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A Role for H-NS in the Thermo-Osmotic Regulation of Virulence Gene Expression in *Shigella flexneri*

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The role of the *hns* gene (coding for the curved-DNA-binding protein H-NS) in the thermo-osmotic regulation of *Shigella flexneri* virulence gene transcription was investigated. Two structural genes, *mxiC* and *icsB*, which are transcribed divergently on the high-molecular-weight virulence plasmid, were found to be transcriptionally inhibited in cultures grown in a low-osmolarity medium, even at the inducing temperature. This repression was relieved by inactivation of the *hns* gene, establishing a role for *hns* in the osmotic as well as the thermal regulation of invasion gene expression. The physiological relevance of this finding is discussed.

Shigella flexneri is the causative agent of bacillary dysentery and harbors a high-molecular-weight plasmid encoding proteins essential for virulence (26). Genetic analysis of the plasmid has identified a complicated system coding for products essential for internalization and spread of the bacteria within and between host cells, for secretion of these internalization proteins to the surface of the bacterium, and for the regulation of virulence gene expression. Expression of these virulence genes is environmentally regulated at the level of transcription, and regulatory proteins controlling their expression are encoded by genes located on the plasmid and on the chromosome (reviewed in reference 10).

The detailed transcriptional organization of the genes is still being elucidated, but some patterns have emerged and are summarized in Fig. 1. The essential transcription control genes *virB* and *virF* possess their own promoters (1, 23, 24, 30, 31, 33), and a structural gene called *virG* (or *icsA*) which is required for intercellular spread is also transcribed independently (6, 17). Genes coding for products involved directly in invasion and spread (*icsB* and *ipaADCB*) are transcribed on one DNA strand of the plasmid, while genes involved in the secretion of these virulence factors are transcribed divergently in at least two operons (*mxiHIJMEDCA* and *spa*) (2–4, 27, 28, 32) (Fig. 1).

Work from several laboratories has indicated that the primary environmental stimulus involved in virulence gene activation is temperature: at 30°C, the genes are repressed and at 37°C they are transcribed (15, 18). Temperature control acts via the plasmid-encoded VirF and VirB proteins in a cascade-like manner. VirF is required to activate transcription of *virB*, and VirB then activates the structural gene promoters (30, 31). The *virG* gene is an exception: it is activated by VirF directly (1, 24).

In addition to being controlled by the plasmid-encoded regulatory proteins, the virulence regulon is regulated negatively by the chromosomally encoded protein, H-NS (8, 16, 19). This is a major component of bacterial chromatin and affects the expression of a large number of unrelated genes, coding for housekeeping as well as virulence functions (14). *S. flexneri* mutants deficient in H-NS express the invasion genes constitutively with respect to temperature.

Expression of the invasion genes is required only inside the host, and then only in the lower digestive tract. It might be expected that the bacteria have evolved a virulence gene control circuit which responds to a range of environmental signals to ensure that the virulence genes remained repressed in all situations except that in which their products are required. According to this rationale, repression at a low temperature makes sense because the environments inhabited by enteric bacteria outside the host are commonly at low temperatures. Low osmolarity also characterizes many of the external niches (for example, fresh water) where enteric bacteria such as *S. flexneri* are found. While there is already some evidence that the growth of *S. flexneri* in a hyperosmotic medium at the inducing temperature enhances invasion gene transcription (5), the effect of low osmolarity on virulence gene expression has not been investigated before (as discussed below).

While H-NS has been established as playing a role in the thermal control of *S. flexneri* virulence gene expression, it is interesting that in other systems it has been classified as an osmotic regulator (9, 13). In this study, the expression of *S. flexneri* virulence genes was investigated in a low-osmolarity medium (LO) and the potential of H-NS to contribute to the osmotic component of *S. flexneri* virulence gene control was investigated.

Virulence gene expression is inhibited at low osmolarity. Luria-Bertani broth (LB) was used as a high-osmolarity medium. It contains 1% NaCl, which is close to the salt concentration of physiological saline (20). LO is identical to LB but contains no NaCl (22). Isosmotic levels of NaCl and sucrose affected *S. flexneri* virulence gene expression in similar manners, showing that the effects associated with NaCl were due to osmolarity and not some other property of the salt (21). Bacteria were grown in Erlenmeyer flasks with vigorous aeration.

The structural genes *mxiC* and *icsB* are transcribed divergently on the high-molecular-weight virulence plasmid of *S. flexneri* (Fig. 1). Strain BS184 harbors an *mxiC-lacZ* transcriptional fusion (*vir83*::Mu dI1734) which has been used extensively as a reporter of virulence gene transcription (5, 8, 19, 22). Expression of this fusion was studied by β-galactosidase assay (20). Transcription of *icsB* was measured by Northern (RNA) hybridization. This combination allowed the expression

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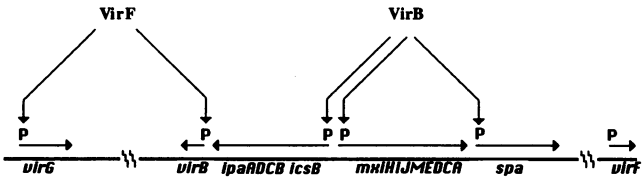

FIG. 1. Genetic map of the virulence region on the 230-kb plasmid of *S. flexneri* 2a. Transcripts are indicated by horizontal arrows. The sites at which the VirF and VirB transcriptional regulators exert positive control are indicated. P, promoter. The map is not drawn to scale.

of both genes to be monitored in the same sample. In the gene expression experiments, 500-ml cultures were grown in 2-liter flasks to an optical density at 600 nm of 0.6 at 30°C. The culture was then transferred to a 42°C water bath and shaken vigorously until the temperature of the broth reached 37°C. Growth was then allowed to continue in an orbital incubator at 37°C. Samples were then removed at fixed time points, and the expression of both genes was measured from the same samples; *mxiC* was tested by β-galactosidase assay, and *icsB* was tested by Northern analysis.

Total RNA extraction involved resuspension of cells from a 50-ml aliquot of culture in 1 ml of boiling RNA extraction buffer (REB; 20 mM sodium acetate, 2% sodium dodecyl sulfate [SDS], 0.3 M sucrose) and incubation at 100°C for 2 min. RNA was isolated by phenol extraction followed by three to four extractions with phenol-chloroform (50:50, vol/vol) and precipitation with ethanol at −70°C for 12 h. RNA was resuspended in water and treated with RNase-free DNase (Boehringer, Mannheim, Germany) according to the manufacturer's instructions. It was then reprecipitated at −70°C for 4 h. RNA concentrations were determined spectrophotometrically at an optical density of 260 nm. Ten micrograms of RNA was denatured in 1× 3-(*N*-morpholino)propanesulfonic acid–50% formamide–2.2 M formaldehyde at 65°C for 5 min. An equal volume of 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) (25) was added to obtain a final concentration of 10× SSC. Each sample was transferred to a Hybond-N nylon filter (Amersham) with a Biodot SF microfiltration apparatus (Bio-Rad), following the procedure recommended by the manufacturers. Equality of loading was also checked by electrophoresing RNA samples on agarose gels, transferring to Hybond-N nylon filters and staining for rRNA with methylene blue (25). Hybridization was performed with an oligonucleotide probe (5′-TTAGCTGCTTCATAGGCGGCAAACGC ACTT-3′) labeled with digoxigenin-labeled dUTP (Boehringer) which was specific for the 5′ end of the mRNA. In separate control experiments, this oligonucleotide was shown to be specific for *icsB* (data not shown). Hybridizations were carried out overnight at 50°C in a mixture of 7% SDS, 50 mM sodium phosphate buffer, 50% formamide, 2% blocking reagent, total yeast RNA (Sigma; 50 μg/ml), 5× SSC, and 0.1% laurylsarcosine. Following stringency washes (2× SSC for 15 min at room temperature followed by 0.1× SSC for 5 min at 50°C, with all washes performed twice), bound probe was detected by using the digoxigenin-AMPPD luminescence detection system (Boehringer) and Hyperfilm-MP X-ray film (Amersham). The exposed films were scanned densitometrically with a Molecular Dynamics phosphorimager. A further check for equality of RNA sample loading was carried out by probing for the transcript of the single-copy kanamycin resistance gene, *kan*, located on the virulence plasmid in BS184. The transcript from this gene was detected using a digoxigenin-labeled 2.7-kb *Bgl*II fragment derived from plasmid pRT733 (which harbors a copy of Tn*phoA*) (29) as a probe specific for its 5′ end. Hybridization conditions were as described for the *icsB* probe except that the temperature used was 68°C; the stringency washes were also performed at 68°C. In this way, equality of RNA loading was assured by (i) spectrophotometric measurement, (ii) probing with an independent, specific probe (*kan*), and (iii) methylene blue staining of the filter following RNA transfer.

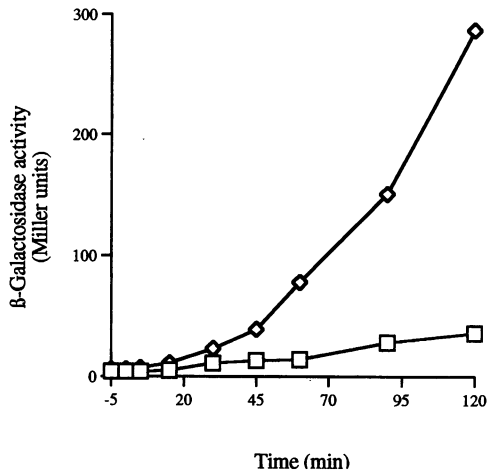

FIG. 2. Effect of low osmolarity on *mxiC* transcription. *mxiC-lacZ* fusion expression in LB-grown (diamonds) or LO-grown (squares) BS184 cultures, measured by β-galactosidase assay. The cultures were shifted from 30 to 37°C conditions at time zero. The initial datum points are for the sample taken 5 min prior to the temperature upshift. Each point is the average of two measurements carried out in triplicate. The standard errors were less than 10%.

Cultures of BS184 were grown to mid-exponential phase in either LO or LB at 30°C and then shifted to 37°C conditions. Samples were removed at fixed time intervals for analysis of *mxiC* expression by β-galactosidase assay and for analysis of *icsB* expression by Northern blotting. In the LB culture, temperature upshift resulted in a 48-fold enhancement of *mxiC* transcription over the preshift value after 120 min (Fig. 2). Significant thermal induction became detectable 45 min after the temperature upshift. However, in LO, temperature upshift resulted in a level of *mxiC* transcription after 120 min that was only 13% of that obtained in LB (Fig. 2). This showed that the absence of the osmolyte from the growth medium had a strong negative effect on *mxiC* expression.

In the case of *icsB*, thermal upshift in the LB culture resulted in an approximately eightfold transcriptional enhancement over the preshift value after 120 min (Fig. 3). Like *mxiC*, significant thermal induction of *icsB* became detectable 45 min after the temperature upshift, indicating that *mxiC* and *icsB* possessed similar induction kinetics. In LO, the effect of temperature on *icsB* transcription was negligible. Only a twofold increase in transcript level was detected 120 min after the thermal upshift (Fig. 3). Thus, like *mxiC*, *icsB* requires an osmotic signal in addition to a thermal signal for optimal transcriptional induction.

Effect of an *hns* mutation on virulence gene expression at low osmolarity. Previous work has established the *hns* gene as a negative regulator of virulence gene expression in *S. flexneri* (8, 16, 19, 31). Inactivation of *hns* results in derepression of virulence gene transcription at 30°C, indicating that H-NS is involved in transcriptional repression at 30°C. Several osmo-

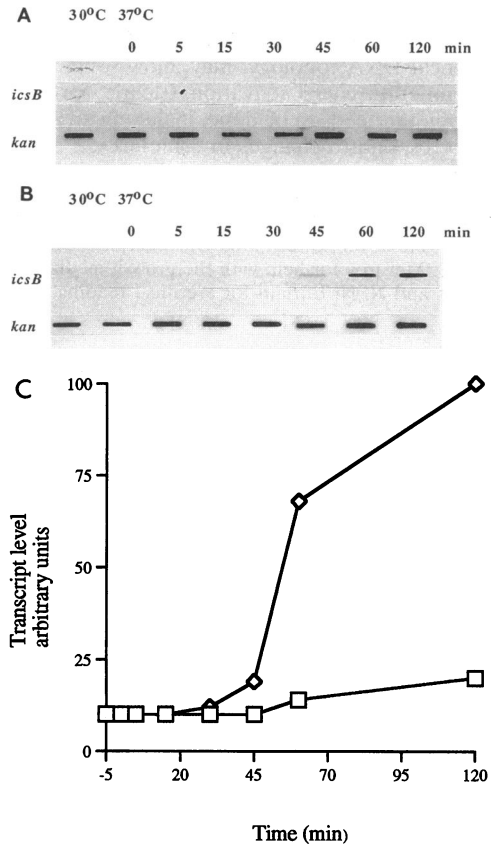

FIG. 3. Effect of low osmolarity on *icsB* transcription. *icsB* transcription in LO-grown (A) or LB-grown (B) BS184 cultures was measured by Northern hybridization. The cultures were shifted from 30 to 37°C conditions at time zero. The initial datum points are for the sample taken 5 min prior to the temperature upshift. The transcript of the *kan* gene associated with the *mxiC-lac* fusion on the virulence plasmid was used as a control for RNA loading. The data from the slot blot analysis were quantified by densitometer tracing of the exposed film and expressed in arbitrary units as a function of time (C). Squares, LO-grown cultures; diamonds, LB-grown cultures. These measurements were carried out four times, and typical data are shown.

regulated systems are also controlled negatively by H-NS in enteric bacteria (9, 13). The data presented above show that the *S. flexneri* virulence genes are expressed poorly in LO, a low-osmolarity medium, but well in the higher-osmolarity medium, LB. It was possible that H-NS might play a role in this osmotic component of virulence gene expression. For this reason, we tested the effect of *hns* inactivation on virulence gene expression in low-osmolarity cultures.

Strain BS185 contains an *hns*::Tn*10* mutation (16). In all other respects it is identical to BS184. In LB cultures at 30°C, the presence of the *hns* mutation resulted in enhanced transcription of both *mxiC* (approximately 30-fold; Table 1) and *icsB* (approximately 3-fold; Table 2), confirming its previously described role as a negative regulator of thermally controlled virulence genes. As described above, transcription of the virulence genes was inhibited in BS184 LO cultures grown at 37°C. Inactivation of *hns* relieved this inhibition, resulting in an approximately 16-fold increase in *mxiC* transcription in LO cultures at 37°C (Table 1) and an approximately 14-fold increase in *icsB* transcription in LO cultures at 37°C (Table 2). In addition, transcription of the virulence genes was enhanced

TABLE 1. Effect of an *hns* mutation on *mxiC* expression

hns status and medium	Expression (Miller units [%])[a] at:	
	30°C	37°C after 120 min
hns$^+$, LB	6 (2)	290 (100)
hns$^+$, LO	4 (1.4)	38 (13)
hns$^-$, LB	197 (68)	550 (190)
hns$^-$, LO	271 (93)	600 (207)

[a] Data are from β-galactosidase assays (20). Each assay was performed with triplicate samples taken from duplicate cultures. Mean values are reported. In all cases, the standard deviation was less than 10%. The values obtained are also reported parenthetically as percentages of that obtained for the *hns*$^+$ strain grown in LB (100%).

in the *hns* mutant in LO cultures at 30°C: over 60-fold for *mxiC* (Table 1) and about 7-fold for *icsB* (Table 2). These data demonstrate that instead of being regarded simply as a thermoregulator, H-NS should be more accurately described as a thermo-osmotic regulator.

The introduction of the *hns*::Tn*10* mutation did not result in identical effects on *mxiC* and *icsB* expression under all conditions. For cultures grown at 37°C in LB, *mxiC* transcription increased in the presence of the *hns* mutation whereas *icsB* transcription decreased (Tables 1 and 2). Thus, while these structural genes are certainly coregulated, their promoters can differ in the detail of their environmental responses when *hns* is mutated. Perhaps these effects are due to an alteration in local DNA topology caused by the loss of the nucleoid-associated protein H-NS.

Conclusions. The thermal regulation of *S. flexneri* virulence genes is well established (reviewed in reference 10). Previous work has indicated that increasing the osmolarity of a defined laboratory medium (medium A) (20) can enhance the effect of temperature on transcription of *mxiC* (5). Bernardini et al. detected 200 Miller units of β-galactosidase activity from the same *mxiC-lacZ* fusion used in our study when the culture was grown in medium A. Adding osmolyte (15% [wt/vol] sucrose) increased expression to 700 units, an increase of 3.5-fold (5). We detected only 38 Miller units when the same strain was grown in LO. This increased to 290 units when the osmolarity of the medium was elevated by adding 1% NaCl, a 7.5-fold enhancement (Fig. 2; Table 1). These data suggested that LO is a lower-osmolarity medium than medium A. Using medium A, Bernardini et al. detected about 280 Miller units of β-galactosidase activity from an osmotically repressible *ompF-lacZ* fusion in *Escherichia coli* (5). This gene is expressed maximally in low-osmolarity medium but becomes transcriptionally repressed as the osmolarity of the medium is increased (9, 11, 12). Using *E. coli* MH513 (11, 12), which harbors the same *ompF-lacZ* fusion in a genetic background identical to that

TABLE 2. Effect of an *hns* mutation on *icsB* expression

hns status and medium	Expression (%)[a] at:	
	30°C	37°C after 120 min
hns$^+$, LB	12	100
hns$^+$, LO	10	24
hns$^-$, LB	34	76
hns$^-$, LO	76	345

[a] Data were obtained by densitometric scanning of exposed films from Northern blots. They are expressed as percentages of the value obtained with the *hns*$^+$ strain grown in LB (100%). These experiments were performed four times, and typical data are shown. Controls used to ensure equal loading of RNA samples are described in the text.

described in reference 5, we detected 620 Miller units of β-galactosidase activity from a mid-exponential-phase LO culture. This too was consistent with the osmolarity of LO being less than that of medium A. To test this hypothesis, the osmolarities of these growth media were measured with a Wescor 5100C vapor pressure osmometer (Wescor Inc.). For each medium, the osmolarity is reported as the mean of three independent measurements. The osmolarity of LO was found to be 89 (± 5) mmol/kg, whereas that of medium A was 276 (± 5) mmol/kg. This finding is fully consistent with our finding that *mxiC-lacZ* expression is lower in LO than that reported for medium A. We found that the level of *mxiC-lacZ* expression in LB (Table 1) was higher than that reported for medium A (5). Consistent with this, the osmolarity of LB (i.e., LO containing 1% NaCl) was found to be 401 (± 5) mmol/kg.

In this work, the effect of growth in the low-osmolarity medium, LO (22), on transcription of two virulence genes in *S. flexneri* was investigated. Low-osmolarity conditions might be encountered by *S. flexneri* when living outside the host, perhaps in an aquatic environment. However, such an environment would also be likely to be at a temperature lower than that of the host. For these reasons, virulence gene expression there would be unlikely. Within the host, the bacterium might be expected to experience a temperature of 37°C at all times. However, not all niches within the host will exert osmotic stress. By regulating its virulence genes in response to at least two environmental signals rather than simply temperature, the likelihood that the genes will be expressed in an inappropriate niche is diminished. A temperature of 37°C and a high osmotic pressure represent excellent indicators that the bacterium has encountered the large intestine of its host.

The effects of growth medium osmolarity and the *hns* mutation on *mxiC* and *icsB* were qualitatively similar. Both genes were transcribed very poorly in the low-osmolarity medium LO, even at the permissive temperature (37°C). Inactivation of *hns* relieved this low-osmolarity inhibition, showing that both genes are subject to repression by H-NS at some level (as described below) and that this repression is modulated by osmotic stress and by temperature. Transcription of both genes could be further enhanced by a thermal signal in either LO or LB. This showed that inactivation of *hns* did not relieve fully the low-temperature repression and is consistent with thermal control also acting through regulators other than H-NS, such as VirB (see the explanation below). In the *hns* mutant, transcription of *mxiC* and *icsB* was consistently higher in LO than in LB (Tables 1 and 2), indicating that further regulatory features of the system remain to be explored. One possible explanation for this effect is that changes in osmolarity alter the local topology of the DNA at these divergently arranged promoters in ways that are different in the wild type and in the *hns* strain.

The work described here raises the question of how the osmotic signal affects the virulence regulon. One possibility is that increased osmolarity removes H-NS from the *virB* promoter, enhancing the positive effect of VirF. In a low-osmolarity medium such as LO, H-NS may remain resolutely associated with the *virB* promoter, preventing activation. In the *hns*::Tn*10* mutant, H-NS is not available to repress *virB*, so that the thermal signal (acting through VirF) is able to activate *virB* and hence the downstream structural genes. Our data also raise the issue of why an *hns* mutation results in some derepression of virulence gene expression in the absence of a thermal signal. It is possible that VirF may occupy its binding site at the *virB* promoter at all temperatures but requires a thermal signal to overcome H-NS repression. (VirF can certainly bind to *virB* in vitro in the absence of a thermal signal, provided that the DNA is supercoiled [31]). Thus, removal of the negatively acting H-NS, perhaps with a concomitant change in local DNA topology, may allow some VirF-dependent low-temperature *virB* activation. Clearly, further work on the *virB* promoter needs to be done, including an analysis of its potential to respond to changes in osmolarity.

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