JOURNAL OF BACTERIOLOGY, Feb. 2004, p. 699–705 Vol. 186, No. 3
0021-9193/04/$08.00+0 DOI: 10.1128/JB.186.3.699–705.2004

Regulation of IcsP, the Outer Membrane Protease of the *Shigella* Actin Tail Assembly Protein IcsA, by Virulence Plasmid Regulators VirF and VirB

Helen J. Wing, Arthur W. Yan, Seth R. Goldman, and Marcia B. Goldberg*

Infectious Disease Division, Massachusetts General Hospital, Cambridge, Massachusetts 02139

Received 7 August 2003/Accepted 31 October 2003

The *Shigella* outer membrane protease IcsP removes the actin assembly protein IcsA from the bacterial surface, and consequently modulates *Shigella* actin-based motility and cell-to-cell spread. Here, we demonstrate that IcsP expression is undetectable in mutants lacking either of two transcriptional activators, VirF and VirB. In wild-type *Shigella* spp., *virB* expression is entirely dependent on VirF; therefore, to circumvent this regulatory cascade, we independently expressed VirF or VirB in *Shigella* strains lacking both activators and measured both IcsP levels and transcription from the *icsP* promoter. Our results show that VirB significantly enhanced *icsP* transcription, even in the absence of VirF. In contrast, when VirF was induced in the absence of VirB, VirF had variable effects. The regulation of *icsP* is distinctly different from the regulation of the gene encoding its major substrate, *icsA*, which is activated by VirF and not VirB. We propose that the different pathways regulating *icsA* and *icsP* may be critical to the modulation of IcsA-mediated actin-based motility by IcsP.

Shigella spp., gram-negative bacterial pathogens cause severe and bloody diarrhea in their human hosts by invading and spreading through the colonic epithelium. *Shigella* movement within the host cell cytoplasm is dependent on the ability of the bacterium to recruit host cell actin to its surface to form an "actin tail," which propels the bacterium from one cell to another (5, 16, 29). Actin tail assembly is mediated by a single bacterial protein, IcsA, which is found on the outer surface at one pole of the bacterium (17). This asymmetric localization of IcsA ensures that actin assembly occurs in a directional manner. In its mature form, IcsA is comprised of two domains: the α domain (residues 53 to 758) contains the determinant for actin assembly (14) and extends from the bacterial surface into the extracellular environment, whereas the β domain (residues 759 to 1102) is embedded in the outer membrane (33). The amount of IcsA α domain exposed on the bacterial surface correlates with the efficiency of actin tail formation in the cytoplasm of infected cells (21).

IcsP, an outer membrane protease of *Shigella*, cleaves IcsA between Arg^{758} and Arg^{759}, removing the entire IcsA α domain from the bacterial surface (8, 13, 15a, 31). Overexpression of IcsP leads to complete removal of the IcsA α domain from the bacterial cell surface (32), whereas genetic disruption of *icsP* increases the total amount of cell associated IcsA α domain, leading to an increase in the rate of actin-based movement of *Shigella* (31). Although IcsP is not required for polar localization of IcsA (6, 28), it contributes to the maintenance of a tight polar cap of IcsA on the bacterial surface (31). Furthermore, as *Shigella* enter stationary phase, the amount of cell-associated IcsA α domain decreases dramatically, an effect due at least in part to IcsP (18, 32).

These data demonstrate that IcsP plays an important role in modulating the amount of the IcsA α domain present on the bacterial surface and indicate that the amount of IcsA expressed on the bacterial surface correlates with the efficiency of *Shigella* actin-based motility. Given the importance of actin-based motility in *Shigella* pathogenesis, we postulate that it would be advantageous for IcsP to be tightly regulated. Here, we investigate the regulation of IcsP by two regulators of *Shigella* virulence protein expression, VirF and VirB.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The bacterial strains and plasmids used in the present study are listed in Table 1. Bacteria were grown routinely at 37°C in Luria-Bertani (LB) broth (23) with aeration or on LB agar (LB broth containing 1.5% [wt/vol] agar). Antibiotics were added at the following final concentrations: ampicillin, 100 μg ml^{-1}; chloramphenicol, 25 μg ml^{-1}; kanamycin, 50 μg ml^{-1}; and tetracycline, 12.5 μg ml^{-1}. Where appropriate, to ensure that *Shigella* strains had maintained the large virulence plasmid during manipulation, Congo red binding was tested on Trypticase soy broth agar plates containing 0.01% (wt/vol) Congo red (Sigma Chemical Co., St. Louis, Mo.).

Construction of reporter plasmids. The *icsP* reporter plasmid pHJW6 was constructed as follows. The *icsP* promoter (the 1,256-bp sequence located upstream of the *icsP* transcription start site) and *icsP* gene were isolated from the high-copy-number plasmid pAM4 (31) and cloned into the lower-copy-number plasmid pACYC184, so that the *icsP* gene is in the opposite orientation to the disrupted tetracycline resistance cassette. pHJW7, which is derived from pHJW6, carries the *icsP* promoter and the first 48 bp of the *icsP* coding region, cloned upstream of a translation stop site and a promoterless *lacZ* gene, so that expression of *lacZ* is directly regulated by the *icsP* promoter.

Construction of *S. flexneri* strains. The *S. flexneri* 2457T *virF* mutant MBG338 was created as follows. A 570-bp fragment internal to the coding sequence of *virF* (extending from 131 bp to 701 bp of the open reading frame) was amplified by PCR from 2457T template and cloned into the ampicillin-resistant suicide vector pCVD442. The resultant plasmid (pMBG326) was introduced into the tetracycline-resistant *S. flexneri* strain BS109 by conjugation, and transconjugants were selected on ampicillin and tetracycline plates. Integration of the vector into the *virF* locus was verified by Southern blotting. The targeted *virF* disruption was then transduced into the *S. flexneri* wild-type strain by using P1L4 phage transduction and, again, integration at the *virF* locus was verified by Southern blotting.

The *S. flexneri* 2457T *virB* mutant AWY3 was created by moving the kanamycin-resistant locus from YSH6000 *virB*::Tn*5* (gift of C. Sasakawa [1]) into the *S. flexneri* wild-type strain 2457T by transduction (as described above). To create the *virF virB Shigella* mutant AWY7, the ampicillin-resistant locus from MBG338

* Corresponding author. Mailing address: University Park, 65 Landsdowne St., Cambridge, MA 02139. Phone: (617) 768-8740. Fax: (617) 768-8738. E-mail: mgoldberg1@partners.org.

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Description[a]	Source or reference
Strains		
E. coli		
MC4100	Wild type	25
MC4100 *hns*	MC4100 *hns*::Kn^r	38
S. flexneri		
2457T	Wild type serotype 2a	15
BS103	2457T cured of the virulence plasmid	22
AWY3	2457T *virB*::Tn*5*	This work
AWY7	2457T *virF*::pMBG326 *virB*::Tn*5*	This work
AWY8	2457T *virF*::pMBG326 *virB*::Tn*5* P*icsP-lacZ*	This work
MBG338	2457T *virF*::pMBG326	This work
MBG341	2457T *icsP*::Amp^r	31
SY327 λ*pir*	SY327 with integrated *pir*	24
YSH6000	Wild type serotype 2a	1
YSH6000 *virB*	YSH6000 *virB*::Tn*5*	1
Plasmids		
pACYC184	Cloning vector; Tet^r/Cm^r	26
pAM4	pBC-KS-*icsP*	31
pATM324	pBAD-*virB;* Amp^r	30
pBAD18	Arabinose-inducible pBAD expression vector, pBR*ori;* Amp^r	19
pAWY7	pFSV-1-P*icsP-lacZ* Tet^r	This work
pHJW4	pBAD18-*virF;* Amp^r	This work
pCVD442	suicide vector; Amp^r	9
pFSV-1	suicide vector; Tet^r	4
pHJW6	*icsP* promoter and gene cloned into pACYC184	This work
pHJW7	*icsP* promoter transcriptionally fused to *lacZ* in pACYC184	This work
pHJW14	pBAD18-*cat;* Cm^r	This work
pHJW16	pHJW14-*virB;* Cm^r	This work
pHJW17	pBAD18-*tet;* Tet^r	This work
pMBG326	Suicide vector pCVD442 containing internal fragment of *virF*; Amp^r	This work

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

was transduced into the *virB* mutant AWY3. The *icsP* transcriptional reporter strain AWY8 was derived from AWY7 as follows. A fragment carrying the *icsP-lacZ* fusion from pHJW7 was cloned into the suicide plasmid pFSV-1 (a gift of J. Bliska [4]). The resultant plasmid, pAWY7, was introduced into the *Escherichia coli* strain SY327 λ*pir* and then mobilized into AWY7 by conjugation. Transconjugants were selected by plating on tetracycline.

For strains generated in the present study, integration into the appropriate locus and, where appropriate, the presence of *virF*, *virB*, *icsA*, and *icsP* were routinely verified by PCR.

Construction of pBAD derivatives and inducible *virF* and *virB* expression constructs. Where necessary, *virF* and *virB* were supplied in *trans* on the arabinose-inducible expression vector pBAD18. The *virF* or *virB* gene was amplified by PCR from 2457T template and cloned into the multiple cloning site so that its expression was under the control of the L-arabinose-inducible *ara*BAD promoter, thereby generating pBAD-*virF* and pBAD-*virB*. To introduce pBAD-*virF* or pBAD-*virB* into ampicillin-resistant strain backgrounds, tetracycline or chloramphenicol resistance derivatives were generated by cloning the respective resistance genes into the *Sca*I site in the *bla* gene of pBAD18. All pBAD plasmids carrying *virF* or *virB* were checked for their ability to complement the Congo red minus phenotype of a *virF* mutant or a *virB* mutant, respectively, when grown in the presence of 0.2% (wt/vol) L-arabinose.

Quantification of IcsP levels in *Shigella*. Throughout this study, IcsP expression was measured in mid-exponential-phase cultures because preliminary experiments had shown that IcsP is first detected in wild-type cells under these conditions (data not shown). Cells were routinely back-diluted 1:100 from an overnight culture and grown for 4 to 5 h in LB (at which point the A_{600} was ~2.5). To examine the effect of expressing *virF* or *virB* from the pBAD vectors, cells were instead back-diluted 1:100 in 5 ml of LB medium containing 0.2% (wt/vol) glucose and, after 4 to 5 h were harvested, washed with an equivalent volume of LB medium and diluted 5-fold into LB medium containing either 0.08% (wt/vol) L-arabinose or 0.08% (wt/vol) D-arabinose. Cultures were then grown for an additional 2 h before being harvested.

Whole-cell protein extracts were prepared as described previously (32). Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Equivalent amounts of protein were loaded by normalizing the harvest volume to cell density. Western blot analysis was performed with an affinity-purified IcsP rabbit antiserum (32) and by enhanced chemiluminescence (Pierce). Integrated density measurements were performed by using Quantity-One software on a Bio-Rad Gel Doc 2000 system.

Quantification of transcription from the *icsP* promoter. Transcription from the *icsP* promoter was determined by measuring β-galactosidase activity (as described previously [20] by using the Miller protocol) in AWY8 or strains carrying pHJW7. Routinely, transcription was analyzed in mid-exponential-phase cultures. Cells were back-diluted 1:100 and grown for 4 to 5 h in LB medium. To examine the effect of expressing *virF* or *virB* from the pBAD vectors, cells were instead grown as described above before being harvested for β-galactosidase quantification.

RESULTS AND DISCUSSION

IcsP expression is dependent on virulence plasmid encoded loci. IcsP is encoded by a monocistronic operon on the large (230-kb) virulence plasmid of *Shigella* (13, 31). Many virulence plasmid loci are regulated by factors encoded by the virulence plasmid. To examine IcsP expression in strains with or without the virulence plasmid, a low-copy-number plasmid carrying the *icsP* operon (pHJW6) was introduced into both *S. flexneri* 2a strain 2457T cured of the virulence plasmid (BS103) and a 2457T *icsP* mutant. Since both strains lacked the native *icsP* gene but carried the *icsP* reporter plasmid (pHJW6), direct comparison of IcsP expression could be made. Approximately

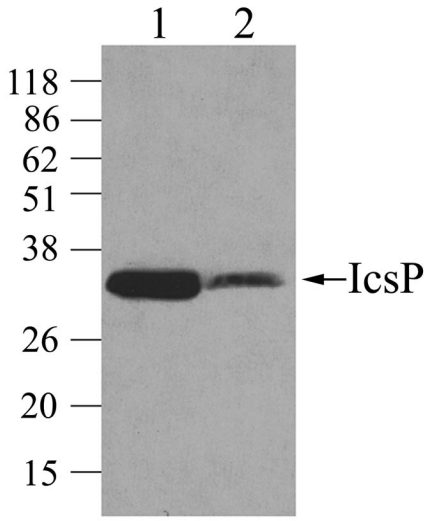


FIG. 1. Comparison of IcsP levels in *Shigella* strains with or without the virulence plasmid. Western blot analysis of the *icsP* mutant (MBG341) (lane 1) and virulence plasmid-cured *Shigella* (BS103) (lane 2), each carrying the *icsP* reporter plasmid (pHJW6), was performed with IcsP antiserum. Equivalent amounts of total cellular protein were loaded onto the gel for the two strains. The experiment was repeated three times, and representative data are shown. Apparent molecular masses are indicated in kilodaltons.

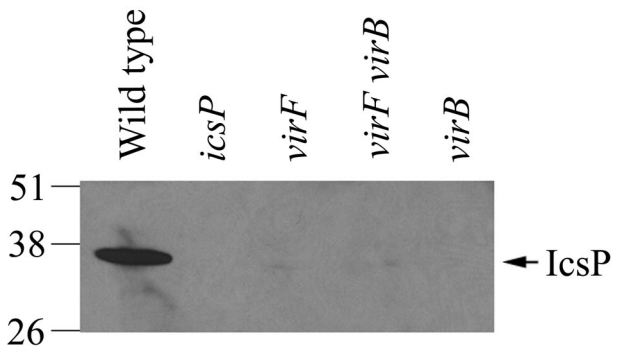


FIG. 2. IcsP levels in the absence of the transcriptional activators VirF and VirB. Western blot analysis of total cellular protein harvested from wild-type *Shigella* (2457T), the *icsP* mutant (MBG341), the *virF* mutant (MBG338), the *virF virB* mutant (AWY7), and the *virB* mutant (AWY3) was performed with IcsP antiserum. Apparent molecular masses are indicated in kilodaltons.

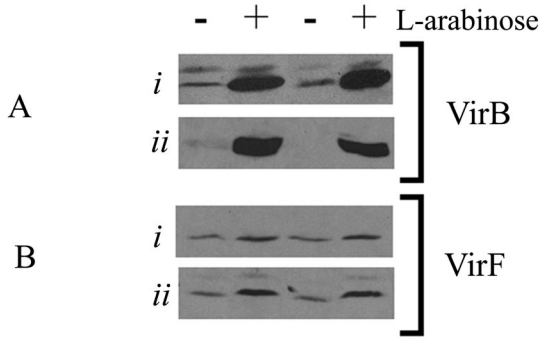
fivefold more IcsP was detected in the strain carrying the virulence plasmid (Fig. 1, lane 1 compared to lane 2). The plasmid copy number was comparable in the two strains. These results indicate that virulence plasmid encoded factors positively regulate IcsP expression.

IcsP is undetectable in *virF* and *virB* mutants of *Shigella*. VirF and VirB (InvE) are virulence plasmid encoded transcription factors that regulate many genes encoded by the *Shigella* virulence plasmid (12). Virulence gene expression is thermoregulated and maximal at 37°C. At this temperature, VirF activates the transcription of *icsA* and *virB* (*invE*). VirB, whose expression is completely dependent on VirF, in turn activates additional genes, most of which are found within the 31-kb invasion locus of the virulence plasmid (12), which encodes the *Shigella* type III secretion apparatus. Since we had demonstrated that the absence of the virulence plasmid results in a decrease in IcsP expression, we investigated whether this phenotype was due to the loss of *virF* and/or *virB*. In mutants lacking *virF* (MBG338), *virB* (AWY3), or both (AWY7), IcsP was markedly reduced (Fig. 2). The absence of IcsP in these strains was due to the lack of either VirF or VirB, because IcsP expression was restored in the *virF* and *virB* mutants by complementation with *virF* or *virB*, respectively (data not shown). Of note, the band corresponding to IcsP in the virulence plasmid cured strain (Fig. 1, lane 2) was more prominent than those in the *virF* (MBG338), *virB* (AWY3), and *virF virB* mutants (AWY7) (Fig. 2). This was likely due to differences in exposure of the blots and the presence of *icsP* on the multicopy reporter plasmid (pHJW6) in the virulence plasmid-cured strain (Fig. 1). Since *virF* mutants do not express VirB (1, 35), these results suggest that VirF has no significant effect on IcsP expression under these experimental conditions (Fig. 2, *virB* mutant) and that VirB increases IcsP expression.

Expression of VirF or VirB independently increases IcsP levels. To investigate whether each activator contributes independently to the expression of IcsP, IcsP levels were measured in strains lacking both *virF* and *virB* but supplied with either *virF* or *virB* in *trans*. Virulence plasmid-cured *Shigella* (BS103) containing the *icsP* reporter plasmid (pHJW6) and the *virF virB Shigella* mutant (AWY7) were each supplied with either *virF* or *virB* under the control of the arabinose promoter on a multicopy vector (P_{BAD}-*virF* and P_{BAD}-*virB*). When *virB* was induced, IcsP levels were increased 7.3-fold in virulence plasmid-cured *Shigella* carrying the *icsP* reporter plasmid, BS103(pHJW6), and by 5.7-fold in the *virF virB* mutant (AWY7; Fig. 3, showing the results of duplicate experiments). These data confirm that VirB positively regulates IcsP in the absence of VirF.

Surprisingly, when *virF* was expressed at high levels in either strain background, IcsP levels increased significantly, albeit by small amounts [1.8-fold in BS103(pHJW6) and 2.4-fold in AWY7; Fig. 3C]. These results contrast with those shown in Fig. 2, where native levels of VirF did not significantly increase IcsP expression in the absence of VirB, and indicate that VirF may regulate IcsP expression independently of VirB. Although in this experiment, the increase in IcsP expression may be caused by supraphysiological levels of VirF, it is possible that regulation of IcsP by VirF may be physiologically relevant under experimental conditions not examined in the present study or may have been relevant at some prior point in *Shigella* evolution. Regardless of whether IcsP was expressed from the *icsP* reporter plasmid (pHJW6 in BS103) or from the native gene (in AWY7), regulation by VirF and VirB followed a similar pattern, indicating the low-copy plasmid encoding *icsP* (pHJW6) was a reasonable reporter of IcsP expression.

VirB significantly enhances transcription of *icsP*. To examine the effect of VirB on *icsP* transcription, we measured β-galactosidase production from a low-copy-number *icsP-lacZ* transcriptional reporter (pHJW7) in virulence plasmid-cured *Shigella* and in the *virF virB* mutant (AWY7), each carrying P_{BAD}-*virB*. When *virB* was induced in each strain, transcription from the *icsP* promoter was significantly increased, indicating that VirB activates *icsP* transcription (Table 2).



C

	Fold induction (mean ± S.D.)	
	VirB	VirF
i. Virulence plasmid-cured pHJW6 (*icsP*$^+$)	7.3 ± 0.7	1.8 ± 0.5
ii. virF virB	5.7±0.02	2.4 ± 0.2

FIG. 3. Effect of independent expression of VirF or VirB on IcsP levels. Western blot analysis of *Shigella* strains deficient for both *virF* and *virB* but supplied in *trans* with either *virF* or *virB* expressed from an arabinose-inducible promoter was carried out. IcsP expression was measured in (i) virulence plasmid-cured *Shigella* (BS103) carrying the *icsP* reporter plasmid (pHJW6) or (ii) the *virF virB* mutant (AWY7). Cells were grown either with (+) or without (−) induction of *virB* (A) or *virF* (B), and data from duplicate experiments are shown. (C) Quantification of bands by integrated densitometry. Approximately fourfold less protein was loaded in the top blot of each panel, due to the presence of *icsP* on a multicopy plasmid in the virulence plasmid-cured strain.

DNA topology has been shown to influence transcription (11). Therefore, we proceeded to measure transcription from the *icsP* promoter in its natural context. An *icsP-lacZ* transcriptional reporter was integrated onto the *Shigella* virulence plasmid at the native *icsP* locus in a *virF virB* mutant. The resultant strain, AWY8, carries both an *icsP-lacZ* transcriptional fusion at the locus normally occupied by the *icsP* gene and a second copy of the *icsP* promoter controlling the native gene downstream of the integrant. Transcription from the *icsP* promoter in AWY8 was increased by 2.6-fold in the presence of VirB (Table 2). Taken together, these data demonstrate that VirB positively regulates the *icsP* at the level of transcription.

TABLE 2. Activation of *icsP-lacZ* transcriptional fusions by VirB

Strain	Genotype[a]	β-Galactosidase expression[b]		Fold activation (mean ± SD)
		Uninduced	Induced	
BS103(pHJW7)	2457T vir. plasmid-cured P*icsP-lacZ*	260	3,996	15.4 ± 0.7
AWY7(pHJW7)	2457T *virF virB* P*icsP-lacZ*	1,284	10,119	7.9 ± 0.9
AWY8	AWY7 P*icsP-lacZ*	5,384	13,749	2.6 ± 0.1

[a] In all backgrounds, *virB* was carried on a pBAD18 derivative.
[b] In Miller units. Data are the means of three experiments.

Interestingly, activation of the *icsP* promoter by VirB was significantly greater in virulence plasmid-cured *Shigella* (BS103) than in the *virF virB* mutant (AWY7), suggesting that the presence of the virulence plasmid inhibits transcription from the *icsP* promoter. One possibility is that VirB activation of *icsP* is modulated by a virulence plasmid factor. Alternatively, since the intracellular concentration of VirB has been shown to correlate with levels of virulence gene expression (3), reduced levels of activation by VirB in the presence of the virulence plasmid could be caused by titration of VirB by other VirB binding sites. It was also notable that *icsP* promoter activation from the integrated reporter (in AWY8) was lower than *icsP* promoter activation from the plasmid-borne reporters (in BS103 and AWY7), suggesting the context of the *icsP* promoter modulates its activation by VirB.

Although our data do not distinguish whether the effect of VirB on the *icsP* promoter is direct or indirect, VirB increases transcription from the *icsP* promoter in both virulence plasmid-cured *Shigella* (BS103; Table 2) and *E. coli* (data not shown), a finding consistent with a direct effect. Recently, a consensus binding site has been described for VirB in *S. sonnei* (34). Our analysis of the *icsP* promoter region has revealed five sites that are similar to those identified in *S. sonnei*, with one (GAGAAAT), located 172 bp upstream of the proposed transcription start site, having a complete match to the consensus (A/G)(A/T)G(G)AAAT sequence (13). It is not yet known whether any of these putative VirB binding sites is required for the regulation of *icsP* by VirB.

The *icsP* promoter is repressed by H-NS and derepressed by VirB. The *virB* gene lies immediately adjacent to the *ipa-mxi-spa* region of the virulence plasmid. Previously, VirB has been shown to increase transcription from three promoters in the *ipa-mxi-spa* region of the virulence plasmid (P*icsB*, P*ipgD*, and P*spa* [1, 10, 37]), as well as from the *virA* promoter, which is found in a distinct region on the virulence plasmid (36). The *icsP* gene is located close to the origin of replication of the virulence plasmid, which is distant from and directly opposite the *ipa-mxi-spa* locus on a circular map. Therefore, whereas *virB* and the *ipa-mxi-spa* locus were likely incorporated into the virulence plasmid as a single evolutionary event, it seems likely that *icsP* and *virA* were acquired in distinct events, implying that VirB regulation of *icsP* and *virA* may have evolved recently.

Each of the previously described VirB-regulated promoters is repressed by the nucleoid structuring protein H-NS, leading to the proposal that the role of VirB at these promoters is one of derepression rather than activation (2). To examine whether the *icsP* promoter was also repressed by H-NS and derepressed by VirB, we examined both *icsP* transcription in the presence or absence of H-NS and the effect of VirB on *icsP* transcription under these conditions. β-Galactosidase production from the *icsP*::*lacZ* fusion plasmid (pHJW7) was compared for an *E. coli* MC4100 *hns* mutant (MC4100 *hns*::Kn) and the wild-type strain MC4100 after growth at either 30 or 37°C with or without induction of VirB expression from the P_{BAD}-*virB* plasmid. At both temperatures, regardless of whether VirB was present, *icsP* transcription was increased in the *hns* mutant compared to the wild type (Table 3), a finding consistent with H-NS repression of the promoter. Furthermore, in the wild-type background when VirB was induced transcription was significantly

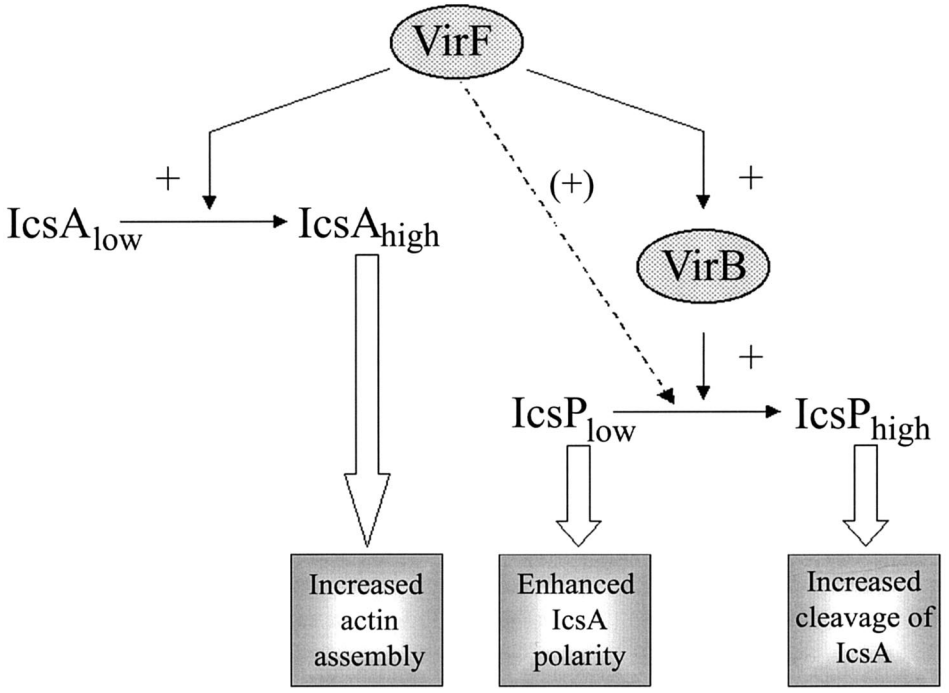


FIG. 4. Model of the distinct regulatory pathways that modulate IcsP and IcsA expression in *Shigella.* VirF positively regulates transcription of *icsA* and *virB*. This increases the amount of IcsA on the bacterial surface and leads to increased levels of VirB. VirB positively regulates transcription of *icsP*, leading to an increase in IcsP from its basal level of expression. VirF may, under certain circumstances, also increase IcsP expression (see the text).

increased at both 30 and 37°C (5.4- and 1.8-fold, respectively). Interestingly, in the *hns* strain no additional increase in promoter activity was observed when VirB was induced, a finding consistent with VirB derepressing the *icsP* promoter rather than activating it per se. It has been reported that the copy number of at least some plasmids is lower in an *hns* mutant background (7); since *icsP* expression was increased in the *hns* mutant, such a difference in copy number could not be responsible for these results. We conclude, therefore, that the *icsP* promoter is repressed by H-NS at both 30 and 37°C and that VirB can overcome this repression to some extent at both temperatures, although more effectively at 30°C (Table 3; 5.4-fold compared to 1.8-fold at 37°C). Thus, the regulation of the *icsP* promoter by H-NS is similar to that previously described for other VirB-regulated promoters.

Role of VirF in transcription of *icsP*. We also examined whether expression of VirF enhanced transcription of *icsP* in the absence of VirB. We observed variable and nonreproducible effects of VirF on expression of the *icsP-lacZ* reporters in each of the strain backgrounds described above. In the presence of VirF, *icsP* transcription was unchanged or increased up to 1.5-fold. Nevertheless, in control experiments, induction of the same *virF* construct increased expression of an *icsA-lacZ* fusion 2.9-fold, similar to the 2.5- to 5-fold activation of *icsA* by VirF that has been reported previously (27), indicating that the *virF* construct was functional. We were unable to define experimental conditions in which activation of *icsP-lacZ* was reproducible. Since small increases in transcription can lead to significant increases in steady-state levels of protein, a minor effect on *icsP* transcription could possibly account for the observed increases in IcsP protein (Fig. 3). Moreover, overall protein expression was not significantly altered in the presence of VirF (data not shown), suggesting increases in IcsP expression by VirF are specific.

Since the possibility remained that VirF might contribute to the regulation of *icsP* in the presence of VirB, we examined

TABLE 3. Repression of *icsP-lacZ* by H-NS and derepression by VirB

Strain	Genotype[a]	Fold activation[b] (mean ± SD) at:			
		30°C		37°C	
		Uninduced	Induced	Uninduced	Induced
MC4100 (pHJW7)	Wild type P*icsP-lacZ*	1.0 ± 0.1	5.4 ± 0.3	0.8 ± 0.1	1.8 ± 0.1
MC4100 *hns* (pHJW7)	*hns*::Kan^r P*icsP-lacZ*	8.8 ± 0.3	7.5 ± 0.4	7.2 ± 0.01	6.0 ± 0.3

[a] In both backgrounds, *virB* was carried on a pBAD18 derivative.
[b] Data have been normalized to the activity obtained in the wild-type strain in the absence of VirB at 30°C and are displayed as the fold activation. The mean and standard deviation of three experiments are presented.

whether VirF might have a more significant effect on *icsP* transcription in the presence of VirB. We compared *icsP-lacZ* transcription after induction of *virF* alone, *virB* alone, or the two together in AWY7. When *virF* and *virB* were induced simultaneously, transcription from the *icsP* promoter was increased slightly and reproducibly, but not significantly, compared to when *virB* was induced alone (data not shown), indicating VirF and VirB do not cooperatively regulate the *icsP* promoter.

The regulation of IcsP by VirB, and possibly VirF, is distinctly different from the regulation of the major substrate of IcsP, IcsA. IcsA is transcriptionally activated by VirF but is unaffected by VirB (1, 27). Because VirB depends on VirF for its own activation, the different pathways that lead to the expression of IcsA and IcsP may reflect subtle differences in the timing and levels of expression of the two proteins during infection. Since the balance between levels of IcsA and levels of IcsP is a critical determinant of the ability of the organism to undergo actin-based motility, differential regulation of the two genes enables the organism to fine-tune this balance, thereby modulating actin-based motility. We propose a model in which VirB activation of IcsP expression leads to increased cleavage of the IcsA actin assembly domain (α domain) from the bacterial surface (Fig. 4). We postulate that this, in conjunction with the distinct pathway of IcsA activation, leads to precise modulation of *Shigella* actin-based motility during infection.

ACKNOWLEDGMENTS

This study was supported by NIH grants AI35817 (M.B.G.) and AI43562 (M.B.G.), the Massachusetts General Hospital Fund for Medical Discovery (H.J.W.), and the Charles H. Hood Foundation, Inc., Boston, Mass. (H.J.W.).

We thank C. Sasakawa, A. Maurelli, S. Busby, and J. Bliska for providing plasmids and strains and J. Butterton and C. Lesser for critical reading of the manuscript.

REFERENCES

1. **Adler, B., C. Sasakawa, T. Tobe, S. Makino, K. Komatsu, and M. Yoshikawa.** 1989. A dual transcriptional activation system for the 230-kb plasmid genes coding for virulence-associated antigens of *Shigella flexneri.* Mol. Microbiol. **3:**627–635.
2. **Beloin, C., and C. J. Dorman.** 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.
3. **Beloin, C., S. McKenna, and C. J. Dorman.** 2002. Molecular dissection of VirB, a key regulator of the virulence cascade of *Shigella flexneri.* J. Biol. Chem. **277:**15333–15344.
4. **Bliska, J. B., K. L. Guan, J. E. Dixon, and S. Falkow.** 1991. Tyrosine phosphate hydrolysis of host proteins by an essential *Yersinia* virulence determinant. Proc. Natl. Acad. Sci. USA **88:**1187–1191.
5. **Brandon, L. D., and M. B. Goldberg.** 2000. Exploitation of mammalian host cell function by *Shigella* spp., p. 175–187. *In* K. A. Brogden et al. (ed.), Virulence mechanisms of bacterial pathogens, 3rd ed. ASM Press, Washington, D.C.
6. **Charles, M., M. Perez, J. H. Kobil, and M. B. Goldberg.** 2001. Polar targeting of *Shigella* virulence factor IcsA in *Enterobacteriacae* and *Vibrio*. Proc. Natl. Acad. Sci. USA **98:**9871–9876.
7. **Deighan, P., A. Free, and C. J. Dorman.** 2000. A role for the *Escherichia coli* H-NS-like protein StpA in OmpF porin expression through modulation of *micF* RNA stability. Mol. Microbiol. **38:**126–139.
8. **d'Hauteville, H., R. Dufourc Qlagelouse, F. Nato, and P. J. Sansonetti.** 1996. Lack of cleavage of IcsA in *Shigella flexneri* causes aberrant movement and allows demonstration of a cross-reactive eukaryotic protein. Infect. Immun. **64:**511–517.
9. **Donnenberg, M. S., and J. B. Kaper.** 1991. Construction of an *eae* deletion mutant of enteropathogenic by using a positive-selection suicide vector. Infect. Immun. **59:**4310–4317.
10. **Dorman, C. J., S. McKenna, and C. Beloin.** 2001. Regulation of virulence gene expression in *Shigella flexneri*, a facultative intracellular pathogen. Int. J. Med. Microbiol. **291:**89–96.
11. **Dorman, C. J., and N. Ni Bhriain.** 1993. DNA topology and bacterial virulence gene regulation. Trends Microbiol. **1:**92–99.
12. **Dorman, C. J., and M. E. Porter.** 1998. The *Shigella* virulence gene regulatory cascade: a paradigm of bacterial gene control mechanisms. Mol. Microbiol. **29:**677–684.
13. **Egile, C., H. d'Hauteville, C. Parsot, and P. J. Sansonetti.** 1997. SopA, the outer membrane protease responsible for polar localization of IcsA in *Shigella flexneri.* Mol. Microbiol. **23:**1063–1073.
14. **Egile, C., T. P. Loisel, V. Laurent, R. Li, D. Pantaloni, P. J. Sansonetti, and M. F. Carlier.** 1999. Activation of the CDC42 effector N-WASP by the *Shigella flexneri* IcsA protein promotes actin nucleation by Arp2/3 complex and bacterial actin-based motility. J. Cell Biol. **146:**1319–1332.
15. **Formal, S. B., G. J. Dammin, E. H. LaBrec, and H. Schneider.** 1958. Experimental *Shigella* infections: characteristics of a fatal infection produced in guinea pigs. J. Bacteriol. **75:**604–610.

15a. **Fukuda, I., T. Suzuki, H. Munakata, N. Hayashi, E. Katayama, M. Yoshikawa, and C. Sasakawa.** 1995. Cleavage of *Shigella* surface protein VirG occurs at a specific site, but the secretion is not essential for intracellular spreading. J. Bacteriol. **177:**1719–1726.

16. **Goldberg, M. B.** 2001. Actin-based motility of intracellular microbial pathogens. Microbiol. Mol. Biol. Rev. **65:**595–626.
17. **Goldberg, M. B., O. Barzu, C. Parsot, and P. J. Sansonetti.** 1993. Unipolar localization and ATPase activity of IcsA, a *Shigella flexneri* protein involved in intracellular movement. Infect. Agents Dis. **2:**210–211.
18. **Goldberg, M. B., J. A. Theriot, and P. J. Sansonetti.** 1994. Regulation of surface presentation of IcsA, a *Shigella* protein essential to intracellular movement and spread, is growth phase dependent. Infect. Immun. **62:**5664–5668.
19. **Guzman, L. M., D. Belin, M. J. Carson, and J. Beckwith.** 1995. Tight regulation, modulation, and high-level expression by vectors containing the arabinose pBAD promoter. J. Bacteriol. **177:**4121–4130.
20. **Jayaraman, P. S., T. C. Peakman, S. J. Busby, R. V. Quincey, and J. A. Cole.** 1987. Location and sequence of the promoter of the gene for the NADH-dependent nitrite reductase of *Escherichia coli* and its regulation by oxygen, the Fnr protein and nitrite. J. Mol. Biol. **196:**781–788.
21. **Magdalena, J., and M. B. Goldberg.** 2002. Quantification of *Shigella* IcsA required for bacterial actin polymerization. Cell Motil. Cytoskeleton **51:**187–196.
22. **Maurelli, A. T., B. Blackmon, and R. Curtiss III.** 1984. Loss of pigmentation in *Shigella flexneri* 2a is correlated with loss of virulence and virulence-associated plasmid. Infect. Immun. **43:**397–401.
23. **Miller, J.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
24. **Miller, V. L., and J. J. Mekalanos.** 1988. A novel suicide vector and its use in construction of insertion mutations: osmoregulation of outer membrane proteins and virulence determinants in *Vibrio cholerae* require. *toxR.* J. Bacteriol. **170:**2575–2583.
25. **Pogliano, J. A., and J. Beckwith.** 1994. SecD and SecF facilitate protein export in *Escherichia coli.* EMBO J. **13:**554–561.
26. **Rose, R. E.** 1988. The nucleotide sequence of pACYC184. Nucleic Acids Res. **16:**355.
27. **Sakai, T., C. Sasakawa, and M. Yoshikawa.** 1988. Expression of four virulence antigens of *Shigella flexneri* is positively regulated at the transcriptional level by the 30 kiloDalton *virF* protein. Mol. Microbiol. **2:**589–597.
28. **Sandlin, R. C., and A. T. Maurelli.** 1999. Establishment of unipolar localization of IcsA in *Shigella flexneri* 2a is not dependent on virulence plasmid determinants. Infect. Immun. **67:**350–356.
29. **Sansonetti, P. J., and C. Egile.** 1998. Molecular bases of epithelial cell invasion by *Shigella flexneri.* Antonie Leeuwenhoek **74:**191–197.
30. **Schuch, R., R. C. Sandlin, and A. T. Maurelli.** 1999. A system for identifying post-invasion functions of invasion genes: requirements for the Mxi-Spa type III secretion pathway of *Shigella flexneri* in intercellular dissemination. Mol. Microbiol. **34:**675–689.
31. **Shere, K. D., S. Sallustio, A. Manessis, T. G. D'Aversa, and M. B. Goldberg.** 1997. Disruption of IcsP, the major *Shigella* protease that cleaves IcsA, accelerates actin-based motility. Mol. Microbiol. **25:**451–462.
32. **Steinhauer, J., R. Agha, T. Pham, A. W. Varga, and M. B. Goldberg.** 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.
33. **Suzuki, T., M. C. Lett, and C. Sasakawa.** 1995. Extracellular transport of VirG protein in *Shigella.* J. Biol. Chem. **270:**30874–30880.
34. **Taniya, T., J. Mitobe, S. Nakayama, Q. Mingshan, K. Okuda, and H. Watanabe.** 2003. Determination of the InvE binding site required for expression of IpaB of the *Shigella sonnei* virulence plasmid: involvement of a ParB boxA-like sequence. J. Bacteriol. **185:**5158–5165.
35. **Tobe, T., S. Nagai, N. Okada, B. Adler, M. Yoshikawa, and C. Sasakawa.**

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.

36. **Uchiya, K., T. Tobe, K. Komatsu, T. Suzuki, M. Watarai, I. Fukuda, M. Yoshikawa, and C. Sasakawa.** 1995. Identification of a novel virulence gene, *virA*, on the large plasmid of *Shigella*, involved in invasion and intercellular spreading. Mol. Microbiol. **17:**241–250.
37. **Watanabe, H., E. Arakawa, K. Ito, J. Kato, and A. Nakamura.** 1990. Genetic analysis of an invasion region by use of a Tn*3*-lac transposon and identification of a second positive regulator gene, invE, for cell invasion of *Shigella sonnei*: significant homology of *invE* with ParB of plasmid P1. J. Bacteriol. **172:**619–629.
38. **Yamada, H., T. Yoshida, K. Tanaka, C. Sasakawa, and T. Mizuno.** 1991. Molecular analysis of the *Escherichia coli* gene encoding a DNA-binding protein, which preferentially recognizes curved DNA sequences. Mol. Gen. Genet. **230:**332–336.