and 1 insertion

[a] aa, amino acid(s); NA, not applicable.

effective transepithelial migration of PMNs *in vitro* (7). During infection, the transepithelial migration of PMNs disrupts the integrity of the epithelial layer and facilitates the passage of *Shigella* to the basolateral surface of the colonic epithelium, the primary site of epithelial cell invasion.

In this study, we characterized the promoter region of the *ospZ* gene in *S. flexneri* serotype 2a. Our initial analysis of the *icsP* and *ospZ* intergenic region revealed a 219-bp open reading frame immediately upstream of the *ospZ* gene that terminates 1 nucleotide prior to the beginning of the *ospZ* gene. Although the location and juxtaposition of this ORF to *ospZ* raised the possibility of a bicistronic operon, this hypothesis was not supported by our 3′ truncation analysis or the transcription start site mapping, which placed the only active promoter(s) in this region within this 219-bp ORF. Upon closer inspection, the 219-bp ORF was found to be similar to ORF III of insertion sequence 3 (IS*3*) (31); a partial IS*3* sequence was found in this genetic locus previously (32). Interestingly, the putative −35 element pinpointed by our studies was found to lie within this partial IS*3* element (described in reference 32), demonstrating the composite nature of the *ospZ* promoter and implicating insertion sequences in the evolution of this virulence gene promoter (Fig. 2B). Based on our analyses of the *ospZ* promoter region, we conclude that the 219-bp ORF does not encode protein, the *ospZ* message is monocistronic, and it is transcribed from a single transcription start site located 28 nt upstream of the *ospZ* gene.

TABLE 4 Effect of 45-bp deletion between −270 and −225 relative to the *ospZ* translation start site

Reporter plasmid	Avg β-galactosidase activity (nmol/min/mg) ± SD		Ratio of activity (WT vs. *virB* mutant)
	WT[a] (2457T)	*virB* mutant (AWY3)	
pospZ_5′t(−1613)	232 ± 5[b]	85 ± 2	2.72
pospZ_Δ45	180 ± 6[b]	68 ± 3	2.65
Promoterless control (pMAP07)	19 ± 2	19 ± 1	0.97

[a] WT, wild type.
[b] $P < 0.05$.

Type III effector proteins are typically tightly regulated (6, 33), so it seemed unlikely that *ospZ* would be constitutively expressed. Based on our studies, the *ospZ* promoter is repressed by H-NS and partially derepressed by VirB. Two VirB binding sites, organized as an inverted repeat and located 397 bp upstream of the *ospZ* TSS, increase the activity of the *ospZ* promoter 2-fold. Removal of the two VirB binding sites by site-directed mutagenesis or through 5′ truncation analysis of the *ospZ* promoter region resulted in a complete loss of VirB-dependent regulation. These findings are consistent with those of Le Gall et al., who reported a modest increase in *ospZ* (*orf212*) expression in response to a shift from 30 to 37°C (5). Since VirB is maximally produced at 37°C and not at 30°C

(28), it seems likely that VirB was responsible for the effect reported by Le Gall et al. Even though a 2-fold VirB-dependent increase in *ospZ* promoter activity appears to be a modest level of regulation, the observed increase in promoter activity is reproducible and statistically significant (Fig. 3 and 4). Furthermore, this increase is completely dependent upon the VirB protein and the two VirB binding sites (Fig. 3). If *ospZ* mRNA is long-lived, a 2-fold change in *ospZ* promoter activity at 37°C could considerably increase OspZ production in *Shigella*. To our knowledge, the half-life of *ospZ* mRNA has not yet been determined. Alternatively, it is possible that *ospZ* expression may be further upregulated by another, as-yet-uncharacterized factor. MxiE, a transcription factor that upregulates many genes encoding type III effector proteins (5), does not appear to be involved, based on our studies (data not shown).

The VirB binding sites implicated in the regulation of the *ospZ* promoter were previously found to be necessary for the VirB-dependent regulation of the divergent *icsP* promoter (15). We therefore conclude that the VirB binding sites that lie within the intergenic region between *icsP* and *ospZ* on the *S. flexneri* 2a virulence plasmid are involved in the bidirectional regulation of these genes. To our knowledge, this is only the second example of divergent genes or operons on the *Shigella* virulence plasmid being regulated by the same VirB binding sites. The first example was observed in the 31-kb invasion locus of the virulence plasmid, between the divergent genes *icsB* and *ipgD* (3, 34, 35). At this locus, the intergenic region containing the VirB binding sites is much shorter, placing the VirB binding sites only 114 bp upstream of P*icsB* and 105 bp upstream of P*ipgD* (34, 35). At the *icsP* and *ospZ* genetic locus, the VirB binding sites are located unusually far upstream of the promoters they regulate: centered 397 bp upstream of the *ospZ* TSS and 1,137 bp upstream of the *icsP* TSS. This clearly demonstrates that VirB breaks some of the paradigms associated with bacterial transcription factors like the cAMP receptor protein (CRP; also known as the catabolite activator protein [CAP]), which typically bind within a 200-bp window of the genes they regulate (36). Clearly, novel mechanisms of gene regulation are at play on the *Shigella* virulence plasmid. Furthermore, although the VirB binding sites lie much closer to P*ospZ* than P*icsP* (Fig. 1A), their effect on P*ospZ* is much less dramatic than at P*icsP*: VirB upregulates the *ospZ* promoter approximately 2-fold, while it increases activity of the *icsP* promoter over 12-fold (15). These findings demonstrate that the proximity of VirB binding sites to *Shigella* promoters does not necessarily correlate with the level of VirB-dependent regulation. This is an important consideration for others studying virulence gene regulation in *Shigella* and mechanisms of transcriptional repression and derepression in other bacterial pathogens.

VirB is an unusual regulator of transcription that displays 39% identity with the P1 plasmid partitioning protein ParB, a DNA-binding protein involved in segregation of plasmid prophage during cell division. Despite this structural similarity, there is no evidence to suggest that VirB functionally contributes to the maintenance or segregation of the virulence plasmids found in *Shigella* today (32, 37). Instead, this protein appears to function solely to upregulate the transcription of virulence gene expression in *Shigella* species. Rather than functioning as an activator of transcription, VirB functions to counter transcriptional repression mediated by the nucleoid structuring protein H-NS (4, 14, 35). In this work, we demonstrate that VirB functions to partially relieve repression mediated either directly or indirectly by H-NS and that VirB has no impact on *ospZ* promoter activity in an *hns* mutant. These data are consistent with our findings (14) and those of others (21) that indicate that VirB functions solely to relieve H-NS-mediated transcriptional repression of genes that are encoded by the *Shigella* virulence plasmid.

To further understand the molecular interplay between H-NS and VirB at the *ospZ* promoter, we chose to characterize the sequences within the *icsP* and *ospZ* intergenic region required for H-NS-mediated repression. Sequences between −412 and −731 (relative to the ATG) were found to be required for full H-NS-mediated repression, but a partial loss of repression was observed as we truncated through the promoter to position −495 (Fig. 4A; compare −731, −495, and −412). The observed increase in promoter activity upon removal of sequences located between −731 and −412 was unlikely to be caused by transcription from a second cryptic promoter, because removal of the *ospZ* promoter elements identified in this study (potential −10 element and TSS) resulted in a complete loss of activity in the −412 deletion construct (data not shown). Although the H-NS high-affinity binding site 5′-TCGATAAATT-3′ has been reported to facilitate the cooperative binding of H-NS to DNA (38, 39), a complete match to the high-affinity binding sequence was not discovered between −731 and −412. The data presented in Fig. 4A are consistent with two regions being required for the H-NS-mediated repression of the *ospZ* promoter: one between −731 and −495 and the other between −495 and −412 (relative to the ATG). Alternatively, it is possible that the entire region located between −731 and −412, which is characteristically AT rich (66%, compared to the entire intergenic region at 63%), binds H-NS uniformly, thereby leading to a gradual loss of repression when 5′ sequences are eliminated in our truncation series. Future work will establish which of these scenarios is more likely.

The relative spacing between DNA sites occupied by VirB and H-NS is likely to be important if we are to understand the molecular interplay between VirB and H-NS on the virulence plasmid. Our work at the *icsP* and *ospZ* genetic locus reveals that sequences required for the H-NS-mediated repression and VirB-dependent derepression of the *ospZ* promoter lie adjacent to one another. Sequences located between −412 to −731 relative to the beginning of the *ospZ* gene are required for full H-NS-mediated repression, and an inverted repeat, displaying a 6/7 and 7/7 match to the reported VirB binding sites (34, 35) and centered at position −425, is required for VirB-dependent derepression. At the *icsB* and *ipgD* locus, the VirB binding sites (an inverted repeat) are also located adjacent to the region that binds H-NS (35). Furthermore, a recent study that sought to relieve H-NS-mediated repression of the *E. coli proU* promoter by transplanting VirB binding sites into different locations within the promoter demonstrated that antagonism of H-NS by VirB occurred optimally when the VirB binding sites were positioned immediately upstream of and adjacent to the upstream regulatory element (URE), one of two regions in this promoter region known to bind H-NS (40). Our findings at the *icsP* and *ospZ* locus therefore provide another example of a genetic locus where VirB and H-NS binding sites lie adjacent to one another. Although the importance of the juxtapositioning of these sites is not fully understood at this time, it appears to be an essential feature of promoters that are repressed by H-NS and transcriptionally derepressed by VirB. It is possible that when VirB binds next to regions occupied by H-NS, it destabilizes or dis-

places H-NS from the DNA. Currently, the molecular details that lead to transcriptional derepression require greater elucidation. This will be a major focus of future research in our laboratory.

Finally, we examined the *ospZ* promoter of other *Shigella* strains and species and compared them to that of the *Shigella flexneri* serotype 2a type strain, 2457T. Regardless of the strain examined, our analysis revealed complete sequence identity of the VirB binding sites, promoter elements, and most of the intergenic regions. The only exceptions were found in *S. dysenteriae* serotype 2 [(10)4279] and *S. boydii* serotype 4 (Sb227, YP_406371), which have a 45-bp deletion downstream of the VirB binding sites (−425) and the region required for H-NS-mediated repression (−731 to −412). Although neither of these strains is currently in our possession, an identical 45-bp deletion did not affect the VirB-dependent regulation of promoter activity (Table 4) when created in our P*ospZ-lacZ* reporter (pospZ_Δ45). Taken together, these data strongly suggest that regardless of the *Shigella* strain background, the *ospZ* gene is upregulated by VirB.

In summary, this study has characterized the *ospZ* promoter and demonstrated that this gene, which is implicated in the pathogenicity of *Shigella*, is repressed by the nucleoid structuring protein H-NS and derepressed by the virulence gene regulator VirB. The DNA sequences needed for this regulation have been identified and found to lie adjacent to one another. As the scientific community continues to develop an interest in the dynamic role that nucleoid associated proteins like H-NS play in shaping the genome and influencing gene expression, it will become important to understand how events that remodel H-NS-DNA complexes are orchestrated and which molecular mechanisms are at play. The mapping of regions required for repression and derepression obtained from this study will provide a framework for future studies seeking to elucidate the molecular mechanism of transcriptional repression and derepression. Since transcriptional repression and derepression can be considered as the molecular switch that controls virulence in many important pathogens, including *Shigella*, we believe that these findings will be met with general interest.

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