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An extended role for the nucleoid structuring protein H-NS in the virulence gene regulatory cascade of *Shigella flexneri*.

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Summary

The H-NS nucleoid structuring protein has been shown previously to play a negative role in controlling virulence gene expression in *Shigella flexneri* by repressing transcription of the *virF* and *virB* regulatory genes and the VirF-dependent *icsA* structural gene under non-permissive growth conditions. Here we show that H-NS also acts at the promoters of the VirB-dependent structural genes in the regulatory cascade. H-NS protein binds to the promoter regions *in vivo* and *in vitro*. The promoters were shown physically and by *in silico* analysis to contain regions of DNA curvature, a feature of H-NS binding sites. H-NS binding sites were determined by DNase I footprinting at the *icsB* and the *virA* promoters. The locations of these sites were consistent with a role for H-NS as a transcription repressor. The VirB-dependent structural gene promoters were found to respond directly to the H-NS repressor, revealing a level of control that is additional to that exerted by the H-NS-dependent *virB* activator gene. Moreover, the promoters were sensitive to the level of VirB protein in the cell, requiring a threshold level of VirB to be reached before becoming active. A model is discussed in which the levels of expression of the structural genes reflect the outcome of competition between the countervailing regulatory activities of the H-NS and VirB proteins.

Introduction

Shigella flexneri and closely related bacteria are the etiological agents of bacillary dysentery in humans. The bacterium is a facultative intracellular pathogen and the genes required for virulence are located within a 31-kbp region of a 230-kbp plasmid (Maurelli *et al.*, 1985; Sasakawa *et al.*, 1988) (Fig. 1). This part of the plasmid is known as the entry region and consists of two divergently transcribed operons coding for the secreted Ipa proteins and the *mxi* and *spa* genes that code for a type III secretion system. The Ipa proteins are secreted through the type III secretion system upon contact with host cells lining the lower gut. Ipa-mediated cytoskeletal rearrangements within the host cell direct the uptake of the bacteria into the otherwise non-phagocytic epithelia. The bacterium then escapes from the vacuole that is created during invasion and is free in the host cell cytosol (reviewed in Philpott *et al.*, 2000). The *ipa* genes are in an operon with the *icsB* and *ipg* genes and the promoter is located immediately upstream of *icsB* (Porter and Dorman, 1997a). The *mxi* genes are in an operon with the *ipgDEF* genes with the promoter immediately upstream of *ipgD* (Porter and Dorman, 1997a). The *spa* genes are encoded on the same DNA strand as the *ipg-mxi* operon and have their own promoter preceding *spa47* (Sasakawa *et al.*, 1993) (Fig. 1).

At a transcriptional level, the virulence genes are regulated in response to temperature, osmolarity, and pH. Optimal expression occurs at 37°C (Maurelli *et al.*, 1984), at moderate levels of osmolarity (Porter and Dorman, 1994), and at pH 7.4 (Nakayama and Watanabe, 1995; 1998). Two plasmid-located genes, *virF* and *virB*, code for essential regulatory proteins. The VirF protein, an AraC-like transcription factor, activates the *virB* gene and the VirB protein in turn binds to the structural gene promoters and activates them (Adler *et al.*, 1989; Beloin *et al.*, 2002; Tobe *et al.*, 1991). Chromosomal loci also contribute to the control of the virulence genes (reviewed in Dorman *et al.*, 2001). Among these are the genes coding for the DNA binding

proteins Fis, IHF and H-NS. The Fis protein regulates transcription of the *virF* regulatory gene positively (Falconi *et al.*, 2001) while IHF is a positive regulator of the *virF* and *virB* regulatory genes and of *icsA*, a VirF-dependent structural gene lying outside the entry region on the 230-kbp plasmid (Porter and Dorman, 1997a). In contrast, the contributions made by the H-NS protein are negative (Hromockyj and Maurelli, 1992; Porter and Dorman, 1994).

H-NS is a DNA binding protein of approximately 15 kDa molecular mass (Atlung and Ingmer, 1997; Bertin *et al.*, 1999). It is a major component of the bacterial nucleoid and is present in concentrations of up to 10^5 monomers per cell (Williams and Rimsky, 1997). DNA binding by H-NS appears to be largely independent of nucleotide sequence at the binding site. Instead DNA structure, particularly curvature, is important in determining the affinity of this protein for a particular site (Bracco *et al.*, 1989; Owen-Hughes *et al.*, 1992; Yamada *et al.*, 1990). The H-NS protein can condense DNA (Dame *et al.*, 2000) and constrain supercoils in DNA *in vitro* (Tupper *et al.*, 2000) and *in vivo* (Higgins *et al.*, 1988). It affects the expression of a wide range of genes, usually negatively and usually at the level of transcription (Hommais *et al.*, 2001). However, the molecular detail of how H-NS influences transcription is lacking in most cases. The ability of the protein to oligomerize is central to its function. The amino-terminal domain of the protein is responsible for oligomerization while DNA binding is a function of the carboxyl-terminus. The two functional domains are connected by a flexible linker (Dorman *et al.*, 1999; Smyth *et al.*, 2000; Ueguchi *et al.*, 1996; 1997; Williams *et al.*, 1996).

In *S. flexneri*, H-NS has been shown previously to bind to the promoter regions of the *virF* and *virB* regulatory genes on the 230-kbp plasmid. Its negative influence at the *virF* promoter is antagonized by the Fis protein (Falconi *et al.*, 2001) while at the *virB* promoter, H-NS mediated repression is counteracted by the VirF protein (Tobe *et al.*, 1993). At *virF*, H-NS binds to two sites separated by a region of DNA curvature. Binding to these sites occurs cooperatively at

temperatures below 32°C but not at 37°C, the permissive temperature for virulence gene transcription. The resulting repression complex is sensitive to the supercoiling of the DNA (Falconi *et al.*, 1998). At *virB*, H-NS binds to a single, extended site that intrudes into the region bound by RNA polymerase (Tobe *et al.*, 1993). Activation of the promoter requires a thermally-induced alteration in local DNA supercoiling in the presence of the VirF protein (Tobe *et al.*, 1995).

Previous work has indicated that the promoters of the structural operons differ in the detail of their responses to environmental signals (Porter and Dorman, 1997a; 1997b). The molecular basis of these differences is unknown and may involve inputs from the H-NS protein. While the VirF-dependent *icsA* gene has been shown to be regulated by H-NS (Prosseda *et al.*, 1998), the possibility that H-NS plays a role in regulating the VirB-dependent structural genes in the regulatory cascade has not been examined previously. We decided to investigate this possibility.

Results

Thermoregulation of VirB protein levels requires H-NS

While the effect of H-NS on *virB* gene transcription has been examined before, the influence of H-NS on the level of VirB protein in the cell has not been examined previously. We compared wild type and *hns* mutant strains for VirB expression at 30°C and 37°C by Western blotting. The experiments were carried out on cultures in the mid-exponential phase or the stationary phase of growth. Growth phase had little effect on VirB protein levels (Fig. 2). In contrast, the levels of this protein were strongly dependent on temperature in the wild type strain and on the presence or absence of the H-NS protein (Fig. 2). Interestingly, VirB protein was detectable in the wild type at the non-permissive temperature (30°C), albeit at greatly reduced levels. Elimination of *hns* gene expression removed the sensitivity of VirB expression to temperature. This was fully consistent with the previously described role of H-NS as a repressor of *virB* gene transcription. It was interesting to note that when H-NS was removed from the cell, the level of VirB protein seen at the non-permissive temperature was equal to that at 37°C. This showed that the H-NS protein played the major role in constraining VirB protein expression at low temperature.

VirB-dependent promoters interact with H-NS in vitro

To examine the possibility that H-NS interacted with the VirB-dependent structural gene promoters, electrophoretic mobility shift assays were carried out with crude cell extracts using a buffer system optimized for DNA binding by H-NS. The labelled DNA fragments used in these experiments were PCR products generated by amplification of the promoter regions of the *virB*, *virA*, and *spa* genes and the intergenic promoters region of the *icsB-ipgD* genes (see Materials and Methods). The crude cell extracts were prepared from wild type cells and from mutants deficient in one of the following proteins: VirB, VirF, H-NS, IHF, Lrp, or StpA. The results

obtained indicated that the promoter regions failed to alter their electrophoretic mobilities only when treated with extracts from the mutant deficient in the H-NS protein (Fig. 3). This provided preliminary evidence that this protein could interact with all of these DNA sequences. However, the possibility remained that H-NS did not interact with the promoters directly, but instead regulated some other cellular component that was absent in the *hns* mutant.

To examine the role of H-NS at the promoters more precisely, electrophoretic mobility shift assays were carried out using purified H-NS protein *in vitro*. These were performed using a mix of each relevant promoter (PCR-amplified) with *TaqI-SspI*-digested pBR322 plasmid restriction fragments as DNA competitors. Plasmid pBR322 contained a copy of the *bla* gene, whose promoter region encompasses a high affinity curved DNA binding site for H-NS (Lucht *et al.*; 1994; Zuber *et al.*, 1994). The DNA fragments were incubated with increasing concentrations of H-NS protein. The results obtained showed that in each case the *bla*, *virB*, *virA*, and *spa* promoters and the *icsB-ipgD* intergenic promoter region were shifted specifically by the H-NS protein while the other plasmid DNA fragments were not (Fig. 4). The interaction of H-NS with the virulence gene promoters was as strong or stronger than that with the *bla* positive control. These data clearly demonstrated that H-NS bound to the virulence gene promoters.

The virulence gene promoters contain regions of DNA curvature

Intrinsic DNA curvature is a feature of many H-NS binding sites. Such sites rarely display strong DNA sequence similarity, and that is true of the virulence gene promoters studied here.

Furthermore, the *virF*, *virB*, and *icsA* promoters on the virulence plasmid that have already been shown to bind H-NS all display intrinsic curvature (Prosseda *et al.*, 1998). When the promoter regions of *virB*, *virA*, *spa*, and *icsB-ipgD* were examined *in silico* using the bend-it computer program (http://www2.icgeb.trieste.it/~dna/bend_it.html), all four were found to possess regions

of curvature (Fig. 5). The promoter sequences were examined physically for curvature by electrophoresis in 8% polyacrylamide at 4°C. Under these conditions curvature caused the DNA fragments to migrate aberrantly in the gel, consistent with results obtained by others working with curved DNA (Ussery *et al.*, 1999; Yamada *et al.*, 1990). This was seen when their actual length in bp was compared with their apparent length as measured electrophoretically. The results obtained (apparent values in parentheses) for each promoter fragment were as follows: *virB* 350-bp (380-bp); *virA* 359-bp (410-bp); *spa* 350-bp (380-bp); *icsB-ipgD* 381-bp (550-bp). All of the sequences were retarded in gel migration under conditions known to enhance the effects of sequence-mediated DNA curvature on electrophoretic mobility. This was consistent with the known preference of H-NS for curved DNA and the H-NS binding data obtained for these sequences in this study.

Footprinting H-NS on virulence gene promoters

To obtain more precise information about the interaction of H-NS with the promoters, we performed DNase I footprint analyses for two of them (*icsB* and *virA*). In the case of *icsB*, the H-NS footprints extended from -88 to +56 with respect to the transcription start site (+1). In the case of *virA*, the footprints extended from -140 to +37 (Fig. 6). In both cases, the areas of H-NS-DNA interaction were consistent with a role for H-NS in impeding the binding of RNA polymerase to the promoter. A similar pattern has been described previously for the *virB* promoter (Tobe *et al.*, 1993).

Direct regulation of the virulence genes by H-NS

Having determined that H-NS binds to the promoter regions of VirB-dependent structural genes, we wanted to assess the consequences of this binding for gene expression. It was important to

avoid confusion arising from the fact that H-NS regulates the structural genes indirectly via its role at the *virB* promoter. Therefore, the experiment was carried out in *E. coli* in the absence of the VirB activator protein. This experiment measures the direct influence of H-NS on the basal level of virulence gene expression when VirB is absent. Each promoter to be analyzed was cloned in the *lacZ* reporter plasmid pQF50 Ω Sm and introduced into wild type *E. coli* strain MC4100 or its *hns* null mutant derivative. Where appropriate, the *hns* null mutation was complemented by introduction of the H-NS expressing plasmid pAF201. This plasmid was compatible with pQF50 Ω Sm and its derivatives. The VirB-independent, H-NS-independent *hupA* promoter was used as a negative control. Knowing from our previous work that plasmid copy number could be affected in *hns* mutant strains (Deighan *et al.*, 2000), β -galactosidase data were normalized with respect to levels of linearized pQF50 Ω Sm plasmid DNA extracted concomitantly from samples used for β -galactosidase assays.

The *virB* promoter was included as a positive control. It was studied in the absence of the VirF activator so only basal activity was detected. As expected, this promoter had higher activity in the absence of H-NS and this elevated activity was repressed when the pAF201 plasmid harbouring a wild type *hns* gene was introduced (Fig. 7). In the absence of the VirF thermo-regulatory protein, the *virB* promoter displayed little thermo-regulation.

The *icsB* promoter was found to retain residual thermo-responsiveness in wild type *E. coli* even in the absence of the VirB activator (Fig. 7). When the *hns* gene was inactivated, *icsB* activity increased. The thermal response was retained. When the *hns* null mutation was complemented, *icsB* activity decreased to low levels, and thermal sensitivity was still detectable.

In contrast to the *icsB* promoter, none of the other three VirB-dependent promoters tested (*ipgD*, *virA*, *spa*) retained thermal responsiveness when studied in wild type *E. coli* strain

MC4100 (Fig. 7). All three showed strong increases in activity at 30°C when the *hns* gene was inactivated. Interestingly, the promoters were now found to be regulated negatively when shifted to 37°C in the *hns* null background. When the pAF201 plasmid was introduced, all three promoters became repressed once again. These unanticipated data showed that not only did H-NS play a role in regulating the VirB-dependent structural gene promoters, in the case of three (*ipgD*, *virA*, *spa*) it influenced the direction of the response to temperature.

The structural gene promoters respond to VirB protein levels

The foregoing experiments had established a role for the H-NS protein in directly modulating the activities of the VirB-dependent structural gene promoters. This leads to a model in which one envisages an antagonistic relationship between the negatively-acting H-NS protein and the positively acting VirB protein at these promoters. We knew that the VirB-dependent promoters were repressed at 30°C and that H-NS played a role in this repression. We also knew that VirB protein levels were very low under these conditions (Fig. 2). It was decided to examine the effect of artificially raising the level of the VirB protein in the cell at 30°C to see if this could overcome the repressive activity of H-NS. This experiment was carried out in *S. flexneri* strain BS184 which harbours a *lacZ* fusion to the *mxiC* gene within the *ipgD* operon (Fig. 1). The *virB* gene was cloned under the control of an arabinose-inducible promoter. VirB protein levels were manipulated by varying the concentration of arabinose inducer added to the 30°C culture and the protein levels were monitored by Western blotting (Fig. 8). A clear positive correlation was seen between VirB protein levels and the level of expression of the *mxiC-lacZ* fusion, a reporter of *ipgD* promoter activity (Fig. 8). This showed that the VirB protein level was the key determinant of virulence gene expression and that artificial overexpression of VirB could overcome all repressive influences under otherwise non-permissive growth conditions.

Discussion

In this study we investigated the interaction of the H-NS nucleoid structuring protein with the promoters of the VirB-dependent virulence genes on the 230-kbp plasmid in *S. flexneri* and the interplay between H-NS and the VirB activator protein at those promoters. H-NS was found to bind to the structural gene promoters and to affect directly their function. In addition, new information was obtained on the expression of the VirB protein and its effects on virulence gene transcription. This information allows a refinement of current understanding of the *S. flexneri* virulence gene regulatory cascade.

Using Western blotting, we showed that in wild type cells the level of expression of the VirB protein clearly changes with temperature, as one would anticipate from a consideration of the published data on temperature and the transcription of the *virB* gene (Day and Maurelli, 2001; Porter and Dorman, 1997b; Tobe *et al.*, 1991; 1993; 1995). Specifically, the level of the protein is much higher at 37°C than at 30°C (Fig. 2). However, it is still clearly detectable at the lower temperature. This raises the question of why the VirB-dependent promoters are repressed when the VirB protein is so clearly present? Presumably, there is a threshold level which the VirB protein concentration must reach before structural gene activation is possible. The results of the experiments in which we manipulated artificially the expression of the VirB protein support this hypothesis. Expressing the protein from an arabinose-inducible promoter at 30°C produces a clear correlation between VirB levels and transcription of a reporter gene fused to *mxiC*, a member of the *ipgD* operon (Fig. 8). This is in spite of the fact that the experiment was performed at a temperature at which the virulence genes are usually repressed. This means that the key issue in promoter activation is the VirB concentration, and not a temperature-dependent modification of VirB, or binding by VirB of a ligand only present at elevated temperatures, or temperature-dependent oligomerization of VirB either in solution or on the DNA. This last point

is important in the light of recent data showing that the VirB protein is capable of forming dimers and higher order oligomers *in vivo* and *in vitro* (Beloin *et al.*, 2002). However, it is possible that progressive oligomerization of VirB along DNA forms an important part of the mechanism by which it displaces H-NS and activates transcription (Fig. 9). Our data demonstrate that thermal activation of the cascade occurs upstream of VirB translation, a view that is consistent with the effects of temperature on the promoters of the *virF* and *virB* regulatory genes (Dorman *et al.*, 2001; Falconi *et al.*, 1998; Tobe *et al.*, 1991; 1993; 1995).

Data presented in this study show that the direct effects of the H-NS protein are not restricted to the *virF*, *virB*, and *icsA* promoters (Prosseda *et al.*, 1998), they also encompass the *ipgD*, *icsB*, *spa*, and *virA* promoters. At *virF*, H-NS participates in a cooperative interaction involving two H-NS binding sites that repress the promoter below a critical temperature (32°C). The facility with which this repression complex forms is thought to be determined by thermally-induced conformational changes in the DNA structure (Falconi *et al.*, 1998). At *virB*, H-NS occupies a portion of the DNA that includes the binding site for RNA polymerase. It is thought that the binding of the VirF activator to the *virB* promoter may disrupt the H-NS-DNA repression complex. VirF is present and bound to the DNA at the non-permissive temperature and so it has been proposed that a thermally-induced change in local DNA supercoiling causes a transition in the VirF-DNA complex from a neutral to an activation mode in which H-NS is displaced (Dorman and Porter, 1998; Tobe *et al.*, 1993; 1995). A similar situation may exist at the VirF-dependent *icsA* promoter, although details are lacking (Prosseda *et al.*, 1998).

In this study we present data suggesting that a superficially similar situation may also occur at the VirB-dependent promoters, with the major difference being that the activator protein is VirB and not VirF. It should be stressed that VirB and VirF are structurally completely distinct (Beloin *et al.*, 2002; Porter and Dorman, 2002) so they may be expected to differ in the detail of

their functions in gene activation. In this model, the H-NS protein binds to the VirB dependent promoters at 30°C and represses them (Fig. 9). We have presented data showing that H-NS does indeed bind to these promoters *in vitro* (Fig. 3 and 4) and that these sequences have the structural features that are characteristic of high affinity H-NS binding sites (Fig. 5). Furthermore, the locations of the H-NS binding sites at the promoters subjected to footprint analysis are consistent with H-NS acting to exclude or impede RNA polymerase (Fig. 6). Although the VirB protein is present in the cell at the non-permissive temperature (Fig. 2) it is unable to activate its target promoters because its concentration is below the required threshold level. When the culture is shifted to 37°C, VirB levels increase and reach values compatible with target promoter activation (as discussed above). Activation may involve displacement of the H-NS protein from the promoters, an issue that will be addressed in future research. Alternatively, it might involve a modification of the H-NS-DNA complex such that RNA polymerase can interact productively with the promoter. VirB may also play a direct role in recruiting RNA polymerase to the promoter and it may subsequently be involved in assisting it to form an open complex. Polymerization of VirB along the DNA may contribute to any or all of these events (Fig. 9). Insufficient detail is available at present to allow these issues to be elucidated. What is clear is that VirB can overcome the repressive effects exerted by H-NS at 30°C if VirB is overexpressed by artificial means (Fig. 8).

The VirB-dependent promoters respond in two ways to the absence of H-NS protein when studied in an *hns* null mutant in the absence of VirB (Fig. 7). The first pattern is displayed by the *icsB* promoter. Here removal of H-NS results in increased levels of expression with retention of some temperature control reflective of the thermal regulation seen in wild type *S. flexneri*. The second pattern is shared by *ipgD*, *virA*, and *spa*. In this case, removal of H-NS in a VirB-deficient background results in a strong increase in promoter activity at 30°C which is repressed at 37°C.

This is a reversal of the normal thermal response of these three promoters and may reflect an underlying temperature responsive mechanism, possibly at the level of DNA structure, that requires the presence of H-NS to direct or constrain it. It illustrates the sophistication of this regulatory cascade and the caution that is needed in studying its components in isolation. It also correlates with previous results obtained with these promoters in their native *S. flexneri* background which showed that they differ in the detail of their responses to environmental stimuli (Porter and Dorman 1997b).

It is important to note that the switch controlling expression of the virulence genes is not binary, that is, not simply on or off. Instead, the genes are regulated over a range of values that reflect directly the level of the VirB protein in the cell (Fig. 9). This may be due to the fact that the repressive influence of H-NS must be overcome in a progressive manner. If so, such a mechanism would conspire to constrain the virulence system to be expressed fully only in optimal conditions. In this way, wasteful expression of the type III secretion system, its effector proteins and ancillary factors may be avoided and their full deployment reserved for authentic contact with the host.

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Experimental procedures

Bacterial strains and growth conditions

The bacterial strains and plasmid used in this study are listed in Table 1. Bacteria were routinely grown in Luria-Bertani broth (LB). Appropriate antibiotics were used at the following concentrations: carbenicillin, 50 $\mu\text{g ml}^{-1}$; chloramphenicol, 25 $\mu\text{g ml}^{-1}$; kanamycin, 50 $\mu\text{g ml}^{-1}$; spectinomycin, 50 $\mu\text{g ml}^{-1}$; streptomycin, 50 $\mu\text{g ml}^{-1}$; tetracycline, 10 $\mu\text{g ml}^{-1}$.

Plasmid construction

All polymerase chain reactions (PCR) for cloning were performed with the proof-reading Pfx polymerase purchased from the Gibco BRL/*In vitro* gene company using a colony of the BS184 strain of *Shigella flexneri* 2a. The *virB*-bearing plasmid pBAD33*virB* (Table 1) was constructed by cloning a blunt-end/*SalI* fragment digested from a 1,006-bp PCR-based amplified region of *virB* using the primer pair *virB5* (5'-CTT GCT CAC ACT GCA TTT AAC-3') and *virB3* (5'-ATC AGT gtc gac TGT TTA TGA AGA CGA TAG ATG-3', *SalI* site in lower case) and ligated into *SmaI/SalI*-digested sites within pBAD33. The integrity of the *virB* gene in pBAD*virB* was verified by sequencing.

Oligonucleotide primer pairs for the promoter sequences were engineered to contain *BclI* and *XbaI* (*virB*), or *BglII* and *XbaI* (*hupA*, *spa*), or *BglII* and *NcoI* (*icsB*, *ipgD*, *virA*) restriction sites for cloning into the promoterless *lacZ* vector pQF50 Ω Sm (Table 1). Each PCR product was digested and ligated into pQF50 Ω Sm digested by *BglII* and *XbaI* (*virB*, *hupA*, *spa*) or *BglII* and *NcoI* (*icsB*, *ipgD*, *virA*). The integrity of the different cloned regulatory regions was verified by

sequencing. Plasmids were electrotransformed into *E. coli* MC4100, PD32 and CJD1631 (PD32pAF201) (Table 1).

Curvature predictions and analysis

Curvature predictions of regulatory regions of the *virB*, *virA*, *spa*, *icsB-ipgD* genes/operons were performed using the bend-it program (http://www2.icgeb.trieste.it/~dna/bend_it.html). The curvature is calculated as a vector sum of dinucleotide geometries (roll, tilt and twist angles) using the BEND algorithm of Godsell and Dickerson (1994), and expressed as degrees per helical turn ($10.5^\circ/\text{helical turn} = 1^\circ/\text{basepair}$). Experimentally tested curved motifs produce curvature values of 5 to $25^\circ/\text{helical turn}$, while straight motifs give values below $5^\circ/\text{helical turn}$. The intrinsic curvature of PCR-amplified DNA fragments was studied by comparing their electrophoretic mobility to that of a non-bent marker fragment (Promega 100-bp DNA ladder) on a 8% polyacrylamide gel run in 0.5X TBE (45 mM Tris-HCl, 45 mM H_3BO_3 , 1,25 mM Na_2EDTA) at 4°C (Ussery *et al.*, 1999; Yamada *et al.*, 1990). The regulatory regions analyzed correspond to: *spa* promoter nt -260 to +90 with respect to the +1 transcription initiation site (350-bp); *virA* promoter nt -248 to +111 (359-bp); *icsB-ipgD* intergenic region nt -94 to +286 with regards to the transcription initiation site of *icsB* (380-bp); *virB* promoter nt -265 to +85 (350-bp).

Preparation of crude protein extracts

Crude protein extracts were prepared from 100 ml cultures of BS184 and its *virB*, *virF*, *hns*, *ihf*, *lrp* and *stpA* mutant derivatives (Table 1). Cultures of the respective strains were grown overnight, sub-inoculated 1:500 into fresh LB broth and grown at 37°C to an OD_{600} of 1. The cells were harvested by centrifugation and resuspended in 1 ml ice cold sonication buffer (10%

sucrose-50 mM Tris HCl (pH 7.5)-100 mM NaCl-1 mM EDTA-5 mM dithiothreitol (DTT)-0.1 mM phenylmethanesulfonyl fluoride (PMSF)). Upon resuspension 7.5 μ l of lysozyme (10 mg ml^{-1}) was added to each sample and the mixture was frozen overnight at -20°C . Following thawing on ice, the mixture was sonicated with a MSE Soniprep sonicator (Sanyo) with 3 pulses at an amplitude setting of 5 for 30 seconds, allowing 30 seconds cooling between each pulse, cell debris were pelleted by centrifugation at 4°C . Lysate supernatants were mixed with an equal volume of 100% glycerol and stored at -20°C . Protein concentration of the lysates was determined using the Bradford assay (Bradford, 1976).

Electrophoretic mobility shift assays

Crude extracts electrophoretic mobility shift assays were performed as follows: PCR fragments of the various promoters under study were amplified using Pfx polymerase. They corresponded to the same fragments used in the intrinsic curvature analysis. They were end-labelled using $[\gamma\text{-}^{32}\text{P}]\text{dATP}$ and purified from unincorporated label by using the High Pure PCR Product Purification kit (Roche). Approximately 1 ng of probe was used for each 20 μ l of reaction mixture in 25 mM Tris HCl (pH 7.5)-50 mM potassium glutamate- 0.1 mM EDTA-10% glycerol-0.5 mM DTT-0.1 mM PMSF. Reactions were initiated by the addition of 12.5 μ g of bacterial lysate and incubated for 10 min at room temperature. The samples were electrophoresed through a 6% polyacrylamide gel in 0.5X TBE at 4°C . Radioactive fragments were visualized by autoradiography with Hyperfilm-MP film (Amersham).

pBR322 competitive electrophoretic mobility shift assays were performed as follows: PCR fragments used in the crude extract mobility shift assay were mixed in an equimolar ratio with DNA fragments (~ 100 ng of each fragment) obtained by digestion of plasmid pBR322 with *TaqI*

and *SspI* restriction enzymes and incubated for 20 min at room temperature with native pure H-NS (a gift from Dr Sylvie Rimsky) in 20 μ l of reaction mixture containing 40 mM HEPES (pH 8)-100 mM potassium glutamate-10 mM magnesium aspartate-10% glycerol-0.022% NP40-0.1mg ml⁻¹ BSA (H-NS binding buffer). Protein-DNA complexes were resolved through a 3% Molecular Screening (MS) agarose gel (Roche) in 0.5X TBE at room temperature. After migration the gels were stained using ethidium bromide.

DNase I footprinting

A fragment of the *icsB* and *virA* regulatory regions was amplified by PCR using the following primers: *icsB*, *icsB5* (5'-ATT TCA gaa ttc AAC GAG CAC TAC ATA TGC-3', *EcoRI* site in lower case) and *icsB3* (5'-TAT AGG gga tcc TGC GTC AAT-3', *BamHI* site in lower case) leading to a PCR fragment corresponding to nt -94 to +113 with respect to the transcription initiation site (+1) of *icsB* transcription; *virA*, *virA5* (5'-CAC CCA gga tcc CTA TCT GCC CCT TGA TGT-3', *BamHI* site in lower case) and *virA3* (5'-CAG TTG gaa ttc ATG ATG AGT CAT TTC TTT-3', *EcoRI* site in lower case) leading to a PCR fragment corresponding to nt -191 to +44 with respect to position +1 of *virA* transcription. PCR fragments were gel-purified and after overnight digestion with *BamHI* or *EcoRI*, the DNA was labelled with [α -³²P]dATP by Klenow polymerase. Unincorporated label was removed by using the High Pure PCR Product Purification Kit (Roche).

Increasing amounts of H-NS were incubated for 15 min at 30°C with labelled *icsB* or *virA* promoter region DNA in 20 μ l of H-NS binding buffer. DNase I was added (2 μ l of a 0.1 U μ l⁻¹ of RQ1 DNase I in 10 mM Tris HCl (pH 8)-10 mM CaCl₂-10 mM MgCl₂-125 mM KCl) and the reaction incubated at 30°C for a further 1 min. Reaction was stopped by transferring the tube to

ice and adding 150 μl of ice cold stop solution (400 mM sodium acetate [pH 5.2]-2.5mM EDTA [pH 8]-50 $\mu\text{g ml}^{-1}$ yeast tRNA-5 $\mu\text{g ml}^{-1}$ sonicated salmon sperm DNA). DNA was extracted with 250 μl of phenol and ethanol precipitated before being analyzed through a 6% polyacrylamide sequencing gel. Protected bands were identified by comparison with a DNA sequence ladder generated with appropriate primers.

β -galactosidase assays

Transcription of *hupA*, *virB*, *icsB*, *ipgD*, *virA* and *spa-lacZ* fusions constructed in the pQF50 Ω Sm plasmid and of the *mxiC-lacZ* fusion in strain BS184 were monitored by β -galactosidase assay (Miller, 1992) of cells cultured overnight in LB broth. Assays were performed in duplicate, and the data are expressed as the means of two measurements. The standard deviation was calculated. Experiments were performed at least three times. β -galactosidase activity specified by derivatives of pQF50 Ω Sm was normalized with respect to the plasmid copy number. Plasmid copy number was estimated by comparison of the levels of linearized pQF50 Ω Sm derivatives plasmid DNA extracted from each strain concomitantly with samples for the β -galactosidase assay. As a control, chromosomal DNA was also extracted from the same samples and was at the same concentration for each strain. For each plasmid *lacZ* fusion the data were expressed as relative β -galactosidase activities with the activity measured in MC4100 (wt for *hns*) at 30°C being set to 1.

Immunodetection of VirB

Total protein extracts were separated through 12% SDS-PAGE. The separated proteins were electro-blotted onto a nitrocellulose membrane using the Biorad mini-protean II system for 1 hour

at 80 V. Nitrocellulose membranes were stained with Ponceau (0.2% Ponceau dye, 3% trichloroacetic acid) to check efficiency of transfer, before being blocked overnight with 5% dried skimmed milk in phosphate buffered saline (PBS). Detection of VirB was performed in PBS containing 1% dried skimmed milk with a primary polyclonal anti-VirB antiserum (1:500) (Beloin *et al.*, 2002) and a secondary goat anti-rabbit HRP-conjugated antiserum (1:10000). Membranes were developed using the chemiluminescent Pierce West Pico Super Signal kit.

Table 1. Bacterial strains and plasmids^a

Strain or plasmid	Genotype ^b	Reference or source
Strain		
<i>Shigella flexneri</i> 2a		
BS184	<i>mxiC</i> ::Mdl1734 (<i>lacZ</i> -Kan ^r)	Maurelli <i>et al.</i> , 1984
BS185	BS184 <i>hns</i> ::Tn10	Maurelli and Sansonetti, 1988
CJD1006	BS184 <i>virF</i> ::Ap ^r	Porter and Dorman, 1997c
CJD1018	BS184 <i>virB</i> ::Ap ^r	Porter and Dorman, 1997c
CJD1103	BS184 <i>ihfA</i> Δ82::Tn10	Porter and Dorman, 1997a
CJD1197	BS184 <i>lrp</i> ::Tn10	Porter, 1998
CJD1199	BS184 Δ <i>stpA</i> ::Tn10	Porter, 1998
<i>Escherichia coli</i>		
MC4100	F ⁻ λ ⁻ <i>araD139</i> Δ(<i>argF-lac</i>)U169 <i>rpsL150 relA1 flbB5301 deoC</i> <i>ptsF25 rbsR</i>	Casadaban, 1976
PD32	MC4100 <i>hns-206</i> ::Ap ^r	Dersch <i>et al.</i> , 1993
CJD1631	PD32pAF201	This laboratory
Plasmid		
pBR322	Cloning vector	Bolivar <i>et al.</i> , 1977
pBAD33	Expression vector, arabinose inducible promoter	Guzman <i>et al.</i> , 1995
pAF201	pACYC184 <i>hns</i>	Free, 1995
pBAD <i>virB</i>	<i>virB</i> gene cloned in pBAD33	This study
pQF50ΩSm	<i>lacZ</i> promoter less pQF50 based vector, Sp ^r /Sm ^r	Deighan <i>et al.</i> , 2000
pQFpro <i>hupA</i>	pQF50ΩSm derivative carrying the <i>hupA</i> regulatory region (nt -263 to +149) ^c	This study
pQFprovirB	pQF50ΩSm derivative carrying the	This study

	<i>virB</i> regulatory region (nt -372 to +73) ^c	
pQFpro <i>icsB</i>	pQF50ΩSm derivative carrying the <i>icsB</i> regulatory region (nt -294 to +94) ^c	This study
pQFpro <i>ipgD</i>	pQF50ΩSm derivative carrying the <i>ipgD</i> regulatory region (nt -182 to +206 regards to P1) ^c	This study
pQFpro <i>virA</i>	pQF50ΩSm derivative carrying the <i>virA</i> regulatory region (nt -540 to +74) ^c	This study
pQFpro <i>spa</i>	pQF50ΩSm derivative carrying the <i>spa-47</i> regulatory region (nt -200 to +69) ^c	This study

^a Ap^r, ampicillin resistance; Kan^r, kanamycin resistance; Sp^r, spectinomycin resistance; Sm^r, streptomycin resistance

^b nt, nucleotide.

^c positions given referred to +1 of transcription.

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FIGURES

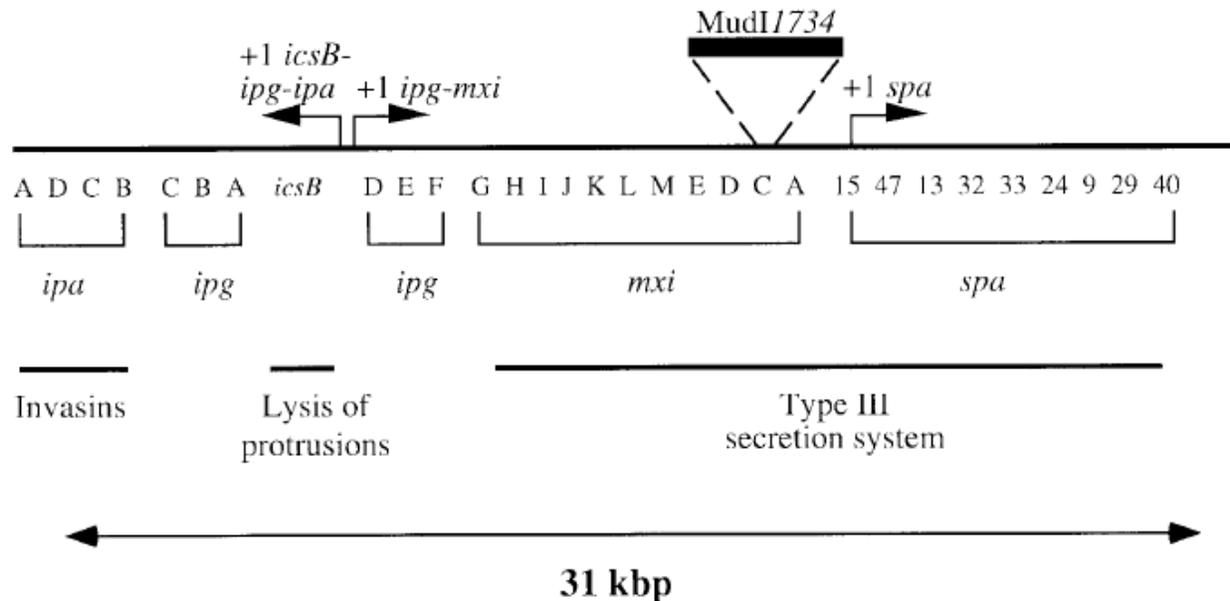


Fig. 1. Schematic map of the 31-kb virulence entry region of *Shigella flexneri* 2a strain BS184. The locations of the promoters of the three operons encompassed in this region are indicated by angled arrows (Sasakawa *et al.*, 1993; Porter and Dorman, 1997a). The position of the *MdI1734* transcriptional fusion to *mxiC* is shown by dashed lines. Not to scale.

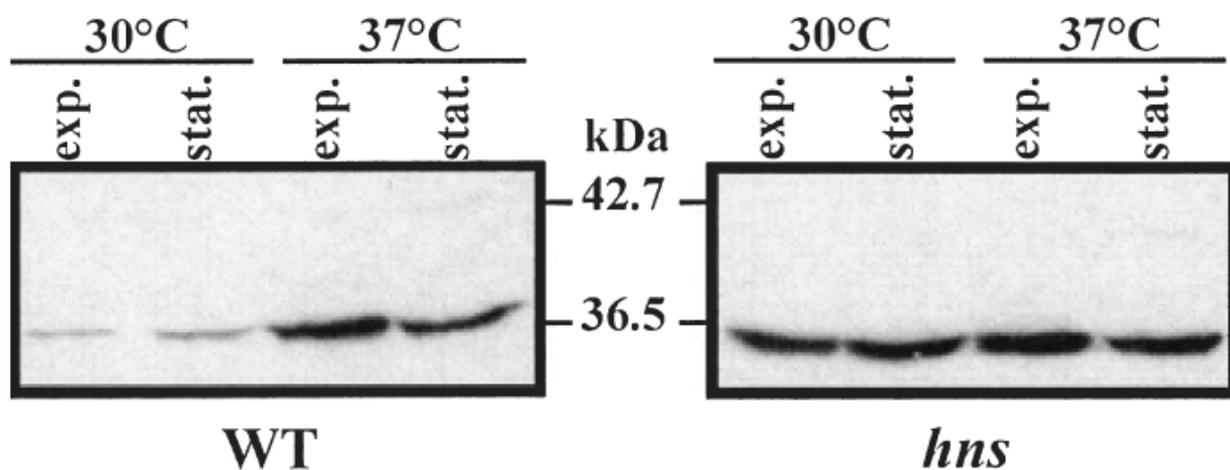


Fig. 2. Temperature and H-NS-dependent production of the VirB protein. *S. flexneri* BS184 (WT)

and BS185 (*hns*) were cultured in LB broth to mid-exponential phase or stationary phase at 30°C or 37°C. Crude protein extracts were then prepared and proteins separated through a 12% SDS-PAGE. VirB protein was visualized by immunoblotting with polyclonal anti-VirB antiserum.

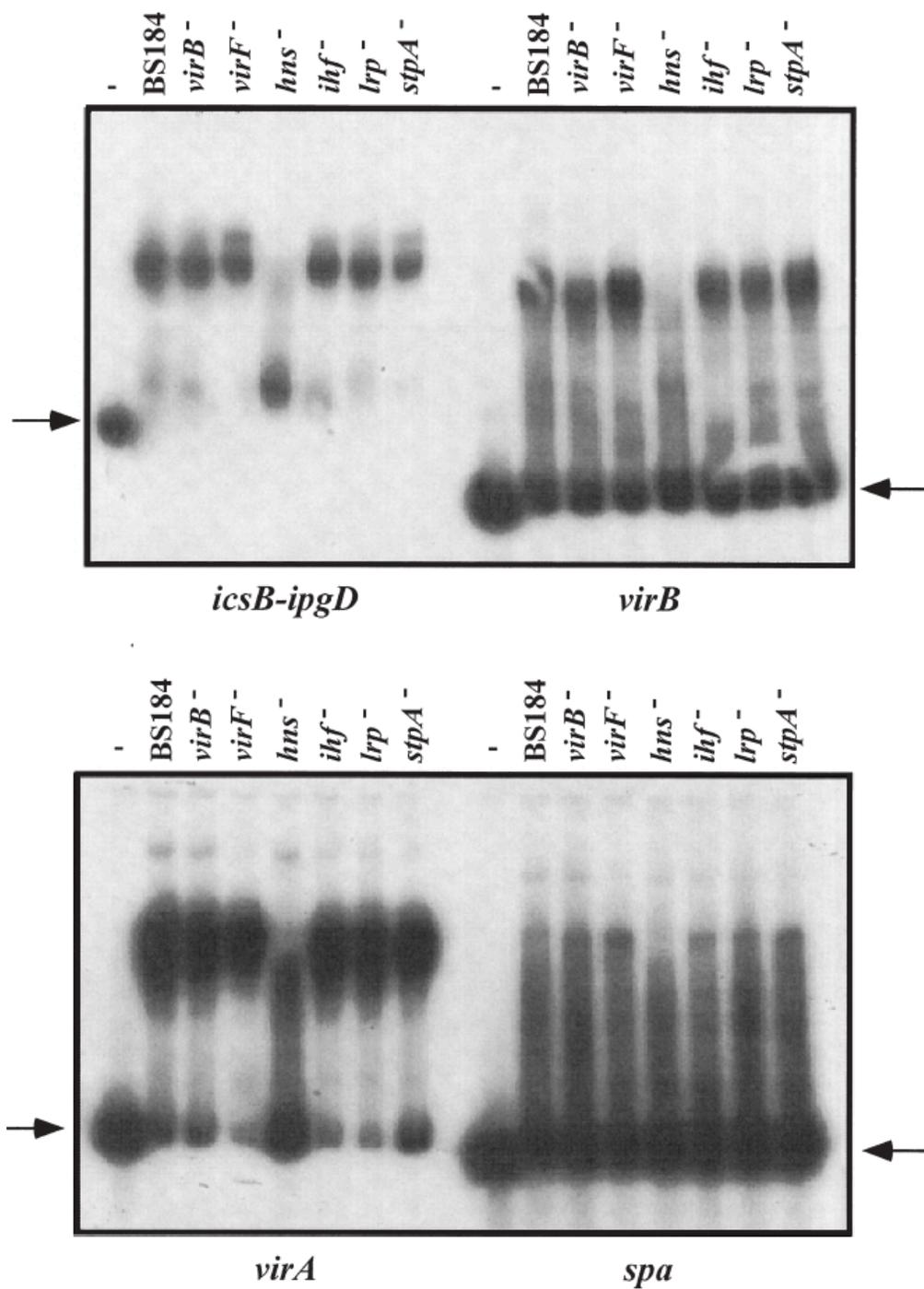


Fig. 3. Retardation of fragments of the *icsB-ipgD* intergenic region and *virB* (upper panel), and *virA* and *spa* (lower panel) promoter regions with crude cell extracts from BS184 and its *virB*, *virF*, *hns*, *ihf*, *lrp* and *stpA* mutant derivatives (Table 1). Complexes were resolved through a 6% polyacrylamide gel in 0.5X TBE at 4°C. Arrows correspond to unbound DNA.

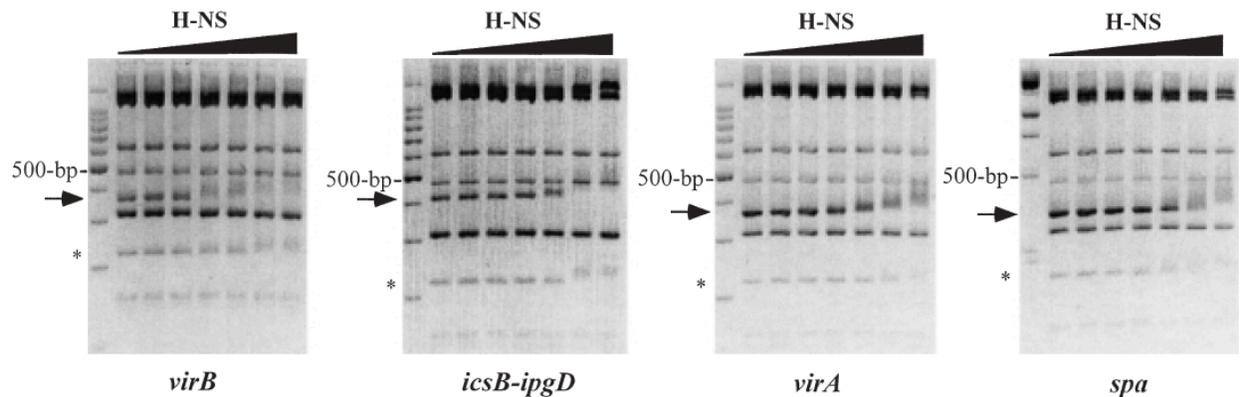
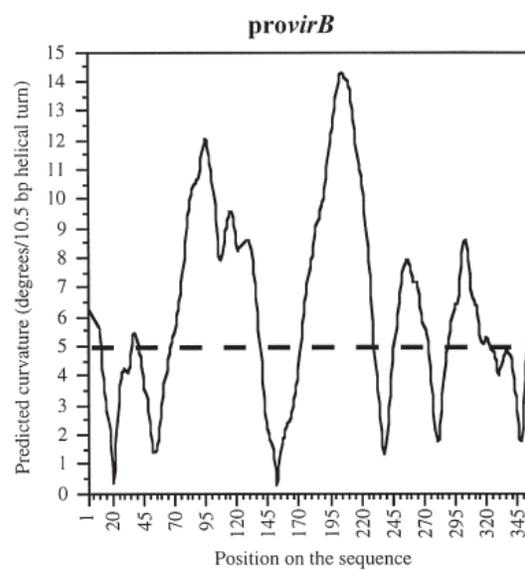
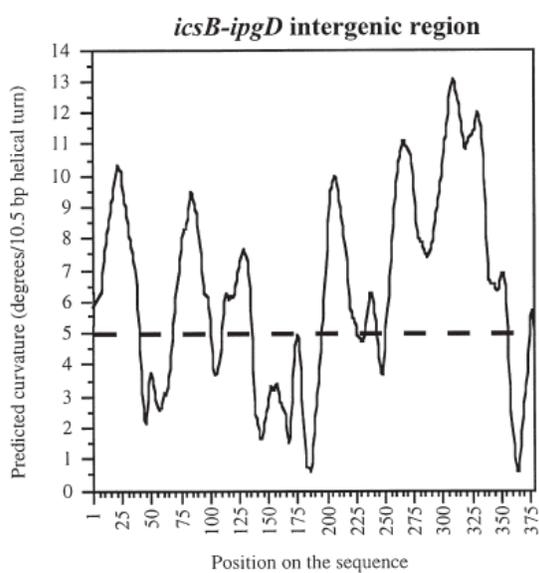
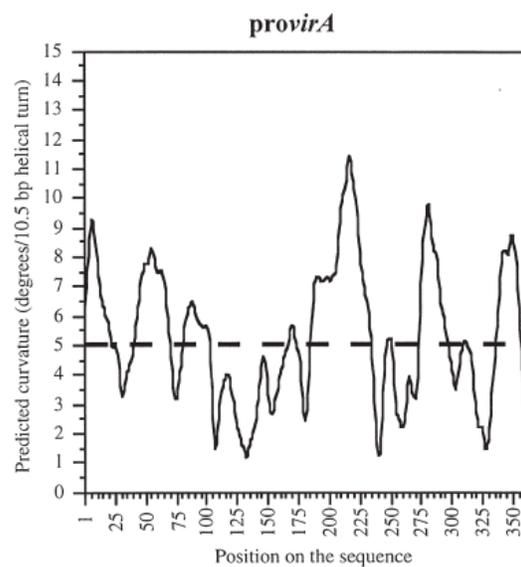
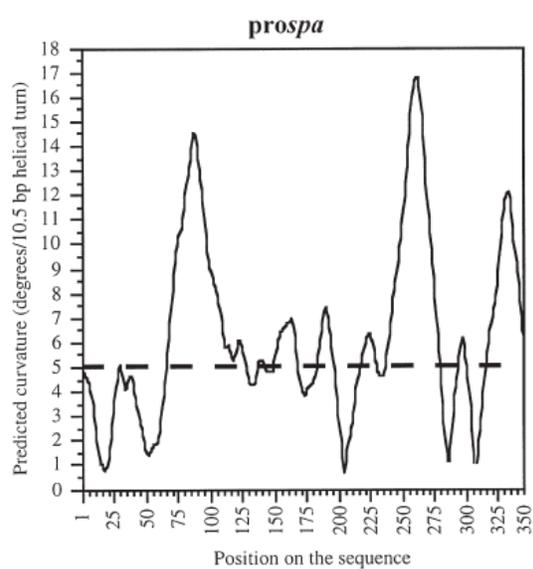


Fig. 4. Competitive electrophoretic mobility shift assay with H-NS and mixtures of the *virB*, *icsB-ippD*, *virA*, or *spa* promoter regions and restriction fragments derived from plasmid pBR322. The plasmid pBR322 DNA was digested by *TaqI* and *SspI* and the restriction fragments resulting from these digestions were mixed with PCR fragments of the different promoter regions. The DNA mixture was incubated with increasing concentrations of H-NS (0, 0.05, 0.1, 0.2, 0.5, 1 and 2 μM). After protein-DNA complex formation, the fragments were resolved in a 3% MS agarose gel. The arrow indicates the position of the different *S. flexneri* virulence gene promoters whereas the asterisk indicates the position of the fragment containing the curved *bla* promoter. The latter is a positive control known to bind H-NS. The other plasmid DNA bands are not thought to bind the protein with high affinity and are negative controls. The first lane corresponds to a 100-bp DNA ladder and the 500-bp marker is indicated at the left of each panel.

A



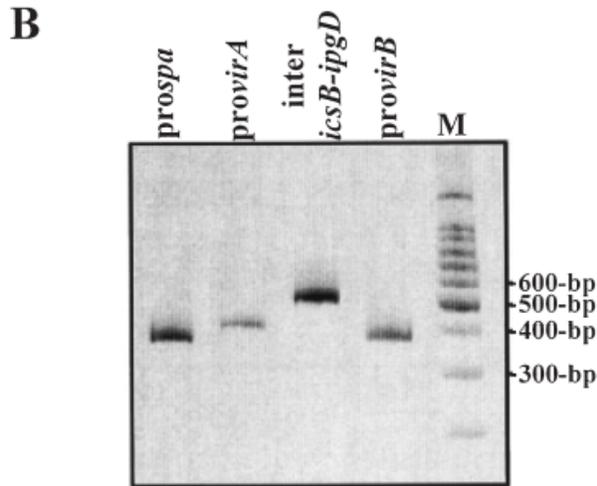


Fig. 5. Analysis of the intrinsic curvature of *virB*, *icsB-ipgD*, *virA*, *spa* promoter regions.

A. Computer prediction of the intrinsic curvature of *spa*, *virA*, and *virB* regulatory regions and *icsB-ipgD* intergenic region was performed using the bend-it program

(http://www2.icgeb.trieste.it/~dna/bend_it.html). Predicted curvature was expressed in degrees/helical turn and plotted against the position on the sequence. Motifs with values below 5 degrees/helical turn (dashed line) correspond to straight motifs whereas motifs with values above 5 degrees/helical turn can be considered as curved.

B. The PCR-generated *spa* (*prospa* 350-bp), *virA* (*provirA* 359-bp), *icsB-ipgD* (*intericsB-ipgD* 380-bp) and *virB* (*provirB* 350-bp) regulatory regions were run on a 8% polyacrylamide gel in 0.5X TBE at 4°C. Lane M corresponds to non-bent DNA marker fragments.

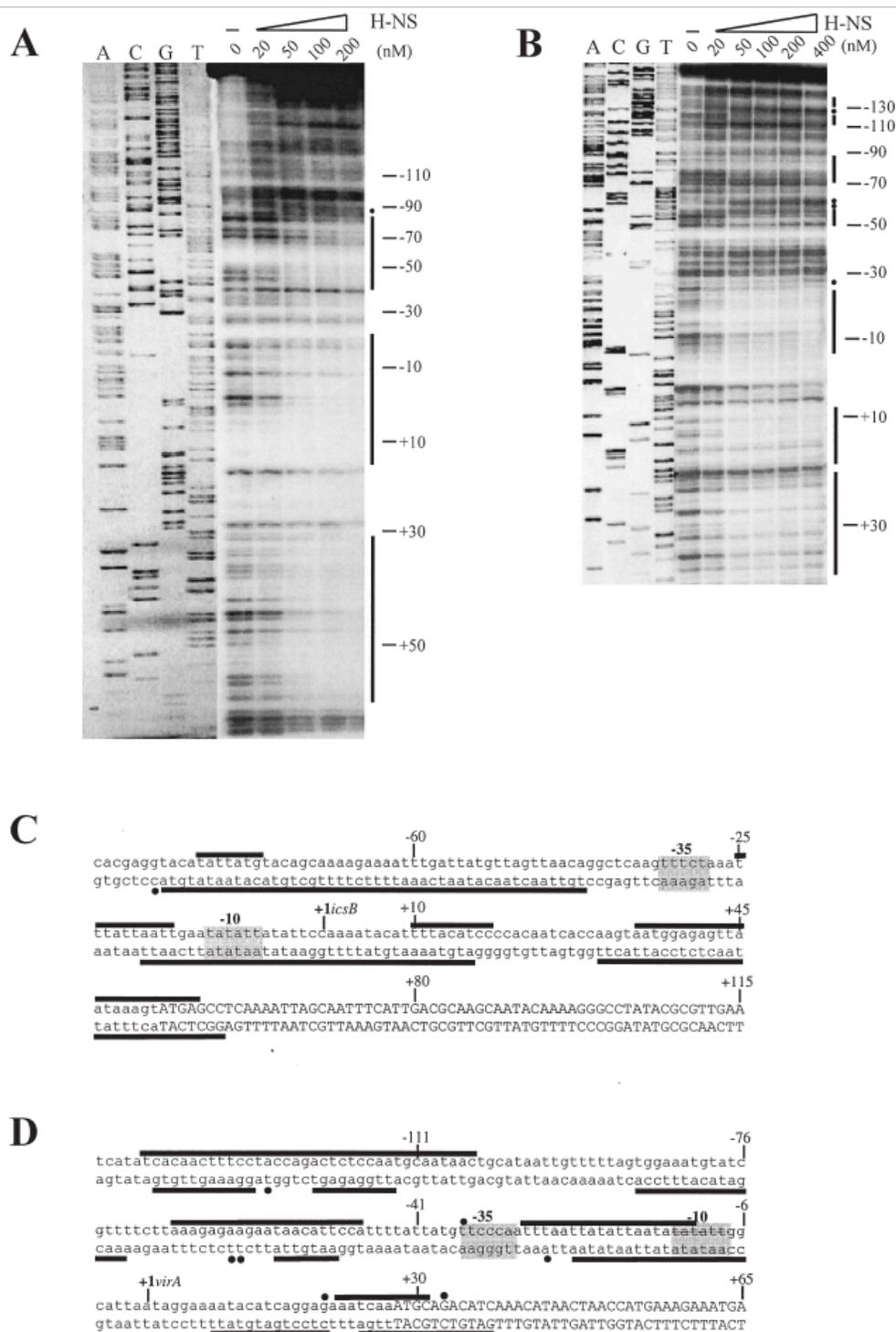


Fig. 6. Analysis of H-NS binding to the *icsB* and *virA* regulatory regions by DNase I footprinting assays. The labelled DNA fragment represents the non-coding strand of the *icsB* regulatory region (A) and the non-coding strand of the *virA* regulatory region (B). Reaction mixtures were

migrated alongside sequencing reactions of the labelled fragments (lanes A, C, G and T). Sequences of the regulatory regions of the *icsB* gene (C) and of the *virA* gene (D) are presented. The nucleotides are numbered relative to the transcriptional start site (+1). The positions of the -10 and -35 boxes are shaded. Footprints obtained on both coding and non-coding strands are illustrated. In (A) to (D) the regions protected by H-NS are marked by solid lines and the sites hypersensitive to DNase I are indicated by filled circles.

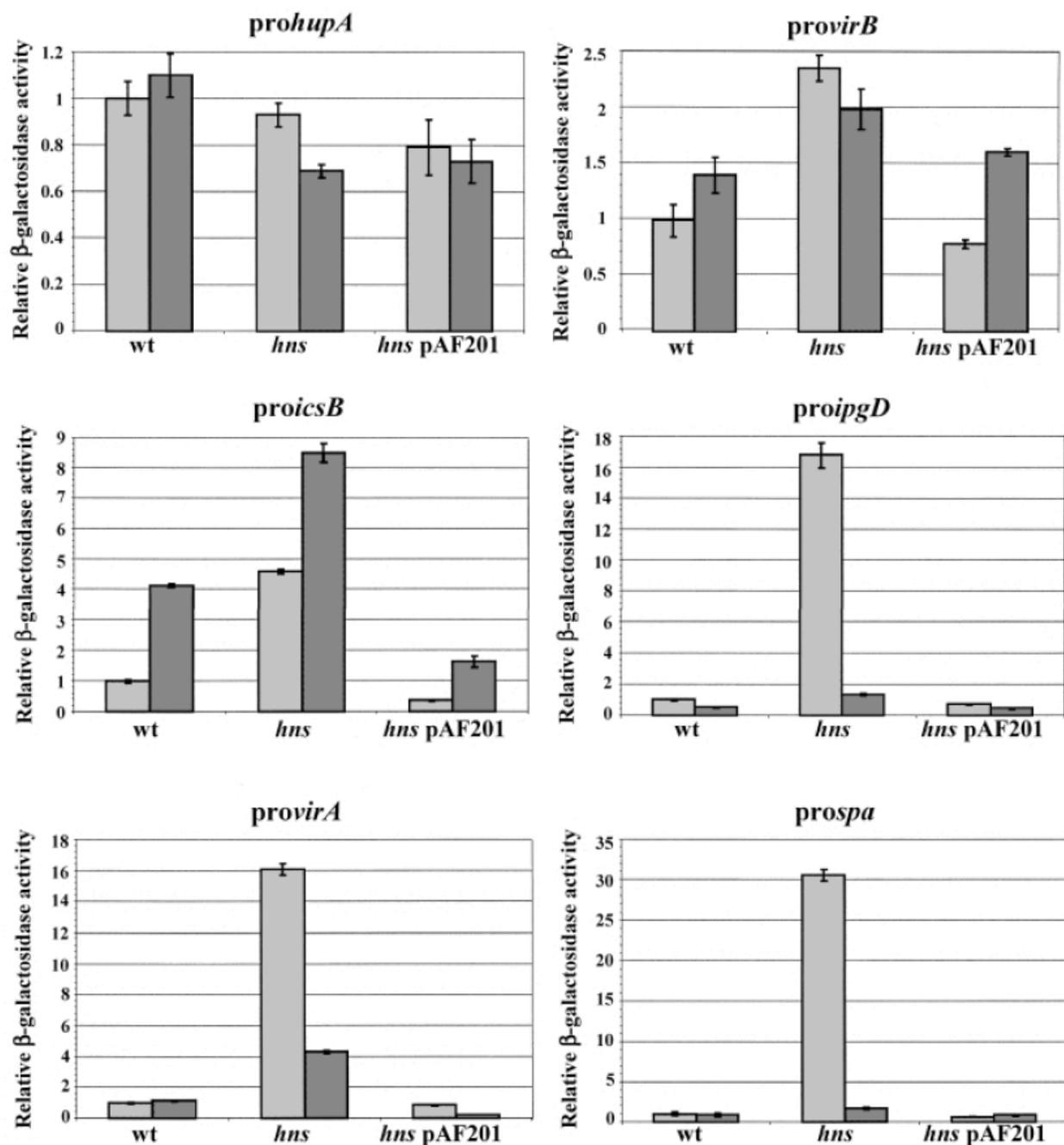


Fig. 7. Observation of the direct effect of H-NS on the expression of *hupA*, *virB*, *icsB*, *ipgD*, *virA* and *spa-lacZ* transcriptional fusions. Histograms show β -galactosidase activity in wild type *E. coli* MC4100 (wt), corresponding *hns* and *hns* complemented (*hns* pAF201) MC4100 strains carrying the different pQF50 Ω Sm plasmid derivatives. Strains were cultured overnight in LB

broth at 30°C (light grey) or at 37°C (dark grey). β -galactosidase activities were normalized with respect to the derivatives of pQF50 Ω Sm plasmid copy number (see Experimental procedures). For each plasmid-located *lacZ* fusion, the data were expressed relative to the β -galactosidase activity measured in the wild type strain (wt) at 30°C (this value was set to 1). The data represent an average of three independent experiments. Error bars represent standard deviations.

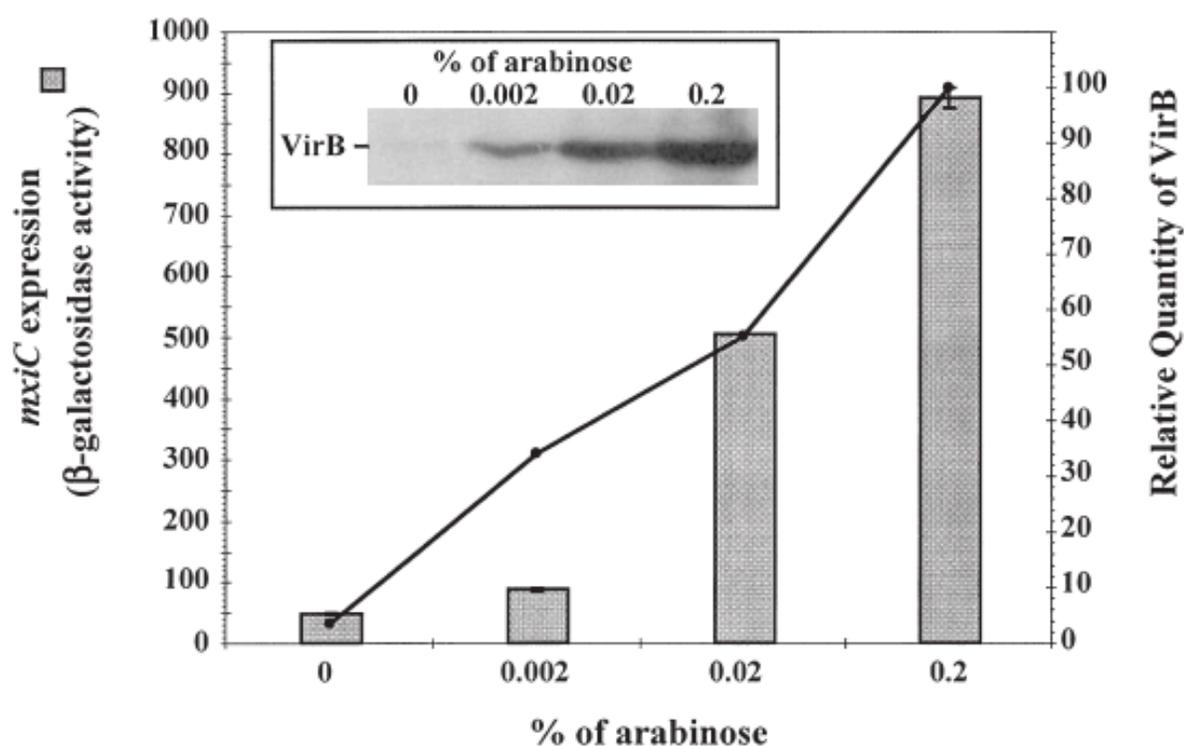


Fig. 8. Virulence gene expression is proportional to VirB protein levels in the cell. Strain BS184pBAD*virB* was cultured overnight at 30°C in LB broth with an increasing concentration of arabinose. The expression of the *mxiC-lacZ* fusion present on the virulence plasmid in this strain was measured and represented by an histogram. The data are expressed in Miller units and represent the average of three experiments. Error bars represent standard deviations. Crude protein extracts were prepared and proteins separated through a 12% SDS-PAGE. VirB protein

was visualized by immunoblotting with polyclonal anti-VirB antiserum. The result of a typical immunoblot is shown (see inset). Densitometric analyses of the VirB level were performed and the relative quantity of VirB was plotted as a function of arabinose concentration (plain line). The quantity of VirB in the culture containing 0.2% arabinose was set to 100.

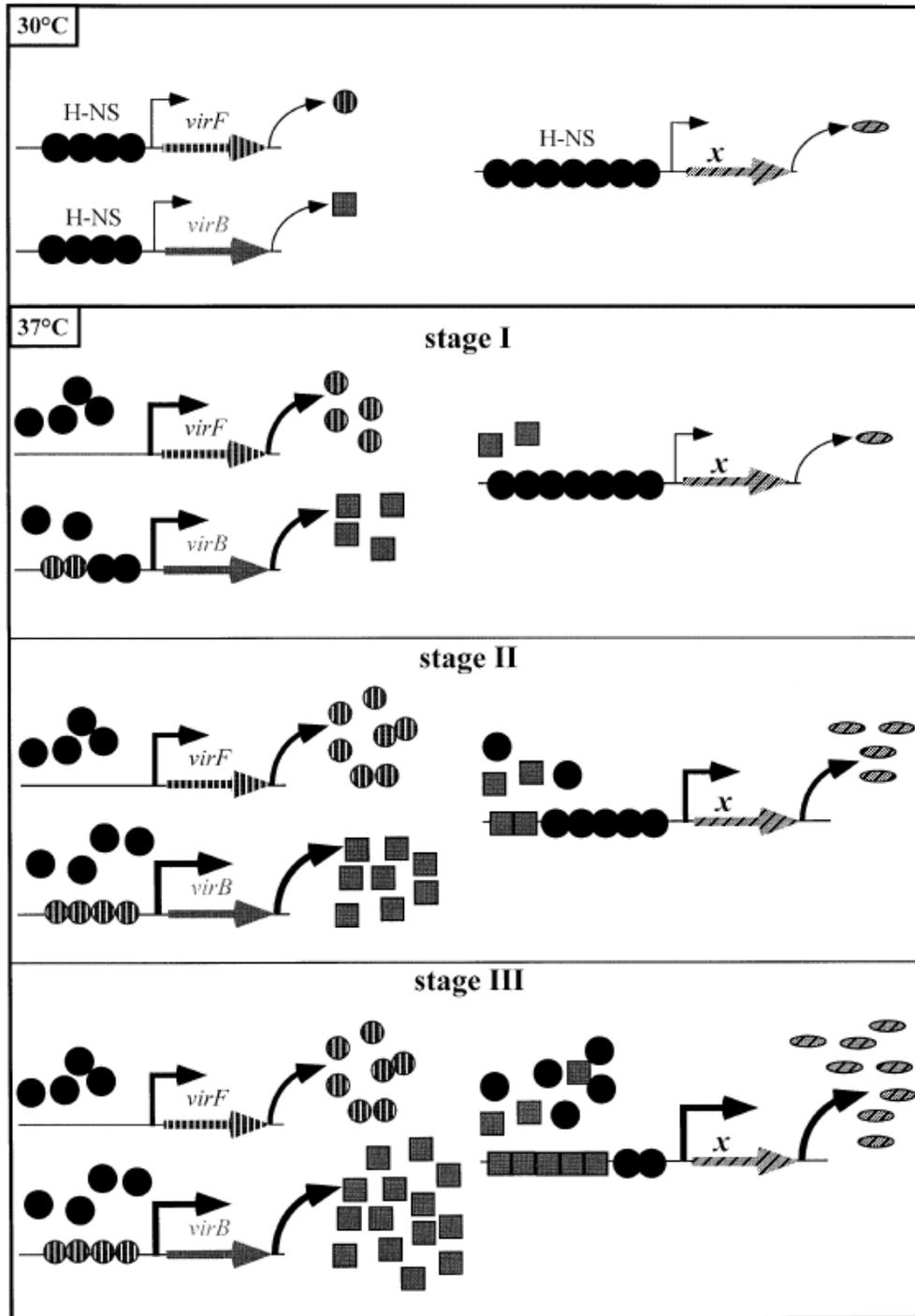


Fig. 9. Schematic overview of the virulence gene regulatory cascade. At 30°C, H-NS (black discs) represses both the *virF* and the *virB* promoters, and only low levels of the gene products are expressed. Similarly, a typical VirB-dependent structural gene or operon (like the *ipg-mxi* or

icsB-ipg-ipa operons), named *x*, is weakly expressed due to H-NS repression and the low level of VirB protein in the cell. At 37°C, the repression complex at the *virF* promoter is disrupted, and VirF protein overcomes the H-NS repression at *virB*. The level of VirB in the cell begins to increase but is not yet sufficient to activate promoter *x* (stage I). Full activation of the *virB* promoter is achieved by binding of VirF and displacement of H-NS. As the level of VirB protein increases, it binds to and polymerizes along the structural gene promoter, displacing H-NS (stage II). This results in enhanced structural gene transcription. Progressive accumulation of VirB in the cell leads to more of the protein bound and oligomerized along the structural gene promoter, leading to maximal activation of gene *x* (stage III).