Molecular Microbiology (2018) 00(00), 00–00 ■
doi:10.1111/mmi.13932
First published online 2018

Insights into transcriptional silencing and anti-silencing in *Shigella flexneri*: a detailed molecular analysis of the *icsP* virulence locus

**Natasha Weatherspoon-Griffin,[†§] Michael A. Picker,[§] Krystle L. Pew, Hiromichi S. Park, Daren R. Ginete, Monika MA. Karney, Pashtana Usufzy, Maria I. Castellanos, Juan Carlos Duhart, Dustin J. Harrison, Jillian N. Socea, Alexander D. Karabachev, Christopher T. Hensley, Amber J. Howerton,[‡] Rosa Ojeda-Daulo, Joy A. Immak and Helen J. Wing *

School of Life Sciences, University of Nevada, Las Vegas, Las Vegas, NV 89154-4004, USA.

Summary

Transcriptional silencing and anti-silencing mechanisms modulate bacterial physiology and virulence in many human pathogens. In *Shigella* species, many virulence plasmid genes are silenced by the histone-like nucleoid structuring protein H-NS and anti-silenced by the virulence gene regulator VirB. Despite the key role that these regulatory proteins play in *Shigella* virulence, their mechanisms of transcriptional control remain poorly understood. Here, we characterize the regulatory elements and their relative spacing requirements needed for the transcriptional silencing and anti-silencing of *icsP*, a locus that requires remotely located regulatory elements for both types of transcriptional control. Our findings highlight the flexibility of the regulatory elements' positions with respect to each other, and yet, a molecular roadblock docked between the VirB binding site and the upstream H-NS binding region abolishes transcriptional anti-silencing by VirB, providing insight into transcriptional anti-silencing. Our study also raises the need to re-evaluate the currently proposed VirB binding site. Models of transcriptional silencing and anti-silencing at this genetic locus are presented, and the implications for understanding these regulatory mechanisms in bacteria are discussed.

Accepted 14 February, 2018. *For correspondence. E-mail helen.wing@unlv.edu; Tel. (+702) 895 5382; Fax (+702) 895 3956. Present addresses: [†]Emerging Pathogens Institute, University of Florida, Gainesville, FL 32610-0009, USA. [‡]School of Liberal Arts and Sciences, Nevada State College, Henderson, NV 89002, USA. [§]These authors contributed equally to this work.

Introduction

Nucleoid structuring proteins (NSPs) mediate transcriptional silencing of bacterial genes by binding, compacting and organizing DNA, activities that frequently render promoter regions inaccessible (Dorman and Deighan, 2003; Dorman, 2007). Transcriptional anti-silencing proteins relieve this repression through poorly characterized mechanisms, although it is clear that remodeling of the NSP-DNA complex is involved (reviewed in [Stoebel *et al.*, 2008]). Using the human bacterial pathogen *Shigella flexneri* as a model, our goal is to further characterize mechanisms of transcriptional silencing and anti-silencing of target genes found on the large (222 kb) virulence plasmid. At temperatures below 37°C, many of these target genes are transcriptionally silenced by the NSP H-NS (Hromockyj and Maurelli, 1989; Beloin and Dorman, 2003). Upon a switch to 37°C, VirF, the master activator of virulence genes, triggers the production of the anti-silencing protein VirB (Tobe *et al.*, 1991), which subsequently functions to relieve H-NS-mediated silencing of virulence genes that is not relieved by the temperature increase alone (Wing *et al.*, 2004). Despite the importance of the interplay between H-NS and VirB for *Shigella* virulence, mechanistic insight into transcriptional silencing and anti-silencing of virulence genes in *Shigella* is limited (Beloin *et al.*, 2002; Turner and Dorman, 2007; Gao *et al.*, 2013).

The transcriptional silencing activity of H-NS can be explained by its DNA binding preference and the resulting nucleoprotein complexes that form (reviewed in [Picker and Wing, 2016]). H-NS preferentially binds AT-rich DNA (Williams and Rimsky, 1997; Navarre *et al.*, 2006; Bouffartigues *et al.*, 2007), which is a common feature of bacterial promoters (Landick *et al.*, 2015) and horizontally acquired genetic loci (Navarre *et al.*, 2007), including virulence genes. Although a high-affinity

binding site for H-NS has been proposed (Bouffartigues *et al*., 2007; Lang *et al*., 2007), AT-rich DNA tracts that produce narrow minor groove widths primarily govern the DNA binding preference of H-NS (Gordon *et al*., 2011). Once bound to DNA, H-NS oligomerizes along the helix into regions with lower binding affinities (Lang *et al*., 2007; Fang and Rimsky, 2008), leading to the formation of large H-NS-DNA complexes. Two H-NS-DNA complexes have been described: H-NS nucleoprotein filaments that coat long, contiguous stretches of DNA, and H-NS bridging complexes that bring two discrete regions together by direct H-NS-H-NS interactions (Dame *et al*., 2000, 2005, 2006; Arold *et al*., 2010). Both of these nucleoprotein complexes have been implicated in the silencing of virulence genes in *Shigella* (Tobe *et al*., 1993; Falconi *et al*., 1998; Prosseda *et al*., 2004; Turner and Dorman, 2007).

The anti-silencing protein VirB belongs to the ParB protein superfamily (Turner and Dorman, 2007; Taniya *et al*., 2003) that contains both chromosomal and plasmid partitioning factors, including SopB and Spo0J. Consequently, VirB appears to have been co-opted to a new role in *Shigella* where it serves to relieve transcriptional silencing mediated by H-NS and its family members (Turner and Dorman, 2007; Stoebel *et al*., 2008; Picker and Wing, 2016). VirB is a bona fide anti-silencing protein because it up-regulates transcription of its target genes in the presence of H-NS, but has little to no effect on these genes in its absence ([Wing *et al*., 2004; Turner and Dorman, 2007; Basta *et al*., 2013] and reviewed in [Stoebel *et al*., 2008]). The DNA binding activity of VirB is necessary for its role as a transcriptional regulator (Beloin *et al*., 2002; Gao *et al*., 2013). Even though a DNA recognition site for VirB, 5′-RWG(G)AAAT-3′, has been proposed (Taniya *et al*., 2003), this site is only loosely supported by *in vitro* assays (Taniya *et al*., 2003), making the validity of the proposed site uncertain.

To date, the best studied example of a transcriptionally silenced and anti-silenced virulence gene locus in *Shigella* is the *icsB* promoter, which controls the *ipa* operon located within the invasion locus on the large virulence plasmid (Taniya *et al*., 2003; Turner and Dorman, 2007). Here, DNA sequences required for both transcriptional silencing by H-NS and anti-silencing by VirB are promoter-proximal, located within 150 bp upstream of the *icsB* transcription start site (TSS; [Turner and Dorman, 2007]). The VirB binding site at this genetic locus is organized as a near-perfect inverted repeat separated by a single base pair. Interestingly, only the upstream half (known as Box 2) is reported to be necessary for VirB-dependent regulation of the *icsB* promoter *in vivo* and VirB binding *in vitro* (Turner and Dorman 2007). As such, this site appears to be consistent with the currently proposed VirB binding site (Taniya *et al*., 2003).

To improve our understanding of transcriptional silencing and anti-silencing and how this process regulates virulence gene expression in *Shigella*, our work has focused on characterizing another H-NS and VirB-regulated gene, *icsP* (Wing *et al*., 2004; Castellanos *et al*., 2009; Africa *et al*., 2011; Hensley *et al*., 2011), which encodes the IcsA-specific outer membrane protease (Wing *et al*., 2005; Egile *et al*., 1997; Shere *et al*., 1997; Steinhauer *et al*., 1999). Several features of the *icsP* locus make it different from the *icsB* locus and justify our interest in characterizing its silencing and anti-silencing. These include its regulation by two promoters (Hensley *et al*., 2011), its location outside of the invasion locus (100 kb away) and the unusually long, 1.5 kb intergenic region upstream of the *icsP* gene (Castellanos *et al*., 2009). Our previous studies of *icsP* reveal that H-NS silences the transcription of *icsP* at 37°C in the absence of VirB (Wing *et al*., 2004). Consistent with its role as an anti-silencing protein, VirB has little to no effect on *icsP* promoter activity in the absence of a functional *hns* gene (Wing *et al*., 2004). VirB-dependent anti-silencing of *icsP* relies on a DNA sequence (Castellanos *et al*., 2009) resembling that found at the *icsB* promoter: a near-perfect inverted repeat, with each half bearing similarity to the proposed VirB binding site (Taniya *et al*., 2003). Unlike the VirB regulatory site at the *icsB* promoter, however, both halves of the inverted repeat are required for VirB-dependent regulation of *icsP* (Castellanos *et al*., 2009). Strikingly, and in contrast to the *icsB* promoter, these sites are remotely located, centered 1137 bp upstream of the primary *icsP* TSS (Castellanos *et al*., 2009). These differences raise questions about the mechanism of transcriptional anti-silencing mediated by VirB.

Here, we focus on the detailed characterization of the remotely located regulatory elements controlling the expression of *icsP* and their relative spacing requirements to improve our understanding of the mechanisms of transcriptional silencing and anti-silencing of virulence gene expression in *Shigella* (Wing *et al*., 2004; Stoebel *et al*., 2008; Picker and Wing, 2016). We present a model of transcriptional regulation by H-NS and VirB at the *icsP* locus and discuss the implications of our findings for those studying mechanisms of transcriptional silencing and anti-silencing in other bacteria.

Results

Remote DNA sequences located between −900 and −436 are required for H-NS-mediated silencing of the icsP *promoter*

Previous studies revealed that sequences located between −1232 (full-length *icsP* intergenic region) and −351 relative to the primary TSS (+1) of *icsP* were

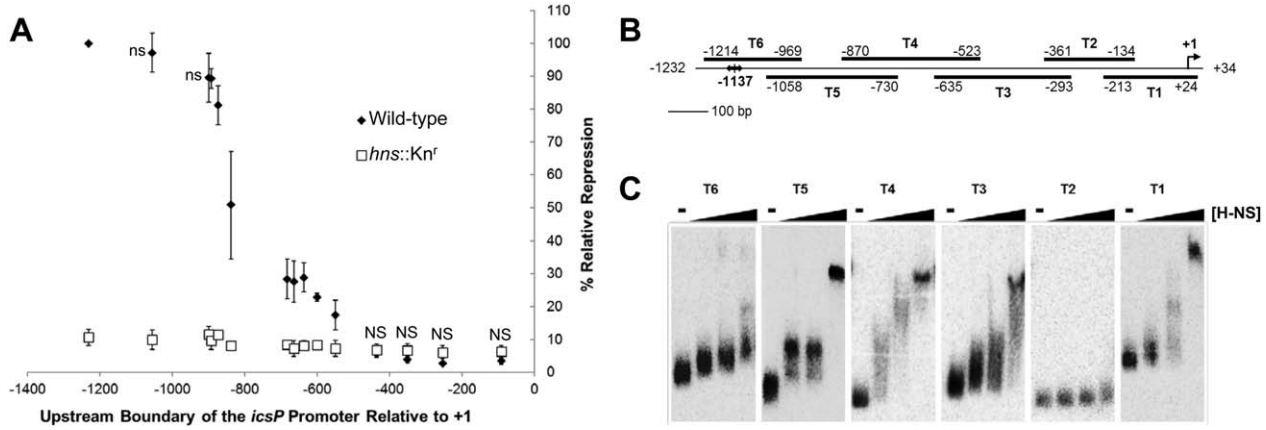


Fig. 1. Precise mapping of DNA sequences required for H-NS-mediated silencing of P*icsP* are capable of binding H-NS.
A. Activities of the P*icsP-lacZ* and 5′ truncation derivatives in wild-type *E. coli* MC4100 and its isogenic *hns*::Kn^r mutant. β-Galactosidase activities are expressed as a percentage of repression exhibited by the full-length P*icsP-lacZ* in wild-type MC4100. Upstream boundary of constructs relative to the primary TSS (+1) (Hensley *et al.*, 2011) are listed from left to right: −1232 (full-length), −1056 bp, −900 bp, −893 bp, −874 bp, −838 bp, −683 bp, −665 bp, −637 bp, −601 bp, −550 bp, −436 bp, −351 bp, −254 bp and −92 bp. All data generated in the wild-type (MC4100) background, except those annotated ns, are statistically different from the full-length promoter ($P < 0.001$). All data generated in the *hns*::Kn^r mutant, except those annotated NS, are statistically different from the identical construct measured in the wild-type strain ($P < 0.05$).
B. Schematic of the six DNA targets (T1–T6) used in EMSAs to identify H-NS binding regions. All coordinates given are relative to the primary *icsP* TSS (Hensley *et al.*, 2011). Inverted arrows represent the VirB boxes essential for VirB-dependent regulation (Castellanos *et al.*, 2009).
C. *In vitro* binding of purified, recombinant H-NS-His$_6$ to six DNA targets (T1–T6) as determined by EMSAs. Each radiolabeled target was incubated with final concentrations of 0, 10.7, 13.4 or 16.1 μM (for each panel, lanes 1–4, respectively) of H-NS-His$_6$. DNA and the resulting nucleoprotein complexes were separated by polyacrylamide gel electrophoresis.

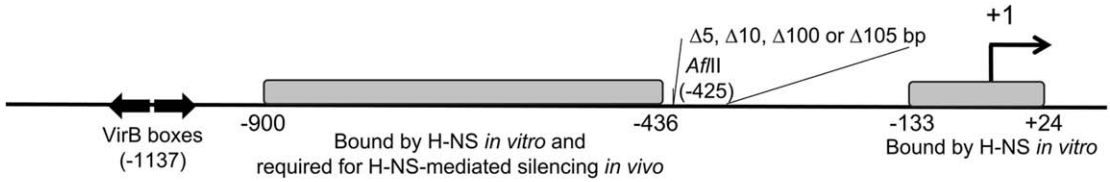
required for full silencing of the *icsP* promoter, with partial silencing observed when sequences up to −665 were present (Castellanos *et al.*, 2009). However, it was unclear if this silencing was mediated by H-NS, and the sequences involved needed to be more precisely mapped. To address this, a 5′ truncation series of the *icsP* intergenic region was constructed, extending as far as −92 (Egile *et al.*, 1997; Hensley *et al.*, 2011). Subsequently, each DNA fragment was introduced into our P*icsP-lacZ* transcriptional reporter pAFW04 (Basta *et al.*, 2013), and the resulting constructs were assayed for β-galactosidase activity in the *Escherichia coli* strain MC4100 and an isogenic mutant lacking a functional *hns* allele (MC4100 *hns*::Kn^r). These strains have been routinely used to investigate the role of H-NS in the regulation of *Shigella* promoters (Beloin and Dorman, 2003; Wing *et al.*, 2004; Basta *et al.*, 2013; Picker and Wing, 2016) because they avoid the genetic instability routinely exhibited by *Shigella hns* mutants (Schuch and Maurelli, 1997) and eliminate interference arising from the dysregulation of *virB* in the absence of *hns* (Tobe *et al.*, 1993; Falconi *et al.*, 1998). Our data show that DNA sequences located between −900 and −436 are needed for H-NS-mediated silencing of the *icsP* promoter *in vivo* (Fig. 1A). In wild-type cells, 90% repression was observed when this region is present in its entirety, and 5′ truncations from −436 to −92 displayed repression levels similar to those observed in the *hns* mutant. These data raise the possibility that H-NS binds throughout this remote region to confer direct H-NS-mediated silencing of the *icsP* promoter.

H-NS directly binds to two discrete regions upstream of the icsP *gene*

To determine if H-NS binds directly to the DNA located upstream of the *icsP* gene, electrophoretic mobility shift assays (EMSAs) were used. The full intergenic region upstream of *icsP* (1.2 kb) was divided into six nearly equal length DNA targets (Fig. 1B). Each radiolabeled target was incubated with increasing concentrations of purified, His-tagged H-NS, and the resulting DNA-protein complexes were resolved by polyacrylamide gel electrophoresis (Fig. 1C). Because H-NS oligomerizes along DNA (Lang *et al.*, 2007), H-NS-DNA complexes do not appear as discrete shifted bands in EMSAs, but instead, appear more diffuse (Azam and Ishihama, 1999; Doyle *et al.*, 2007). Nonetheless, we fully expected that H-NS would interact with some of the six DNA fragments at lower concentrations than others.

H-NS caused pronounced shifts of T4 and T5 at the lowest concentration used (10.7 μM; Fig. 1C), although moderate shifts of T1, T3 and T6 were also observed with this concentration. The only targets, however, to solely exhibit discrete shifts at the highest concentration of H-NS (16.1 μM) were T1, T4 and T5 (T3 showed a

A



B

	PicsP-lacZ construct					
	Full-length	Δ5	Δ10	Δ100	Δ105	Promoterless *lacZ*
Average Fold Change in Activity (*hns* mutant/WT)	12.48 ± 2.07	12.48 ± 4.57	12.07 ± 3.41	11.6 ± 4.81	12.03 ± 3.86	1.07 ± 0.26*

Fig. 2. Helical phasing and spacing requirements between H-NS binding regions at the *icsP* promoter.
A. Schematic showing the size and location of deletions made in the *icsP* promoter region relative to key regulatory elements and regions (not drawn to scale).
B. Effect of deletions between the two regions binding H-NS *in vitro*. Fold change in promoter activity of the P*icsP-lacZ* reporter and deletion derivatives when compared in the *hns*::Kn^r mutant and wild-type backgrounds. The asterisk represents data that are statistically different from the fold change observed with full-length P*icsP-lacZ*, ($P < 0.05$).

mixture of discrete and non-discrete shifts). In contrast, T2 failed to shift even in the presence of the highest H-NS concentration used (16.1 μM). Taken together, these data are consistent with our 5' truncation analysis because the sequences bound by H-NS *in vitro* (Fig. 1B and C; T3–T5) overlap with those required for H-NS-mediated silencing *in vivo* (Fig. 1A; −900 to −436), thus supporting our hypothesis that H-NS directly binds to the *icsP* intergenic region to silence the *icsP* promoter. Strikingly, these data also reveal that T1 displays high affinity for H-NS *in vitro*, a region demonstrated not to be sufficient for H-NS mediated silencing of *icsP* (Fig. 1A). Notably, this DNA target contains *icsP* promoter elements and has the highest AT content of all six targets used in our EMSAs (70% AT-rich), which likely contributes to its affinity for H-NS (Williams and Rimsky, 1997; Navarre *et al*., 2006; Bouffartigues *et al*., 2007).

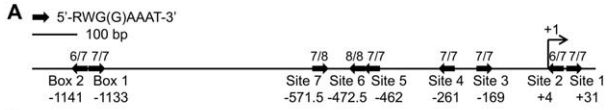
Spacing and helical phasing requirements between the two discrete regions displaying high affinity for H-NS in vitro

The presence of two discrete H-NS binding regions (T5–T3 and T1) identified by our EMSAs suggests that H-NS silences the *icsP* promoter by bridging these two regions of DNA, a demonstrated activity of H-NS (Dame *et al*., 2005). A similar arrangement of H-NS binding regions at the *virF* promoter is crucial for the bridging of two regions by H-NS, resulting in transcriptional silencing (Falconi *et al*., 1998; Prosseda *et al*., 2004). At this locus, bridging can be disrupted by changing the helical phasing of the two H-NS binding regions with respect to each other (Prosseda *et al*., 2004). To determine if H-NS-mediated silencing of the *icsP* promoter is also affected by the spacing between and/or helical orientation of the regions bound by H-NS, four derivatives of the P*icsP-lacZ* construct (pAFW04) were created. Each construct, containing either a change in a half helical turn (5 or 100 bp deletion) or full helical turn (10 or 105 bp deletion) immediately downstream of the *Afl*II site at position −425 (Fig. 2A), was introduced into MC4100 and the isogenic MC4100 *hns* mutant strain, and β-galactosidase activities were measured.

Regardless of the deletion tested, the average fold change in β-galactosidase activity between the *hns* mutant strain and wild-type remained approximately 12-fold (Fig. 2B), demonstrating that none of these deletions affected H-NS-mediated silencing of the *icsP* promoter. Thus, we conclude that, unlike the *virF* promoter, the spacing and helical phasing of the *icsP* regulatory elements with respect to each other can be altered without affecting H-NS-mediated silencing.

Seven sites matching the proposed VirB binding site do not significantly contribute to VirB-dependent regulation in vivo

Having mapped the DNA sequences required for H-NS-mediated silencing of the *icsP* promoter, we next turned our attention to the sequences required for VirB-dependent regulation. Two putative VirB binding sites (known as boxes 1 and 2), each matching the proposed recognition site (Taniya *et al*., 2003; Turner and Dorman, 2007), are required for the regulation of the *icsP* promoter by VirB *in vivo* (Castellanos *et al*., 2009). Notably, these boxes are organized as an inverted repeat and are positioned over 1 kb upstream of the primary *icsP* TSS (Egile *et al*., 1997; Hensley *et al*., 2011).



B

	Promoter Description	Average Relative β-Galactosidase Activity (%)		Average Fold Change
		WT *S. flexneri*	*virB* mutant	
	P*icsP-lacZ*	100 ± 0	6 ± 0*	15 ± 1
Mutated Site(s)	Boxes 1 and 2	7 ± 2*	8 ± 1*	1 ± 0^
	Box 2	6 ± 1*	7 ± 1*	1 ± 0^
	Box 1	5 ± 1*	7 ± 1*	1 ± 0^
	Site 7	97 ± 33	6 ± 1*	16 ± 3
	Site 6	96 ± 34	6 ± 1*	14 ± 3
	Site 5	92 ± 30	6 ± 1*	14 ± 4
	Site 4	93 ± 26	7 ± 1*	14 ± 2
	Site 3	114 ± 21	9 ± 1*	14 ± 3
	Site 2	102 ± 15	8 ± 1*	12 ± 3
	Site 1	101 ± 12	7 ± 0*	14 ± 2
	Sites 1-7	85 ± 10	9 ± 0*	9 ± 1
	Promoterless	11 ± 3*	11 ± 1*	1 ± 0^

Fig. 3. Investigating the role of seven sites that closely match the proposed VirB binding site *in vivo*.
A. Schematic showing the location of seven sites displaying at least a 6/7 or 7/8 match to the proposed VirB binding site 5′-RWG(G)AAAT-3′ (Taniya *et al.*, 2003). The central base pair of each site is indicated.
B. Activities of P*icsP-lacZ* reporter and derivatives carrying transversion mutations made in each of the seven sites, all seven sites and the previously characterized box 1, box 2 or both. Average β-galactosidase activities are expressed as percentage activity relative to the full-length P*icsP-lacZ* in wild-type *S. flexneri* (2457T). Average fold changes between *S. flexneri* wild-type and isogenic *virB* mutant are also provided. The asterisk represents data that are statistically different from the average relative activity observed in wild-type *S. flexneri* carrying P*icsP-lacZ*. ^, represents data that are statistically different from the fold change observed with full-length P*icsP-lacZ*, ($P < 0.05$).

Interestingly, seven other putative binding sites with at least a 6/7 match to the proposed recognition site were also identified in the intergenic region immediately upstream of the *icsP* gene ([Castellanos *et al.*, 2009]; Fig. 3A), but their contribution to VirB-dependent regulation had not been tested.

To assess the involvement of these seven sites in VirB-dependent regulation of the *icsP* promoter, each site was subjected to site-directed mutagenesis using transversion mutations. The eight resulting promoter fragments carrying mutations of each of the seven putative binding sites, or a combination of all seven mutated sites, were cloned upstream of the *lacZ* gene in our transcriptional reporter, pHJW20 (Supporting Information Table S1). The resulting reporter constructs were then introduced into wild-type *S. flexneri* strain 2457T or a *virB* mutant derivative, and β-galactosidase activities were measured.

Mutagenesis of these putative binding sites alone or in combination did not significantly alter the VirB-dependent regulation of the *icsP* promoter (Fig. 3B). In contrast, mutagenesis of either or both of the required boxes 1 and 2 completely abolished VirB-dependent regulation (Fig. 3B), as demonstrated previously (Castellanos *et al.*, 2009). Based on these data, we conclude that the seven sites displaying at least a 6/7 match to the proposed VirB-binding site do not contribute significantly to VirB-dependent regulation of the *icsP* promoter. Interestingly, none of these seven sites were organized as an inverted repeat separated by a single base pair.

VirB binds DNA sites required for VirB-dependent regulation

Next, we chose to study the direct interaction of VirB with the remotely located VirB boxes. To do this, a combination of *in vitro* electrophoretic mobility shift assays and DNase I protection assays were used to test binding of purified His-tagged VirB to radiolabeled DNA products of the *icsP* promoter containing either wild-type or mutated boxes 1 and 2. A 54 bp DNA fragment containing wild-type boxes was retarded when VirB was added at the lowest concentration (0.94 μM), while retardation of an identically sized DNA fragment containing mutated boxes began at the second lowest concentration (1.88 μM) (Fig. 4A). With increasing concentrations of VirB, the formation of discrete VirB-DNA complexes in the middle of the gel, as well as higher order complexes in the gel wells, are evident on the DNA bearing wild-type boxes (Fig. 4A and B panel i). In contrast, only higher order VirB-DNA complexes form with the DNA fragment containing mutated boxes (Fig. 4A and B, panel ii). These data suggest that the wild-type boxes are required for the formation of specific, discrete VirB-DNA complexes and that higher order complexes form through non-specific interactions. We therefore conclude that VirB displays higher binding affinity and specificity for the DNA fragment containing the wild-type boxes than the mutated boxes.

Next, to precisely map sequences protected by VirB at the *icsP* promoter, DNase I protection assays were used. VirB protection was observed between positions −1154 to −1122 (relative to the primary TSS of *icsP*) at the two highest protein concentrations used, 0.62 and 1.2 μM (Fig. 4C, panel i and Supporting Information Fig. S1, panel i). This protection is consistent with the region that contains the VirB boxes required for VirB-dependent regulation of the *icsP* promoter. In contrast, VirB was unable to protect the DNA fragment containing mutated boxes at the concentrations needed to protect the wild-type VirB binding site (Fig. 4C, compare panels i and ii, and Supporting Information Fig. S1). Interestingly, with just a two-fold increase in the concentration of VirB needed to protect the wild-type boxes (0.77–1.54 μM), an extended region of protection was observed in

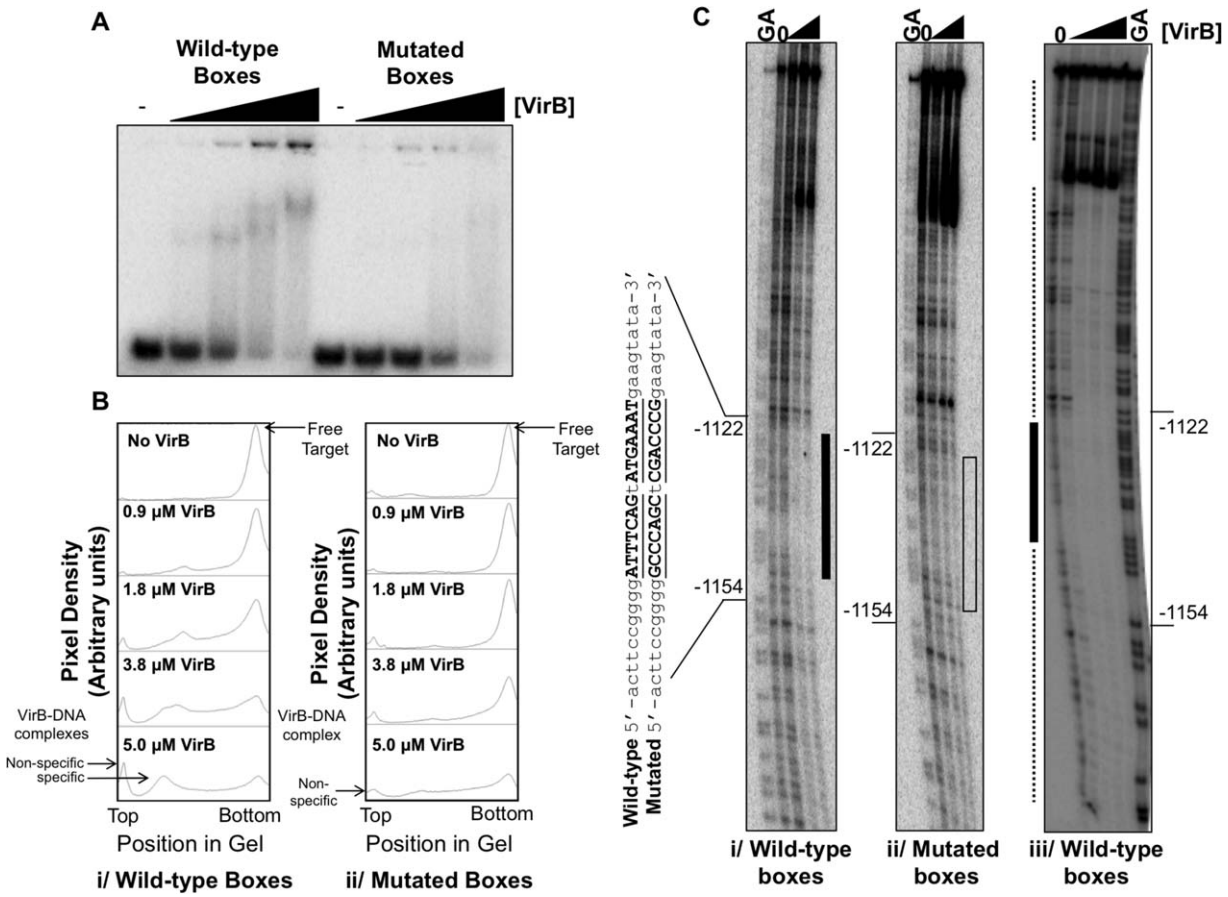


Fig. 4. *In vitro* binding of VirB to DNA sites required for VirB-dependent regulation.
A. EMSA of recombinant VirB-His_6 with 54 bp DNA targets carrying either centrally located wild-type boxes or mutated boxes. Increasing amounts of purified recombinant VirB-His_6 at final concentrations of 0, 0.94, 1.88, 3.75 and 5.00 μM (from left to right) were incubated with radiolabeled DNA.
B. Densitometric trace analysis of each lane in (A). Panels i and ii show traces of DNA targets containing wild-type and mutated boxes respectively. Concentrations of recombinant VirB-His_6 are indicated.
C. DNase I protection analysis of VirB-His_6 bound to a 250 bp DNA fragment carrying either wild-type boxes or mutated boxes (coding strand radiolabeled). Panels i and ii show sequencing gels of DNA products after DNase I cleavage of complexes formed with increasing final concentrations of VirB-His_6 (0, 0.31, 0.62 and 1.2 μM) on DNA bearing wild-type boxes and mutated boxes respectively. Panel iii shows a similar gel of DNA products after DNase I cleavage of complexes formed on DNA bearing wild-type boxes with increasing final concentrations of VirB-His_6 (0, 0.77, 1.54, 3.08 and 4.62 μM). A Maxam-Gilbert ladder (GA) was used to identify coordinates relative to the primary *icsP* TSS. The solid bars indicate initial regions of protection by VirB, the open bar highlights lack of protection observed on DNA bearing mutated boxes, and the dotted line represents an extended region of protection observed.

both directions away from the initially protected site (Fig. 4C, panel iii). These findings are consistent with the oligomerization of VirB along the DNA. Cumulatively, these data indicate that VirB specifically binds to the remote boxes required for VirB-dependent regulation of the *icsP* promoter, and may oligomerize along DNA bi-directionally from these sites.

The remote VirB binding sites are cis*-acting elements needed for VirB-dependent regulation*

Since it is unusual for bacterial transcriptional regulators to modulate transcription from remotely located DNA sites (>1 kb upstream of the regulated promoter), we next chose to investigate how these remote VirB binding sites were used to modulate *icsP* promoter activity. To start, we sought to determine if the binding sites function as bona fide, remote *cis*-acting elements necessary for the transcriptional regulation of *icsP* or if these sites function *in trans* by controlling the production of a small protein or sRNA that subsequently regulates *icsP* promoter activity.

Two existing full-length P*icsP-lacZ* reporter constructs (Supporting Information Table S1) bearing either wild-type VirB binding sites (pHJW20) or mutated sites (pMIC18) (Castellanos *et al.*, 2009) were introduced into wild-type *S. flexneri* and an isogenic *virB* mutant. Subsequently, pBR322 or derivatives carrying either DNA

Table 1. Effect of inserting base pairs between the two halves of the VirB binding site.

	Average relative β-galactosidase activity (%)	
	Wild-type *S. flexneri* (2457T)	*virB* mutant (AWY3)
P*icsP-lacZ*	100	6 ± 1*
2 bp insertion	8 ± 1*	6 ± 1*
3 bp insertion	7 ± 1*	6 ± 1*
4 bp insertion	7 ± 1*	7 ± 1*
Promoterless *lacZ*	3 ± 0*	6 ± 1*

All data are statistically different (*t*-test) from the average relative activity observed in wild-type *S. flexneri* carrying P*icsP-lacZ*, ($P < 0.05$).

sequences upstream of −665 (pKLP09) or upstream of −255 (pADK05) relative to the primary TSS of the *icsP* promoter was also introduced. The activities of the *lacZ* reporters were then measured using β-galactosidase assays, and data were expressed as fold change in the VirB-dependent regulation of the *lacZ* reporter (Supporting Information Fig. S2). Our data show that the VirB binding sites and the downstream sequences contained in pKLP09 or pADK05 do not significantly influence VirB-dependent regulation of the P*icsP-lacZ* reporter bearing mutated sites when placed *in trans* (Supporting Information Fig. S2). Consequently, we conclude that the VirB binding sites found in the *icsP* intergenic region function as remote, *cis*-acting elements that are required for VirB-dependent regulation of *icsP*.

The VirB boxes function as a single cis*-acting element*

The finding that mutagenesis of either box 1 or box 2 resulted in a complete loss of VirB-dependent regulation ([Castellanos *et al*., 2009]; Fig. 3B) raised the possibility that, rather than functioning as two distinct binding sites, the two boxes actually function together as a single *cis*-acting element. To test this, base pair insertions (2, 3 and 4 bp) were made in between the two binding sites ordinarily organized as a near-perfect inverted repeat separated by a single base pair. Three promoter fragments bearing these insertions were then introduced into the P*icsP-lacZ* transcriptional reporter (pHJW20), and β-galactosidase activities were measured in wild-type *S. flexneri* and an isogenic *virB* mutant (Table 1).

Insertion of two, three or four base pairs between the two sites abolished VirB-dependent activity of the *icsP* promoter (Table 1). Thus, in combination with the previous data presented in this work (Fig. 4, Supporting Information Figs S1 and S2), and in contrast to the site that regulates the *icsB* promoter (Turner and Dorman, 2007), we conclude that the remote VirB binding site that regulates the *icsP* promoter functions as a single *cis*-acting element comprised of a near-perfect inverted repeat separated by a single base pair. Based on these findings, it is likely that VirB binds to this site as a dimer.

Plasticity of the spacing and helical phasing of the VirB binding site with respect to the promoter-distal H-NS binding region

An earlier study revealed that a DNA fragment carrying a VirB binding site transplanted upstream of a H-NS binding region in the *E. coli proU* promoter allowed VirB to relieve silencing of this promoter by H-NS (Kane and Dorman, 2011). This was striking not only because VirB is not naturally produced by *E. coli*, but also because changes in the spacing of the VirB binding site relative to the H-NS binding region did not alter VirB-dependent regulation at this artificial *proU* promoter construct. To investigate these requirements at the naturally occurring *icsP* promoter, three deletions of increasing size (Δ5, Δ10 and Δ50 bp) were created (upstream of −1038) between the VirB binding site and downstream sequences, including the promoter-distal region required for H-NS-mediated silencing and the promoter elements (Supporting Information Fig. S3A). Each of the resulting constructs were then introduced into wild-type *S. flexneri* and an isogenic *virB* mutant, and β-galactosidase activities were measured (Supporting Information Fig. S3B). None of the deletions compromised VirB-dependent regulation of the *icsP* promoter (Supporting Information Fig. S3B). Furthermore, because the 5 bp deletion places the VirB binding site on the opposite face of the DNA helix with respect to the downstream H-NS binding region and promoter elements, we conclude that the helical phasing of the VirB binding site relative to these features is not critical for its role as a transcriptional anti-silencer of this locus.

A molecular roadblock, LacI, placed between the VirB binding site and the upstream region bound by H-NS blocks VirB-mediated anti-silencing

To further probe the relationship between the VirB binding site and the promoter-distal H-NS binding region, we took inspiration from a study that investigated the oligomerization along DNA of ParB, the closest homologue of VirB, at the P1 plasmid centromere (Rodionov *et al*., 1999). In that study, oligomerization of ParB along DNA was blocked by docking a DNA binding protein to its introduced binding site, which was positioned close to, but not overlapping, the initial ParB binding site. Like ParB, VirB is known to form oligomers *in vivo* (Beloin *et al*., 2002) and in addition, our DNase I protection

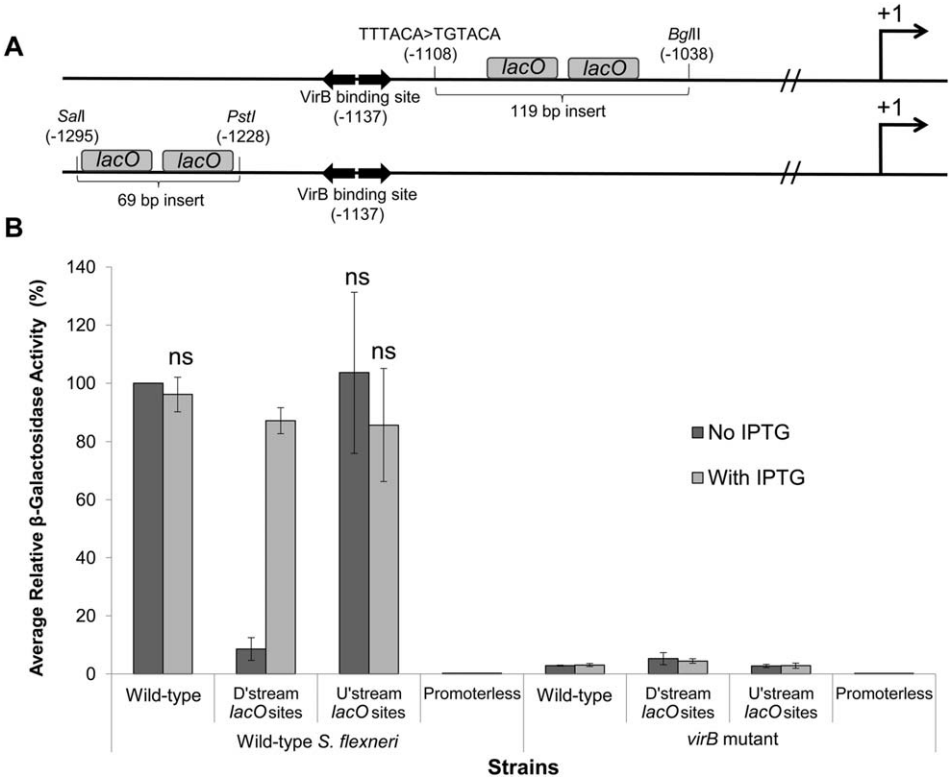


Fig. 5. A LacI molecular roadblock placed downstream, but not upstream, of the essential VirB binding site abolishes VirB-dependent anti-silencing of the *icsP* promoter.
A. Schematic showing the inserts bearing tandem *lacO* sites located either 87 bp upstream or 80 bp downstream of the VirB binding site (not drawn to scale). Double forward slashes represent intergenic sequences not drawn.
B. Effect of *lacO* sites in the presence or absence of LacI on VirB-dependent regulation of the *icsP* promoter. Average β-galactosidase activities are expressed as percent activity relative to the full-length P*icsP-lacZ* in wild-type *S. flexneri* (2457T). All data, except those labeled ns, are statistically significant from the average relative activity observed in wild-type *S. flexneri* carrying the wild-type P*icsP-lacZ* reporter in the absence of IPTG ($P < 0.05$).

assays suggest VirB oligomerizes bi-directionally from the VirB binding site (Fig. 4B, panel iii). We therefore reasoned that VirB binding to its recognition site is likely followed by its oligomerization along DNA, and that VirB oligomerization towards the promoter-distal H-NS binding region may be key for the transcriptional anti-silencing of the *icsP* promoter.

To test this hypothesis, two constructs were created that placed two tandem *lacO* sites approximately 80 bp either upstream or downstream of the *cis*-acting VirB binding site (Fig. 5A). The use of pQE2, a medium-copy plasmid that expresses *lacI*^{q}, ensured LacI production and LacI binding to the *lacO* recognition sites in the absence of IPTG and its dissociation in the presence of IPTG. With these constructs, the effect of a DNA binding protein acting as a molecular roadblock, positioned either on the upstream or downstream flank of the VirB binding site, was tested in the context of our P*icsP-lacZ* reporter, pAFW04.

VirB-dependent regulation of the *icsP* promoter was not affected when the tandem *lacO* sites were positioned upstream of the remote VirB binding site, regardless of whether IPTG was present or absent from the growth medium (Fig. 5B). These data indicate that VirB-dependent regulation of the *icsP* promoter is neither impacted by the inserted *lacO* sites nor LacI binding to these sites. Because VirB-dependent regulation was still observed in the absence of IPTG, conditions supporting LacI binding, we conclude that the 87 bp between the LacI binding sites and the VirB binding site provides sufficient room for both proteins to simultaneously dock at their recognition sites.

In the construct where the tandem *lacO* sites were placed 80 bp downstream of the VirB binding site, a modest decrease (less than 1.2-fold effect) in VirB-dependent promoter activity was observed in the presence of IPTG (i.e., absence of bound LacI) when compared to the wild-type P*icsP-lacZ* reporter. In contrast, a dramatic decrease in VirB-dependent regulation was detected in the absence of IPTG (i.e., presence of bound LacI; 17-fold effect), reducing promoter activity to levels similar to those observed in the *virB* mutant (Fig. 5B). These data demonstrate that a LacI molecular roadblock located downstream of the VirB binding site significantly interferes with VirB-dependent regulation of the *icsP* promoter. In combination with our results from DNase I protection assays (Fig. 4B, panel iii), these data support our current hypothesis that VirB oligomerization along DNA toward, but not away from, the region required for H-NS-mediated silencing is required for VirB-dependent transcriptional anti-silencing of the *icsP* promoter. As such, these data provide important insight into transcriptional anti-silencing by VirB.

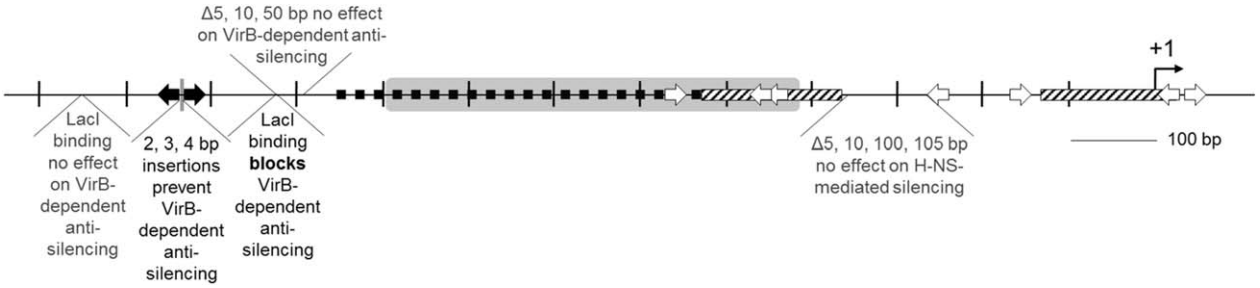


Fig. 6. Schematic of findings presented in this study. The *icsP* intergenic region is depicted. The flexibility of regulatory elements is highlighted by experiments described in grey text. Precise requirements or major effects on regulation of the *icsP* promoter by VirB are highlighted by experiments described in black text. Black dotted line depicts the high affinity H-NS binding region. Hatched boxes depict the lower affinity H-NS binding regions. Dark grey shaded area depicts the region required for H-NS-mediated silencing of P*icsP in vivo*. Light grey shaded box depicts the sequences required for VirB-dependent regulation *in vivo*. Black inverted arrows depict the single, remote, *cis*-acting VirB binding site required for VirB-dependent transcriptional anti-silencing of the *icsP* promoter. White arrows depict sequences strongly matching the proposed VirB binding site (Taniya *et al*., 2003) that do not contribute to VirB-dependent regulation of the *icsP* promoter.

Discussion

The xenogeneic silencing and anti-silencing of virulence genes has had a profound effect on the evolution of many bacterial pathogens and continues to play a key role in controlling the pathogenicity of these organisms (Stoebel *et al*., 2008; Ali *et al*., 2012; Marteyn *et al*., 2012; Picker and Wing, 2016). Many virulence genes on the *Shigella* virulence plasmid are transcriptionally silenced by H-NS and anti-silenced by VirB (Porter and Dorman, 1997; Beloin and Dorman, 2003; Le Gall *et al*., 2005; Turner and Dorman, 2007; Picker and Wing, 2016; Weatherspoon-Griffin *et al*., 2016). Despite VirB being a key regulator of *Shigella* virulence, very little is known about how VirB functions to offset transcriptional silencing mediated by H-NS (Turner and Dorman, 2007). To help address this gap in knowledge, in this study, we thoroughly mapped the genetic elements necessary for transcriptional silencing and anti-silencing of the *icsP* locus located on the large *Shigella* virulence plasmid. Our work demonstrates the necessity of remotely located DNA binding sites while highlighting the flexibility of key regulatory elements required for transcriptional silencing and anti-silencing at this genetic locus. Moreover, our work demonstrates that a molecular roadblock placed between the key regulatory elements (i.e., the VirB binding site and the H-NS-bound region) completely blocks transcriptional anti-silencing by VirB. Finally, our findings support the need to re-evaluate the currently proposed VirB binding site (Taniya *et al*., 2003; Turner and Dorman, 2007). Based on our findings, a model of transcriptional silencing and anti-silencing and a summary diagram of regulatory elements identified by this work are presented in the graphical abstract and Fig. 6, respectively.

Our study reveals that a stretch of DNA located between 900 and 436 bp upstream of the primary *icsP* TSS (AT content 68%) is required for H-NS-mediated transcriptional silencing of *icsP*. This is corroborated by our EMSAs because three of the six DNA targets (targets 3, 4 and 5), each containing at least part of this region, bind H-NS *in vitro* with relatively high affinity. Strikingly, a 100 bp, 79% AT-rich region located in targets 4 and 5 (–844 to −744) contains a 20 bp, 95% AT-rich stretch starting at −782. Taken together, the DNA sequences identified by our *in vivo* 5' truncation analysis and EMSAs are consistent with established binding characteristics of H-NS (Navarre *et al*., 2006; Bouffartigues *et al*., 2007; Lang *et al*., 2007). Another AT-rich region (79%; −133 to +24; found in target 1) was found to display relatively high affinity for H-NS in our EMSAs. The binding of H-NS to this region, which contains *icsP* promoter elements, is consistent with other findings that demonstrate H-NS commonly binds to bacterial promoter regions *in vivo* (Landick *et al*., 2015). Nonetheless, our 5' truncation analysis of the *icsP* promoter region reveals that the promoter-proximal sequences alone are not sufficient for H-NS mediated silencing, bringing into question their role in this regulatory process.

Based on previous work (Basta *et al*., 2013) and data presented here, a repression loop involving the H-NS bound promoter-distal and promoter-proximal sites does not seem likely for three reasons. First, removal of the upstream H-NS binding region causes a complete loss of H-NS-mediated silencing (Fig. 1A). So, unless the upstream region functions to stabilize the H-NS interaction with the promoter region, a mechanism of promoter occlusion that directly involves the promoter region seems unlikely. Secondly, analysis of the *ospZ* promoter, which lies divergent to the *icsP* promoter, revealed that there was no requirement for *icsP* promoter-proximal sequences for H-NS-mediated silencing (Basta *et al*.,

2013). Instead, DNA sequences overlapping the region necessary for H-NS-mediated silencing of *icsP* were required (Basta *et al*., 2013). Finally, both small and large deletions that place the two regions that bind H-NS *in vitro* on the same or opposite faces of the DNA helix do not impact H-NS-mediated silencing (Fig. 2B), suggesting that if a H-NS bridge complex does form, it must tolerate these spatial changes. As such, we favor a model of transcriptional silencing where H-NS forms a nucleoprotein filament along the *icsP* intergenic region from the upstream H-NS binding region identified by this study. Future studies aimed at fully characterizing the nature of the H-NS-DNA complex that forms at the *icsP* promoter, as well as identifying which step in transcription is inhibited by H-NS, are underway.

Regarding VirB-dependent regulation of the *icsP* promoter, our previous work had shown that remote sites located over 1 kb upstream of the *icsP* promoter were required for VirB-dependent regulation (Castellanos *et al*., 2009). This same study identified seven additional sites further downstream, each displaying at least a 6/7 match to the proposed VirB binding site (Taniya *et al*., 2003). However, despite being closer to the *icsP* gene than the required VirB binding site (Fig. 3A), none of these sites contribute to VirB-dependent regulation (Fig. 3B). This finding not only validates our previous result that VirB regulates transcription from a remote site, but also challenges our understanding of the proposed VirB binding site sequence; clearly not all sites resembling the currently proposed site are involved in VirB-dependent regulation of *icsP* (Taniya *et al*., 2003; Turner and Dorman, 2007). When we expanded our search on the large virulence plasmid for the previously proposed 7–8 bp VirB binding site (Taniya *et al*., 2003), ~250 perfect matches were identified, and this number increases to ~4000 if a single base pair mismatch is tolerated. As such, it seems highly unlikely that all of these sites are involved in VirB binding and VirB-dependent regulation of *Shigella* virulence genes. Due to the functional differences in the VirB binding sites found at the *icsB* (Turner and Dorman, 2007) and *icsP* promoters highlighted by this study, it is clear that there is a pressing need to improve our understanding of VirB-DNA interactions on the virulence plasmid *in vivo*. Further, it will be important to determine which of these DNA-protein interactions are needed for the anti-silencing of virulence genes in this important human pathogen.

It is unusual for bacterial transcription factors to influence promoter activity from more than 250 bp upstream of a bacterial promoter (Collado-Vides *et al*., 1991), and yet, the essential *cis*-acting VirB binding site at the *icsP* locus is centered at −1137 relative to the primary *icsP* TSS. This location places the VirB binding site only 237 bp upstream of the region required for H-NS-mediated silencing (Fig. 1B). Our data show that small or large deletions that maintain or alter the helical phasing of these regulatory elements do not impact VirB-dependent transcriptional anti-silencing of the *icsP* promoter (Supporting Information Fig. S3). These findings demonstrate a level of flexibility inherent to transcriptional anti-silencing complexes, a finding consistent with previous work (Kane and Dorman, 2011). Given our work reveals that VirB oligomerizes along DNA *in vitro* (Fig. 4C), it is possible that these extended VirB-DNA interactions facilitate this flexibility. Going forward, it would be interesting to probe the limits of this apparent spatial flexibility.

New insights into the VirB anti-silencing mechanism come from our study where a molecular roadblock was introduced on either side of the essential VirB binding site. Strikingly, we found that the LacI roadblock only interferes with transcriptional anti-silencing when placed in between the VirB binding site and the region required for H-NS-mediated silencing (Fig. 5B). While our finding is consistent with VirB oligomerizing along the DNA towards the region required for H-NS-mediated silencing, which is also supported by the extended footprints (Fig. 4C, panel iii), it remains unclear if VirB oligomerization along DNA occurs *in vivo* or if this activity is needed for VirB-dependent transcriptional anti-silencing. Future experiments that more thoroughly investigate the mechanism of transcriptional anti-silencing by VirB *in vivo* are in progress.

In summary, we have: (i) identified that a remote region is required for H-NS-mediated silencing of *icsP*, (ii) demonstrated that VirB binds directly to a remote, single *cis*-acting site arranged as a near-perfect inverted repeat, (iii) revealed the significant plasticity in the spacing requirements between the two H-NS binding regions as well as the VirB binding site and region required for H-NS-mediated silencing and (iv) determined that a protein docked immediately downstream of the VirB binding site, but upstream of the region required for H-NS-mediated silencing, completely blocks VirB-dependent regulation. More generally, our findings stress the importance of considering the involvement of DNA sequences outside of the canonical promoter region when studying transcription regulation (Collado-Vides *et al*., 1991; Gralla and Collado-Vides, 1996), especially when the regulation is being imparted by NSPs and their counter-silencing proteins. While our work highlights that there can be considerable flexibility in the architecture of nucleoprotein complexes controlling transcriptional silencing and anti-silencing, our work also shows the apparent ease with which transcriptional anti-silencing can be disrupted. This insight may prove useful in the design of new antibacterials that could be broadly applicable because gene regulation mediated by transcriptional silencing and anti-silencing is common in bacteria and central to numerous aspects of bacterial physiology, including virulence.

Table 2. Bacterial strains used in this work.

Strain	Description[a]	Source or reference
E. coli		
MC4100	*E. coli* strain K-12 with *araD* and *lacZ* deletion	(Pogliano and Beckwith, 1994)
MC4100 *hns*	MC4100 *hns*::Kn^r The first 37 amino acids of H-NS are expressed, resulting in a dominant-negative effect on other H-NS-like proteins in the cell.	(Yamada *et al.*, 1991)
S. flexneri		
2457T	*S. flexneri* serotype 2a	(Formal *et al.*, 1958)
AWY3	2457T *virB*::Tn5; Kn^r	(Wing *et al.*, 2004)

[a] Kn^r, kanamycin resistance.

Experimental procedures

Bacterial strains, plasmids and media

The bacterial strains and plasmids used in the present study are listed in Table 2 and Supporting Information Table S1 respectively. *E. coli* strains were grown routinely at 37°C in Luria-Bertani (LB) broth (Miller, 1972) with aeration or on LB agar (LB broth containing 1.5% [w v^{-1}] agar). *Shigella flexneri* strains were routinely grown at 37°C in Tryptic Soy broth (TSB) with aeration or on trypticase soy agar (TSA; TSB containing 1.5% w v^{-1} agar). Where appropriate, antibiotics were added to achieve the following final concentrations: ampicillin, 100 μg ml^{-1}; chloramphenicol, 25 μg ml^{-1} and kanamycin, 50 μg ml^{-1}. To ensure that *Shigella* strains had maintained the large virulence plasmid during manipulation, Congo Red binding was tested on TSA plates containing 0.01% (w v^{-1}) Congo Red (Sigma Chemical Co., St. Louis, Mo.).

Plasmid construction

Plasmids and plasmid constructs used in this study are fully described in the supporting information (Supporting Information Table S1 and Plasmid construction). All constructs had their DNA sequences verified by Sanger sequencing. DNA sequences of oligonucleotide primers and duplexes used in this work are available upon request.

Quantification of icsP promoter activity using the PicsP-lacZ reporter and derivatives

To measure promoter activities, the P*icsP-lacZ* fusion plasmids described in this work were introduced into *S. flexneri* and *E. coli* strains by electroporation. Activities of the *icsP* promoter constructs were determined by measuring β-galactosidase activity as described previously (Wing *et al.*, 2004), using the Miller protocol (Miller, 1972). Overnight cultures were diluted 1:100 and grown for 5–7 h in either TSB medium (*S. flexneri*) or LB (*E. coli*) at 37°C with shaking at 325 rpm (LabLine/Barnstead 4000 MaxQ), prior to cell lysis. Routinely, β-galactosidase levels were measured in early stationary phase cultures grown from three independent transformants because experiments had shown that *icsP* expression significantly increases under these conditions (Hensley *et al.*, 2011). Assays routinely contained three independent biological replicates and were repeated three times. For statistical analyses, a Student's *t*-test assuming equal variance was routinely used.

To assess the effect of LacI binding to *lacO* recognition sites engineered upstream or downstream of the essential VirB binding sites in pAFW04, wild-type *S. flexneri* (2457T) and the isogenic *virB* mutant (AWY3) were simultaneously transformed with pAFW04 or a derivative (pHS27, pDRG01 or MAP07) and the *lacI*^{q}-expressing plasmid, pQE2. Overnight cultures bearing pQE2 and a pAFW04 derivative were back-diluted 1:100 and grown for 2 h at 37°C. Cell cultures were either induced with a final concentration of 250 μM IPTG or not induced and then all cultures were grown for an additional 3 h. Cells were then harvested by centrifugation and resuspended in an equal volume of PBS before lysis. β-galactosidase activities were determined using the Miller protocol (Miller, 1972).

Purification of His-tagged H-NS and VirB proteins

For the purification of C-terminally tagged H-NS-His$_6$ protein, the pQE60 derivative pCTH01 was used. H-NS-His$_6$ was purified as described previously with a few exceptions (Deighan *et al.*, 2003). Briefly, the protein was produced in the *E. coli* strain M15 carrying the plasmid pREP4. The expression of the C-terminally His-tagged proteins were induced in 500 ml cultures growing exponentially with 1 mM IPTG (isopropyl-β-thiogalactopyranoside). After a 2 h induction, the cells were harvested and frozen at −80°C overnight. The cell pellet was then thawed on ice and resuspended in lysis buffer (60 mM imidazole, 500 mM NaCl, 20 mM Tris-HCl [pH 8.0]). Cells were lysed by sonication, and cellular debris was pelleted by centrifugation at 10,000 × g at 4°C. Cell lysates were applied to Ni-NTA columns (Qiagen) pre-equilibrated with lysis buffer. The columns were then washed with 10 bed volumes of wash buffer (100 mM imidazole, 500 mM NaCl, 20 mM Tris-HCl [pH 8.0]). Proteins were eluted by the addition of 2.5 bed volumes of elution buffer (equilibration buffer with 500 mM imidazole). The H-NS-His$_6$ fractions were collected and analyzed on SDS-PAGE followed by Coomassie staining. The H-NS fractions were combined and dialyzed against a storage buffer (350 mM NaCl, 50 mM Tris-HCl [pH 8.0], 1 mM EDTA, 0.5 mM DTT, 0.1 mM PMSF, 30% glycerol). Protein concentration was determined using a Bradford assay. Previous studies using an identical His-tag H-NS fusion protein, produced in a manner similar to that described above, was shown to retain normal function of H-NS in assays (Williams and Rimsky, 1997).

The C-terminally VirB-His$_6$ protein was produced from pAJH03 and purified by Monserate Biotech. The hexa-his tag was not found to interfere with VirB expression or activity because His-tagged VirB was observed to restore *icsP* expression to wild-type levels in a strain lacking *virB in vivo*

(data not shown). SDS-PAGE and western blots of purified proteins used in this work are shown in Supporting Information Fig. S4.

Electrophoretic mobility shift assays

To test H-NS binding to the six DNA fragments taken from the *icsP* intergenic region *in vitro*, 0.25 pmol of ^{32}P-labeled P*icsP* DNA (PCR amplified from pHJW20 with the following primers: W63 and W64 target 1, W65 and W66 target 2, W67 and W68 target 3, W69 and W70 target 4, W71 and W148 target 5, W73 and W74 target 6 and gel purified by electroelution) was incubated at 37°C for 30 min with 0, 107, 134 or 161 pmol of purified His-tagged H-NS protein in a 10 μl reaction containing 50 mM Tris-HCl (pH 8.0), 20 mM KCl, 10% glycerol, 100 μg ml^{-1} BSA and 25 μg ml^{-1} poly (dI-dC). DNA loading dye solution was added to the reaction and directly subjected to 5% polyacrylamide gel electrophoresis (PAGE) in 0.5X TBE running buffer. Radioactive signals were detected using a Typhoon 9410 (Amersham) variable mode imager.

To test VirB binding *in vitro*, two 54 bp *icsP* promoter fragments containing either wild-type or mutated VirB sites were used. To create each target, primer pairs W391/W392 and W393/W394, respectively, were denatured at 95°C for 5 min and subsequently annealed using a cycle that decreased by 1°C every minute until a final temperature of 25°C. Each sample was then gel purified and electroeluted into the surrounding buffer, and the resulting DNA was phenol-chloroform extracted and ethanol precipitated. For radiolabeling of the non-coding strand, 4.8 pmol of each target was single-end labeled using T4 polynucleotide kinase (Promega Cat. No. M4101) according to manufacturer directions using [γ^{32}P] ATP (specific activity, 3000 Ci mmol^{-1}; Perkin Elmer). Unincorporated radionucleotides were removed using Illustra ProbeQuant G-50 Micro Columns according to manufacturer directions (GE Healthcare).

Increasing concentrations of purified VirB-His$_6$ (Monserate Biotech) were incubated with ~0.02 pmol of each *icsP* promoter target at 37°C for 20 min in 1x binding buffer (10 mM potassium phosphate, pH 7.5, 25 mM NaCl, 0.5 mM β-mercaptoethanol, 0.5 mM EDTA, 50% glycerol, 25 ng μl^{-1} herring sperm DNA) in a total reaction volume of 20 μl. The reactions were then resolved on a 6% native polyacrylamide gel in 1x TBE at 120 v for 40 min. The gel was transferred to Whatman paper, covered with plastic wrap, exposed to a phosphor-imaging screen and then scanned with the Typhoon 9410 (Amersham) Variable Mode Imager. For densitometric lane trace analysis, ImageJ software (http://imagej.nih.gov) was used.

DNase I protection assays

DNase I protection assays to identify VirB bound regions of the *icsP* promoter were carried out using PCR amplified DNA fragments. Primers W515 and W516 were used to amplify a 250 bp fragment containing wild-type VirB boxes (amplified using pHJW20 as a template) or mutated VirB boxes (amplified using pMIC18 as a template). Prior to the PCR, one of the primers W515 or W516 was labeled with T4 polynucleotide kinase and [γ-^{32}P]-ATP (^{32}P-labeled W515 allowed detection of the coding strand; ^{32}P-labeled W516 allowed detection of the non-coding strand). To detect the initial binding site of VirB, approximately 0.25 pmol of labeled DNA and 0, 6.2, 12.3 or 24.6 pmol of the purified His-tagged VirB were incubated at 37°C for 20 minutes in a 20 μl reaction containing 50 mM Tris-HCl (pH 8.0), 20 mM KCl, 10% glycerol, 100 μg ml^{-1} BSA and 25 μg ml^{-1} poly (dI-dC). To investigate the potential for VirB oligomerization along DNA, the same conditions were used, but the following amounts of VirB were added; 0, 15.4, 30.8, 61.6 or 92.4 pmol. Samples were treated with 0.06 U of DNase I (New England Biolabs) for 30 seconds followed by phenol-chloroform DNA extraction and ethanol precipitation. DNA was resuspended in a gel loading buffer (40% deionized formamide, 5 M urea, 5 mM NaOH, 1 mM EDTA, 0.025% bromophenol blue and 0.025% xylene cyanol) and analyzed by 6% denaturing PAGE by comparing to an appropriate DNA sequence ladder generated by the Maxam and Gilbert A + G reaction (Maxam and Gilbert, 1986). Radiolabeled DNA fragments were detected using a Typhoon 9410 (Amersham) variable mode imager.

Acknowledgements

We thank David Fujimoto of the Monserate Biotechnology Group for purifying the recombinant VirB protein used in this study and R. Martin Roop, II for guidance with preliminary EMSAs. This work was supported by the National Institute of Allergy and Infectious Diseases of the National Institutes of Health under Award Number R15AI090573 and by NIH grant P20 RR-016464 from INBRE Program of the National Center for Research Resources. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health. MAP was a recipient of a Higher Education Graduate Research Opportunity Fellowship in 2015 from the Nevada Space Grant Consortium (NVSGC) National Aeronautics and Space Administration (NASA) Training Grant #NNX10AN23H and was awarded the Hermsen Fellowship in 2017. RO-D was a recipient of a National Science Foundation (NSF) Research Experience for Undergraduates (REU) scholarship DBI 1005223. PU, MMAK, JCD, MIC were partially supported by the NV INBRE undergraduate research opportunity program (National Institute of General Medical Sciences; 8 P20GM103440-11). CTH was supported in part by the Post-9/11 GI Bill. DJH was a Lieutenant (LT) Medical Service Corps (MSC) United States Navy (USN) microbiologist, NAVMED MPT&E when this work was undertaken. The views expressed in this article are those of the authors and do not necessarily reflect the official policy or position of the Navy, Department of Defense, or U.S. Government. The authors declare no conflicts of interest.

Author contributions

Region required for H-NS-mediated silencing of P*icsP* – MAP, HSP, RO-D, DJH; Spacing between two H-NS binding regions – MAP, KLP; *In vitro* H-NS binding study – NWG, CTH, DJH; Contribution of seven sites matching the proposed VirB binding site – JCD, MIC, PU, MMAK;

Insertions between VirB sites – KLP; Spacing between VirB binding site and region required for H-NS mediated silencing and inverted repeat – KLP; *cis/trans* study – JNS, ADK; Molecular Roadblock – DRG, HSP; Purification of VirB – Monserate Biotechnology Group; *In vitro* VirB binding studies – NWG, MAP, AJH; Writing – HJW, MAP, NWG, JAI; Project management & Funding – HJW.

References

Africa, L.A., Murphy, E.R., Egan, N.R., Wigley, A.F., and Wing, H.J. (2011) The iron-responsive Fur/RyhB regulatory cascade modulates the *Shigella* outer membrane protease IcsP. *Infect Immun* **79**: 4543–4549.

Ali, S.S., Xia, B., Liu, J., and Navarre, W.W. (2012) Silencing of foreign DNA in bacteria. *Curr Opin Microbiol* **15**: 175–181.

Arold, S.T., Leonard, P.G., Parkinson, G.N., and Ladbury, J.E. (2010) H-NS forms a superhelical protein scaffold for DNA condensation. *Proc Natl Acad Sci U S A* **107**: 15728–15732.

Azam, T.A., and Ishihama, A. (1999) Twelve species of the nucleoid-associated protein from *Escherichia coli*. Sequence recognition specificity and DNA binding affinity. *J Biol Chem* **274**: 33105–33113.

Basta, D.W., Pew, K.L., Immak, J.A., Park, H.S., Picker, M.A., Wigley, A.F., *et al*. (2013) Characterization of the *ospZ* promoter in *Shigella flexneri* and its regulation by VirB and H-NS. *J Bacteriol* **195**: 2562–2572.

Beloin, C., and Dorman, C.J. (2003) An extended role for the nucleoid structuring protein H-NS in the virulence gene regulatory cascade of *Shigella flexneri*. *Mol Microbiol* **47**: 825–838.

Beloin, C., McKenna, S., and Dorman, C.J. (2002) Molecular dissection of VirB, a key regulator of the virulence cascade of *Shigella flexneri*. *J Biol Chem* **277**: 15333–15344.

Bouffartigues, E., Buckle, M., Badaut, C., Travers, A., and Rimsky, S. (2007) H-NS cooperative binding to high-affinity sites in a regulatory element results in transcriptional silencing. *Nat Struct Mol Biol* **14**: 441–448.

Castellanos, M.I., Harrison, D.J., Smith, J.M., Labahn, S.K., Levy, K.M., and Wing, H.J. (2009) VirB alleviates H-NS repression of the *icsP* promoter in *Shigella flexneri* from sites over 1 kb upstream of the transcription start site. *J Bacteriol* **191**: 4047–4050.

Collado-Vides, J., Magasanik, B., and Gralla, J.D. (1991) Control site location and transcriptional regulation in *Escherichia coli*. *Microbiol Rev* **55**: 371–394.

Dame, R.T., Luijsterburg, M.S., Krin, E., Bertin, P.N., Wagner, R., and Wuite, G.J. (2005) DNA bridging: a property shared among H-NS-like proteins. *J Bacteriol* **187**: 1845–1848.

Dame, R.T., Noom, M.C., and Wuite, G.J. (2006) Bacterial chromatin organization by H-NS protein unravelled using dual DNA manipulation. *Nature* **444**: 387–390.

Dame, R.T., Wyman, C., and Goosen, N. (2000) H-NS mediated compaction of DNA visualised by atomic force microscopy. *Nucleic Acids Res* **28**: 3504–3510.

Deighan, P., Beloin, C., and Dorman, C.J. (2003) Three-way interactions among the Sfh, StpA and H-NS nucleoid-structuring proteins of *Shigella flexneri* 2a strain 2457T. *Mol Microbiol* **48**: 1401–1416.

Dorman, C.J. (2007) H-NS, the genome sentinel. *Nat Rev Microbiol* **5**: 157–161.

Dorman, C.J., and Deighan, P. (2003) Regulation of gene expression by histone-like proteins in bacteria. *Curr Opin Genet Dev* **13**: 179–184.

Doyle, M., Fookes, M., Ivens, A., Mangan, M.W., Wain, J., and Dorman, C.J. (2007) An H-NS-like stealth protein aids horizontal DNA transmission in bacteria. *Science* **315**: 251–252.

Egile, C., d'Hauteville, H., Parsot, C., and Sansonetti, P.J. (1997) SopA, the outer membrane protease responsible for polar localization of IcsA in *Shigella flexneri*. *Mol Microbiol* **23**: 1063–1073.

Falconi, M., Colonna, B., Prosseda, G., Micheli, G., and Gualerzi, C.O. (1998) Thermoregulation of *Shigella* and *Escherichia coli* EIEC pathogenicity. A temperature-dependent structural transition of DNA modulates accessibility of *virF* promoter to transcriptional repressor H-NS. *Embo J* **17**: 7033–7043.

Fang, F.C., and Rimsky, S. (2008) New insights into transcriptional regulation by H-NS. *Curr Opin Microbiol* **11**: 113–120.

Formal, S.B., Dammin, G.J., LaBrec, E.H., and Schneider, H. (1958) Experimental *Shigella* infections: characteristics of a fatal infection produced in guinea pigs. *J Bacteriol* **75**: 604–610.

Gao, X., Zou, T., Mu, Z., Qin, B., Yang, J., Waltersperger, S., *et al*. (2013) Structural insights into VirB-DNA complexes reveal mechanism of transcriptional activation of virulence genes. *Nucleic Acids Res* **41**: 10529–10541.

Gordon, B.R., Li, Y., Cote, A., Weirauch, M.T., Ding, P., Hughes, T.R., *et al*. (2011) Structural basis for recognition of AT-rich DNA by unrelated xenogeneic silencing proteins. *Proc Natl Acad Sci U S A* **108**: 10690–10695.

Gralla, J.D., and J. Collado-Vides, (1996) Organization and function of transcription regulatory elements. In: *E. coli and Salmonella*. F. Neidhardt (ed). Washington, DC: ASM Press, pp. 1232–1245.

Hensley, C.T., Kamneva, O.K., Levy, K.M., Labahn, S.K., Africa, L.A., and Wing, H.J. (2011) Two promoters and two translation start sites control the expression of the *Shigella flexneri* outer membrane protease IcsP. *Arch Microbiol* **193**: 263–274.

Hromockyj, A.E., and Maurelli, A.T. (1989) Identification of an *Escherichia coli* gene homologous to *virR*, a regulator of *Shigella* virulence. *J Bacteriol* **171**: 2879–2881.

Kane, K.A., and Dorman, C.J. (2011) Rational design of an artificial genetic switch: Co-option of the H-NS-repressed *proU* operon by the VirB virulence master regulator. *J Bacteriol* **193**: 5950–5960.

Landick, R., Wade, J.T., and Grainger, D.C. (2015) H-NS and RNA polymerase: a love-hate relationship? *Curr Opin Microbiol* **24**: 53–59.

Lang, B., Blot, N., Bouffartigues, E., Buckle, M., Geertz, M., Gualerzi, C.O., *et al*. (2007) High-affinity DNA binding sites for H-NS provide a molecular basis for selective

silencing within proteobacterial genomes. *Nucleic Acids Res* **35**: 6330–6337.
Le Gall, T., Mavris, M., Martino, M.C., Bernardini, M.L., Denamur, E., and Parsot, C. (2005) Analysis of virulence plasmid gene expression defines three classes of effectors in the type III secretion system of *Shigella flexneri*. *Microbiology* **151**: 951–962.
Marteyn, B., Gazi, A., and Sansonetti, P. (2012) *Shigella*: a model of virulence regulation *in vivo*. *Gut Microbes* **3**: 104–120.
Maxam, A.M., and Gilbert, W. (1986) A method for determining DNA sequence by labeling the end of the molecule and cleaving at the base. Isolation of DNA fragments, end-labeling, cleavage, electrophoresis in polyacrylamide gel and analysis of results. *Mol Biol* **20**: 581–638.
Miller, J. (1972) *Experiments in Molecular Genetics*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.
Navarre, W.W., McClelland, M., Libby, S.J., and Fang, F.C. (2007) Silencing of xenogeneic DNA by H-NS-facilitation of lateral gene transfer in bacteria by a defense system that recognizes foreign DNA. *Genes Dev* **21**: 1456–1471.
Navarre, W.W., Porwollik, S., Wang, Y., McClelland, M., Rosen, H., Libby, S.J., and Fang, F.C. (2006) Selective silencing of foreign DNA with low GC content by the H-NS protein in *Salmonella*. *Science* **313**: 236–238.
Picker, M.A., and Wing, H.J. (2016) H-NS, its family members and their regulation of virulence genes in *Shigella* species. *Genes (Basel)* **7**: 112.
Pogliano, J.A., and Beckwith, J. (1994) SecD and SecF facilitate protein export in *Escherichia coli*. *Embo J* **13**: 554–561.
Porter, M.E., and Dorman, C.J. (1997) Differential regulation of the plasmid-encoded genes in the *Shigella flexneri* virulence regulon. *Mol Gen Genet* **256**: 93–103.
Prosseda, G., Falconi, M., Giangrossi, M., Gualerzi, C.O., Micheli, G., and Colonna, B. (2004) The *virF* promoter in *Shigella*: more than just a curved DNA stretch. *Mol Microbiol* **51**: 523–537.
Rodionov, O., Lobocka, M., and Yarmolinsky, M. (1999) Silencing of genes flanking the P1 plasmid centromere. *Science* **283**: 546–549.
Schuch, R., and Maurelli, A.T. (1997) Virulence plasmid instability in *Shigella flexneri* 2a is induced by virulence gene expression. *Infect Immun* **65**: 3686–3692.
Shere, K.D., Sallustio, S., Manessis, A., D'aversa, T.G., and Goldberg, M.B. (1997) Disruption of IcsP, the major *Shigella* protease that cleaves IcsA, accelerates actin-based motility. *Mol Microbiol* **25**: 451–462.
Steinhauer, J., Agha, R., Pham, T., Varga, A.W., and Goldberg, M.B. (1999) The unipolar *Shigella* surface protein IcsA is targeted directly to the bacterial old pole: IcsP cleavage of IcsA occurs over the entire bacterial surface. *Mol Microbiol* **32**: 367–377.
Stoebel, D.M., Free, A., and Dorman, C.J. (2008) Anti-silencing: overcoming H-NS-mediated repression of transcription in Gram-negative enteric bacteria. *Microbiology* **154**: 2533–2545.
Taniya, T., Mitobe, J., Nakayama, S., Mingshan, Q., Okuda, K., and Watanabe, H. (2003) Determination of the InvE binding site required for expression of IpaB of the *Shigella sonnei* virulence plasmid: involvement of a ParB box A-like sequence. *J Bacteriol* **185**: 5158–5165.
Tobe, T., Nagai, S., Okada, N., Adter, B., Yoshikawa, M., and Sasakawa, C. (1991) Temperature-regulated expression of invasion genes in *Shigella flexneri* is controlled through the transcriptional activation of the *virB* gene on the large plasmid. *Mol Microbiol* **5**: 887–893.
Tobe, T., Yoshikawa, M., Mizuno, T., and Sasakawa, C. (1993) Transcriptional control of the invasion regulatory gene *virB* of *Shigella flexneri*: activation by *virF* and repression by H-NS. *J Bacteriol* **175**: 6142–6149.
Turner, E.C., and Dorman, C.J. (2007) H-NS antagonism in *Shigella flexneri* by VirB, a virulence gene transcription regulator that is closely related to plasmid partition factors. *J Bacteriol* **189**: 3403–3413.
Weatherspoon-Griffin, N., M.A., Picker, and H.J. Wing, (2016) The genetic organization and transcriptional regulation of *Shigella* virulence genes. In: *Shigella: Molecular and Cellular Biology*. W.D. Picking & W.L. Picking (eds). UK: Caister Academic Press, pp. 65–107.
Williams, R.M., and Rimsky, S. (1997) Molecular aspects of the *E. coli* nucleoid protein, H-NS: a central controller of gene regulatory networks. *FEMS Microbiol Lett* **156**: 175–185.
Wing, H.J., Goldman, S.R., Ally, S., and Goldberg, M.B. (2005) Modulation of an outer membrane protease contributes to the virulence defect of *Shigella flexneri* strains carrying a mutation in the *virK* locus. *Infect Immun* **73**: 1217–1220.
Wing, H.J., Yan, A.W., Goldman, S.R., and Goldberg, M.B. (2004) Regulation of IcsP, the outer membrane protease of the *Shigella* actin tail assembly protein IcsA, by virulence plasmid regulators VirF and VirB. *J Bacteriol* **186**: 699–705.
Yamada, H., Yoshida, T., Tanaka, K., Sasakawa, C., and Mizuno, T. (1991) Molecular analysis of the *Escherichia coli hns* gene encoding a DNA-binding protein, which preferentially recognizes curved DNA sequences. *Mol Gen Genet* **230**: 332–336.

Supporting information

Additional supporting information may be found in the online version of this article at the publisher's web-site.