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VirB Alleviates H-NS Repression of the *icsP* Promoter in *Shigella flexneri* from Sites More Than One Kilobase Upstream of the Transcription Start Site▽

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The *icsP* promoter of *Shigella* spp. is repressed by H-NS and derepressed by VirB. Here, we show that an inverted repeat located between positions −1144 and −1130 relative to the *icsP* transcription start site is necessary for VirB-dependent derepression. The atypical location of this *cis*-acting site is discussed.

Shigella species are gram-negative intracellular pathogens that invade cells of the lower intestinal epithelia of humans and primates, causing bacillary dysentery. All *Shigella* species carry a large virulence plasmid, and many genes carried by these plasmids are thermoregulated. At the nonpermissive temperature of 30°C, the global regulator H-NS (histone-like nucleoid structuring protein) represses transcription of these genes (4, 12, 18). At the permissive temperature of 37°C, H-NS-dependent repression is relieved by temperature-induced changes in DNA topology (9, 19), VirF, or its subordinate regulator VirB (InvE) (reviewed in reference 17). The mechanism that leads to the alleviation of transcriptional repression by H-NS has been coined "antisilencing." Antisilencing is thought to play an important role in controlling the expression of genes acquired through horizontal gene transfer and is common in bacterial pathogens in which a variety of transcription factors function to relieve repression by H-NS (reviewed in reference 29).

The *icsP* (*sopA*) gene is carried on the large virulence plasmid in all *Shigella* species (8, 27) and encodes an outer membrane protease, which belongs to the omptin protease family (11, 14) and cleaves the actin-tail assembly protein IcsA from the surface of *Shigella* (8, 27, 28). Previous studies have revealed that *icsP*, like other *Shigella* virulence plasmid genes, including *virA*, *ospB*, and *phoN2*, and those of the invasion locus, *ipa*, *mxi*, and *spa* (1, 5, 6, 25, 26, 30, 31, 33), is repressed by H-NS and derepressed by VirB (34). In this study, we identify sequences upstream of the *icsP* gene necessary for derepression by VirB, with a view to improve our understanding of the mechanism of transcriptional antisilencing at the *icsP* promoter.

Identification of sequences required for VirB-dependent regulation of the *icsP* promoter. Previous work has demonstrated that VirB regulates an *icsP-lacZ* fusion integrated into the *icsP* locus on the *Shigella* virulence plasmid (34). To identify regions upstream of the *icsP* gene that mediate VirB-dependent derepression, a nested set of *icsP* promoter deletions was created (Fig. 1A) and cloned into a medium-copy-number *lacZ* reporter plasmid pHJW20 (Table 1) to replace the existing 1,232-bp *icsP* promoter fragment. This created eight P*icsP-lacZ* fusions whose upstream limits varied from positions −1232 to −92 relative to the previously annotated transcription start site of the *icsP* gene (8) (Fig. 1A; Table 1). Each promoter construct was introduced into wild-type *Shigella* strain 2457T and a mutant derivative, AWY3 (2457T *virB*::Tn*5*), and β-galactosidase production was measured using the Miller protocol (16). Of the eight promoter fragments tested, only one displayed a >2-fold increase in the presence of VirB (Fig. 2). Surprisingly, this was the longest promoter fragment (found in pHJW20). The activity of this promoter was 17-fold higher in the presence of VirB than in its absence. This increase was unlikely to be caused by sequences in the pACYC184 plasmid backbone, because these sequences would also influence the activity of the other promoter fragments. Furthermore, the increase in promoter activity was unlikely to be caused by the creation of a new VirB binding site at the boundary of the plasmid backbone and the promoter region, because two additional constructs with altered plasmid-promoter boundaries (pMIC01 and pMIC02 [Table 1]) were found to have similar activities to those displayed by pHJW20. The simplest interpretation of these data was that DNA sequences located between positions 1232 and 1056 upstream of the *icsP* transcription start site are required for VirB-dependent regulation of the *icsP* promoter.

Identification and site-directed mutagenesis of putative VirB binding sites responsible for VirB-dependent regulation of the *icsP* promoter. Previous analysis of the *icsB*, *spa15*, and *virA* promoters of *Shigella sonnei* established a consensus binding site for VirB (30). Our analysis of sequences upstream of the *icsP* gene identified nine sites with a match greater than 6/7 to the consensus, 5′-(A/G)(A/T)G(G)AAAT-3′ (Fig. 1B). Three of these sites are located between positions −665 and −351. The location of these sites may explain why small yet significant increases in VirB-dependent promoter activity were observed in wild-type *Shigella flexneri* carrying promoter constructs with upstream boundaries of −665, −893, and −1056

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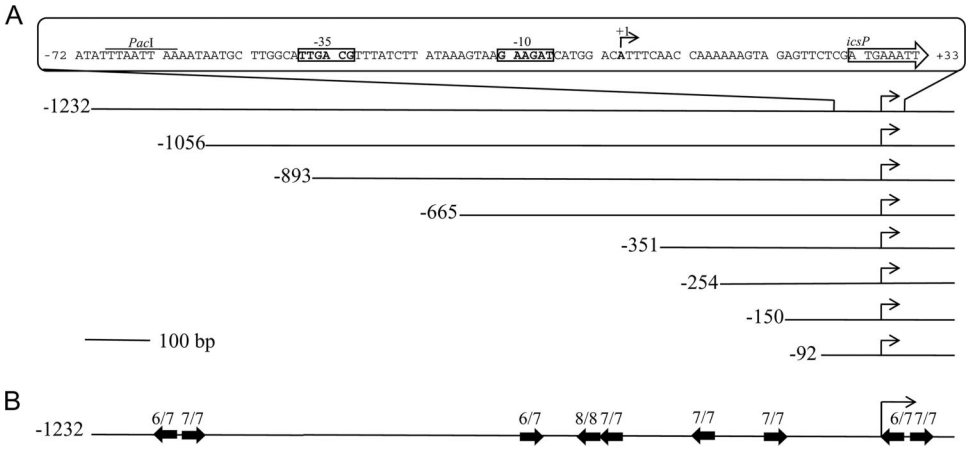

FIG. 1. Promoter elements of the *icsP* promoter and schematic representation of the truncated promoter series. (A) Angled arrows represent the *icsP* transcription start site (+1), determined previously (8). The hexamers at positions −10 and −35 are boxed and shown in boldface type. The translation start site is outlined by an arrow. The truncated promoters are drawn to scale, and the numbers represent the upstream boundary of the *icsP* promoter, relative to the transcription start site (+1). The promoter fragments represented are found in pHJW20, pJS01, pJS02, pDH01, pJS04, pHJW34, pHJW35, and pHJW36. (B) Solid black arrows represent the relative position and orientation of the nine VirB binding sites identified within the promoter fragments used in this work. In each case, the match to consensus sequence 5′-(A/G)(A/T)G(G)AAAT-3′ (30, 32) is given.

relative to the *icsP* transcription start site (+1) (Fig. 2). Two other putative VirB binding sites are located immediately downstream of the transcription start site. Although the location of these sites may explain the small increase in VirB-dependent promoter activity associated with the shortest promoter fragment used in our studies, our data suggest that these sites alone play no significant role in the presence of upstream promoter sequences.

Interestingly, two of the nine sites identified upstream of the *icsP* gene are located between positions −1144 and −1130 and are organized as an inverted repeat (Fig. 1B). Since our truncation analysis indicates that sequences between positions −1232 and −1056 are essential for a 17-fold increase in promoter activity in the presence of VirB (Fig. 2), we chose to analyze these sites further. Seven base pair substitutions were made in either the upstream site (box 2), the downstream site (box 1) or both, using a PCR site-directed mutagenesis method described by Lie and Leigh (15) (Table 2). Each mutated promoter fragment was then introduced into the *lacZ* reporter plasmid pHJW20 to replace the existing wild-type sites, the

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Description[a]	Source or reference
Strains		
S. flexneri		
2457T	*S. flexneri* serotype 2a	10
AWY3	2457T *virB*::Tn5; Kn^r	34
Plasmids		
pACYC184	Cloning vector; p15A replicon Tet^r/Cm^r	24
pHJW7	*icsP* promoter region transcriptionally fused to *lacZ* in pACYC184 Cm^r; carries 1,232 bp of wild-type sequence upstream of the *icsP* transcription start site	34
pHJW20	pHJW7 carrying unique XbaI site upstream of *lacZ* gene; carries 1,232 bp of wild-type sequence upstream of the *icsP* transcription start site	This work
pJS01	pHJW20 carrying 1,056 bp of wild-type sequence upstream of the *icsP* transcription start site	This work
pJS02	pHJW20 carrying 893 bp of wild-type sequence upstream of the *icsP* transcription start site	This work
pDH01	pHJW20 carrying 665 bp of wild-type sequence upstream of the *icsP* transcription start site	This work
pJS04	pHJW20 carrying 351 bp of wild-type sequence upstream of the *icsP* transcription start site	This work
pHJW34	pHJW20 carrying 254 bp of wild-type sequence upstream of the *icsP* transcription start site	This work
pHJW35	pHJW20 carrying 150 bp of wild-type sequence upstream of the *icsP* transcription start site	This work
pHJW36	pHJW20 carrying 92 bp of wild-type sequence upstream of the *icsP* transcription start site	This work
pMIC01	pHJW20 with 33 bp deleted between the SalI and PstI sites in the multiple cloning site of pACYC184	This work
pMIC02	pHJW20 carrying 1,437 bp of wild-type sequence upstream of the *icsP* transcription start site	This work
pMIC13	pHJW20 carrying a 7-bp mutation in box 2 of the putative VirB binding site	This work
pMIC17	pHJW20 carrying a 7-bp mutation in box 1 of the putative VirB binding site	This work
pMIC18	pHJW20 carrying a 14-bp mutation in both box 1 and box 2 of the putative VirB binding site	This work
pMIC21	pHJW20 lacking all *icsP* promoter sequences	This work

[a] Amp^r, ampicillin resistance; Tet^r, tetracycline resistance; Cm^r, chloramphenicol resistance; Kn^r, kanamycin resistance.

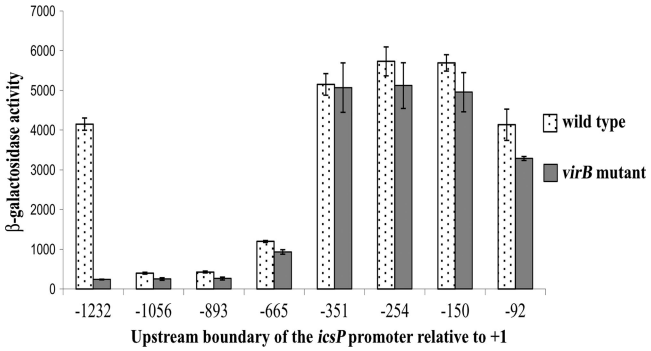

FIG. 2. Activities of the truncated *icsP* promoter series in *Shigella*. Bars indicate β-galactosidase expression of the P*icsP-lacZ* fusions in wild-type *S. flexneri* (2457T) and an isogenic strain lacking *virB* (AWY3). β-Galactosidase activities are expressed in Miller units. Assays were run in triplicate, and the means and standard deviations of the results are shown.

resulting plasmids pMIC13, pMIC17, pMIC18, and pHJW20 and a promoterless control, pMIC21 (Table 1), were introduced into wild-type *Shigella* strain 2457T and the *virB* mutant (AWY3), and β-galactosidase levels were measured (Table 2). Our data revealed that complete mutagenesis of the upstream binding site (box 2), the downstream binding site (box 1), or both, resulted in complete loss of VirB-dependent regulation of the *icsP* promoter. Furthermore, these results were not an artifact of the *lacZ* reporter constructs, because similar patterns of expression were observed when the *icsP* gene was fused to each of our promoter constructs and IcsP levels were measured by Western blotting (data not shown). These data strongly suggest that VirB regulates the *icsP* promoter from sequences located more than 1 kb upstream of the *icsP* transcription start site. To our knowledge, this is the first evidence that VirB can influence promoter activity from such distal sites.

Conserved sequence and location of the two distal VirB binding sites in all known *Shigella* sequences and in EIEC strain HN280. To examine how well conserved DNA sequences located upstream of the *icsP* promoter are among other *Shigella* strains, species, and other enterics, a 2-kb sequence upstream of the *icsP* gene in *Shigella flexneri* 2457T was subjected to BLAST analysis. All known *Shigella* virulence plasmid sequences and the virulence plasmid of the enteroinvasive *Escherichia coli* (EIEC) strain HN280 contained nearly identical sequences (99 to 100% identity) over the entire 2-kb sequence upstream of the *icsP* gene. Furthermore, the upstream inverted repeat identified by our studies was 100% identical in all strains and located in exactly the same position relative to the annotated transcription start site identified in *S. flexneri*. These findings strongly suggest that *icsP* genes found in all *Shigella* spp. and the EIEC strain HN280 are likely to be regulated by VirB from a binding site located more than 1 kb upstream of the gene.

VirB is structurally homologous to plasmid partitioning proteins, which can influence transcription from distances of several kilobases. While it is unusual for transcription factors to influence transcription from distances greater than 200 bp upstream or downstream of the transcription start site in bacteria (2, 7), some examples exist. For example, the enhancer of the *Bacillus subtilis rocG* gene is located 1.5 kb downstream of the promoter and, beyond the end of the *rocG* coding region (3) and the two NtrC binding sites required for the transcriptional activation of *E. coli* σ^{54}-regulated *glnA* promoter, can still function when placed as far as 3 kb from the promoter (20). Furthermore, bacterial plasmid partitioning factors, while not typically considered transcription factors, have also been shown to silence the promoters of genes in the vicinity of their *cis*-acting binding sites from distances of several kilobase pairs (13, 21–23, 35). One of these proteins, the P1 ParB protein, displays structural homology to VirB and has bridging capabilities—the ability to interact with other ParB monomers located at binding sites further up- or downstream. It is therefore possible that the seven other sites with close matches to the VirB consensus binding site play an important role in bridging by VirB and that the resulting DNA topology is central to the alleviation of H-NS-dependent repression of the *icsP* promoter, although this needs to be tested.

In summary, although transcription factors typically bind to sequences located within 200 bp upstream or downstream of the transcription start site (2, 7), here we provide strong evidence that VirB has the capacity to alleviate H-NS-dependent repression of the *icsP* promoter from sites located more than 1 kb upstream of the transcription start site. This raises two important questions. (i) Are other *Shigella* virulence plasmid genes regulated from remote VirB binding sites? (ii) Is it common for transcriptional antisilencing mechanisms to employ distal regulator binding sites? Future studies will address these questions and elucidate the molecular mechanism of

TABLE 2. Summary of mutations introduced into the two boxes that form the upstream inverted repeat and activities of wild-type and mutated *icsP* promoter fragments

Fragment description	Sequence[a]	β-Galactosidase activity[b]	
		virB$^+$	*virB* mutant
WT box 1 and 2	CGGGGATTTCAGTATGAAATGAAGTA	4,412 ± 80	388 ± 10
Mutated box 1	CGGGGATTTCAGTCGACCCGGAAGTA	307 ± 13	326 ± 75
Mutated box 2	CGGGGGCCCAGCTATGAAATGAAGTA	309 ± 27	345 ± 22
Mutated box 1 and 2	CGGGGGCCCAGCTCGACCCGGAAGTA	297 ± 18	341 ± 15
Promoterless *lacZ*		284 ± 13	388 ± 25

[a] 5′→3′ DNA sequences of the wild-type and mutated boxes that form the upstream inverted repeat. Sequences lie between positions −1144 and −1130 relative to the annotated transcription start site of *icsP* (+1) (8). Underlined sequences are box 2 (left) and box 1 (right) sequences.

[b] All promoter fragments were fused to *lacZ*, and β-galactosidase activities were measured in wild-type *S. flexneri* (2457T) and the isogenic strain that lacks *virB* (AWY3). The parent cloning vector with a promoterless *lacZ* gene was included as a negative control. β-Galactosidase activities are expressed in Miller units. Assays were run in triplicate, and the means and standard deviations of the results are shown.

H-NS-dependent repression and VirB-mediated derepression of the *icsP* promoter.

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