Regulation of *Escherichia coli* Hemolysin E Expression by H-NS and *Salmonella* SlyA

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The *Escherichia coli hlyE* gene (also known as *clyA* or *sheA*) codes for a novel pore-forming toxin. Previous work has shown that the global transcription factors FNR and CRP positively regulate *hlyE* expression by binding at the same site. Here in vivo transcription studies reveal that FNR occupies the *hlyE* promoter more frequently than CRP, providing a mechanism for the moderate upregulation of *hlyE* expression in response to two distinct environmental signals (oxygen and glucose starvation). It has been reported that H-NS interacts with two large regions of the *hlyE* promoter (P*hlyE*), one upstream of the −35 element and one downstream of the −10 element. Here we identify two high-affinity H-NS sites, H-NS I, located at the 3′ end of the extended upstream footprint, and H-NS II, located at the 5′ end of the extended downstream footprint. It is suggested that these high-affinity sites initiate the progressive formation of higher order complexes, allowing a range of H-NS-mediated regulatory effects at P*hlyE*. Finally, the identification of a SlyA binding site that overlaps the H-NS I site in P*hlyE* suggests a mechanism to explain how SlyA overproduction enhances *hlyE* expression by antagonizing the negative effects of H-NS.

Recently, a novel pore-forming toxin, designated HlyE, ClyA, or SheA, was identified for *Escherichia coli* and *Salmonella enterica* serovars Typhi and Paratyphi A (2, 6, 7, 10, 16, 17, 21, 22, 35). The three-dimensional (3-D) structure of HlyE shows that it is a mostly α-helical, long (~100 Å), rod-shaped molecule with a hydrophobic two-stranded antiparallel β-sheet at one end that is thought to allow an interaction between HlyE and target membranes (35). The HlyE protein forms pores in target membranes that appear in electron microscopy (EM) images as ring-shaped structures with internal diameters of 50 to 55 Å, when viewed from above, and as 100- to 105-Å spikes in a side view, suggesting that HlyE does not undergo large conformational changes during pore formation (35).

Two members of the CRP family of transcription factors control the expression of *hlyE* in *E. coli* K-12 by binding at the same site centered at −61.5 bp upstream of the *hlyE* transcriptional start (10, 36). Thus, CRP enhances *hlyE* expression in response to glucose starvation (36) and FNR enhances *hlyE* expression in response to oxygen starvation (10, 11, 23). Both CRP and FNR are ~50-kDa homodimers that bind related inverted repeats with a TGANNNNNNTCA core motif (12). At the *hlyE* promoter (P*hlyE*), this site (TTTGATATTTATC ATA) most closely resembles an FNR site (9 of 10 nucleotides match the FNR consensus, TTGATNNNNATCAA, compared to 8 of 10 matches to the CRP consensus, TGTGANNNN NNTCACA; discriminatory bases are underlined). However, it

has been shown that in certain circumstances CRP can recognize FNR sites, although the affinity of CRP for an FNR site is 50-fold lower than that for an equivalent CRP site (27).

A further layer of regulation is provided by the nucleoid structuring protein H-NS (36). The H-NS protein influences the expression of many genes in *E. coli* K-12. It is a small ~15-kDa protein that forms higher order complexes in a concentration-dependent manner (30, 34). The H-NS protein was shown to interact with a large region of P*hlyE* (from −137 to +172, relative to the transcription start site) to repress *hlyE* expression (36) after the observation that an *hns* mutant strain has a hemolytic phenotype (9).

A hemolytic phenotype was also conferred upon *E. coli* K-12 by the overproduction of either the *E. coli* or *Salmonella enterica* transcription factor SlyA (16, 21). The SlyA protein is a member of the MarR family of transcription factors that includes MarR and EmrR (*E. coli*), PecS (*Erwinia chrysanthemi*), HprR (*Bacillus subtilis*), and RovA (*Yersinia enterocolitica*) (20, 24). The 3-D structures of *E. coli* MarR and a SlyA-like protein from *Enterococcus faecalis* provide the structural archetypes for this family of proteins. The 3-D structures show that they are homodimers in which each subunit possesses a winged-helix DNA-binding domain (1, 37). A recent characterization of the *Salmonella* SlyA protein revealed that it is also a homodimer (~32 kDa) that recognizes an inverted repeat sequence in target promoters (32). Site-directed mutagenesis of P*hlyE* led to the suggestion that a GC-rich sequence located between an unusual heptameric −10 element (TATGAAT) and a conventional −35 element might be the site of SlyA action (17). Here we show that the regulation of *hlyE* expression by H-NS is more complex than was previously thought and that the overproduction of SlyA enhances *hlyE* expression by antagonizing the negative effects of H-NS.

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
Strains		
M182	Δ*lac*	5
JRG4830	M182 Δ*lac* Δ*crp*	5
JRG4864	M182 Δ*lac* Δ*hns;* Cm^r	This work
JRG2631	M182 Δ*lac* Δ*fnr* Δ*crp;* Tet^r	Jeff Cole
JRG4865	M182 Δ*lac* Δ*fnr* Δ*hns;* Cm^r Tet^r	This work
JRG4732	M182 Δ*lac* Δ*crp* Δ*hns;* Cm^r	This work
JRG4866	M182 Δ*lac* Δ*fnr* Δ*crp* Δ*hns;* Cm^r Tet^r	This work
JRG4747	M182 Δ*lac* Δ*fnr;* Cm^r	This work
JRG3212	M182 Δ*lac* Δ*fnr* Δ*crp;* Cm^r	This work
JRG4385	JM109 (pGS1482); Ap^r	32
Plasmids		
pRS415	Medium-copy-number vector (15 to 20 copies per cell; pBR322 origin of replication) for construction of *hlyE::lacZ* reporter plasmid pGS1629; Ap^r	29
pGS1629	pRS415 containing P*hlyE* (−299 to +82); Ap^r	This work
pRW50	Low-copy-number (2 to 5 copies per cell; pSC101 origin of replication), *lac* reporter vector; Tet^r	15
pGS1065	pRW50 containing P*hlyE* (−97 to +61); Tet^r	10
pGS1064	pUC118 containing P*hlyE* (−97 to +61, start codon is +1); Ap^r	10
pGS1482	pGEX-KG (13) containing the *slyA* coding region; Ap^r	32
pGS1657	pBluescript containing *S. enterica slyA* coding region and promoter; Ap^r	This work

MATERIALS AND METHODS

Bacterial strains, plasmids, and microbiological methods. Relevant characteristics of the bacterial strains and plasmids used are given in Table 1. Isogenic derivatives of M182 were constructed by P1*vir*-mediated transduction. Bacteria were grown in Lennox broth (L-broth; contains yeast extract, 5 g liter^{-1}; tryptone, 10 g liter^{-1}; and NaCl, 5 g liter^{-1}) at 37°C supplemented with glucose (0.2% [wt/vol]), ampicillin (150 μg ml^{-1}), tetracycline (35 μg ml^{-1}), and chloramphenicol (20 μg ml^{-1}), as appropriate. For β-galactosidase activity measurements (19), anaerobic cultures were grown in sealed bottles filled to the neck with medium, and aerobic cultures were grown in conical flasks (250 ml) containing medium (10 ml), with vigorous shaking (250 rpm) at 37°C. Hemolytic activities were estimated by measuring the areas of hemolysis surrounding individual colonies after 16 h of growth at 37°C on blood agar under aerobic conditions.

Gel shift assays. Initial H-NS gel shifts used a PCR-amplified 696-bp fragment of P*hlyE* containing all of the previously reported sequences protected by H-NS from DNase I digestion (36) plus additional upstream and downstream sequences. In subsequent experiments, this fragment was further resolved into four subfragments by restriction digestion with *Ssp*I, *Bsa*MI, and *Dra*I. Target DNAs (200 to 500 ng) were incubated with H-NS (0 to 6 μM) in 25 mM HEPES, pH 7.6, containing 0.1 mM EDTA, 10% (vol/vol) glycerol, 5 mM dithiothreitol, 50 mM KCl, and 0.01 mg of poly(dI-dC) ml^{-1} for 15 min at 25°C in a total volume of 10 or 20 μl. Complexes were separated in 6% (wt/vol) nondenaturing Tris-borate-EDTA-buffered polyacrylamide gels for the long P*hlyE* fragment or in 12.5% (wt/vol) gels for the dissected P*hlyE* fragments. DNA was visualized by staining with ethidium bromide. The interaction of H-NS and SlyA with P*hlyE* was also investigated by using P*hlyE* DNA amplified by PCR with pGS1064 (10) (Table 1) as the template and with pUC/M13 forward and reverse primers. The product was digested with *Bam*HI and radiolabeled with Klenow enzyme and [^{32}P]α-dGTP (26). For H-NS, ~10 ng of DNA and 0.04 to 2.0 μM H-NS were coincubated for 5 min at 25°C in a solution containing 10 mM Tris-HCl (pH 7.6), 50 mM MgCl$_2$, 2 mM spermidine, and 15 mM potassium glutamate (total incubation volume, 10 μl). For SlyA, ~10 ng of DNA and 0.06 to 0.63 μM SlyA protein were coincubated for 2 min at 25°C in a solution containing 10 mM Tris-HCl (pH 9.0), 50 mM KCl, and 0.1% (vol/vol) Triton X-100 (total incubation volume, 10 μl). Reactions were then loaded onto gels for autoradiographic analysis.

DNase I footprinting. For H-NS, the reactions (total volume, 10 μl) contained radiolabeled P*hlyE* (~10 ng), H-NS (1 μM), 10 mM Tris-HCl (pH 7.6), 50 mM MgCl$_2$, 2 mM spermidine, and 15 mM potassium glutamate. For the SlyA, the reactions (total volume, 10 μl) contained radiolabeled P*hlyE* (~10 ng), SlyA (2.0 and 4.0 μM), 20 mM Tris-HCl (pH 8.0), 10 mM MgCl$_2$, 10 mM dithiothreitol, and 5% (vol/vol) glycerol. The mixtures were incubated for 2 to 5 min at 25°C, followed by digestion with DNase I (1 μl of a 1-U μl^{-1} solution for 5 to 60 s at 25°C). Reactions were stopped by the addition of 200 μl of 0.3 M sodium acetate

(pH 5.2) containing 20 mM EDTA, followed by phenol-chloroform extraction. The DNA was ethanol precipitated and resuspended in 10 μl of loading buffer (80% [vol/vol] formamide, 0.1% [wt/vol] sodium dodecyl sulfate, 10% [vol/vol] glycerol, 8 mM EDTA, 0.1% [wt/vol] bromophenol blue, and 0.1% [wt/vol] xylene cyanol) for electrophoretic fractionation on 6% (wt/vol) polyacrylamide–urea gels and autoradiographic analysis. Maxam and Gilbert G tracks were used to provide a calibration (18).

Other methods. The H-NS protein was provided by C. F. Higgins (MRC Clinical Sciences Centre, Hammersmith Hospital, London, United Kingdom), and the SlyA protein was isolated from *E. coli* strain JRG4385 (Table 1) as previously described (32). The manipulation of DNA, PCR, and plasmid constructions was achieved by conventional methods (26). Plasmid copy numbers were estimated by the method of Taylor and Brose (33).

RESULTS

Effects of the global transcription factors FNR, CRP, and H-NS on *hlyE* expression in liquid culture. Previous studies have shown that FNR, CRP, and H-NS contribute to *hlyE* expression when *E. coli* is grown on a solid medium (10, 36). Both FNR and CRP make positive contributions, whereas H-NS acts negatively under these conditions. Because bacteria grown on solid media are exposed to different microenvironments due to the generation of concentration gradients of nutrients and oxygen within colonies, the starting point for this work was to investigate the effects of all three global regulators on *hlyE* expression in liquid cultures by using a plasmid-based *hlyE::lacZ* gene fusion (pGS1629; Table 1). The reporter plasmid contained a fragment of DNA stretching from −299 bp to +82 bp relative to the *hlyE* transcript start site ligated with pRS415 (Table 1). Initially, transcriptional activity from P*hlyE* was estimated for anaerobic cultures of parental (M182), *fnr*, *crp*, and *hns* strains transformed with pGS1629 (Table 1), after growth in L-broth at 37°C for 16 h. The data revealed that both FNR and CRP made significant positive contributions to *hlyE* expression, whereas the *hns* mutation resulted in slightly reduced *hlyE* transcription under these conditions (Fig. 1a). It was predicted that the addition of glucose (0.2% [wt/vol]) to the medium would abolish the effects of CRP. Accordingly,
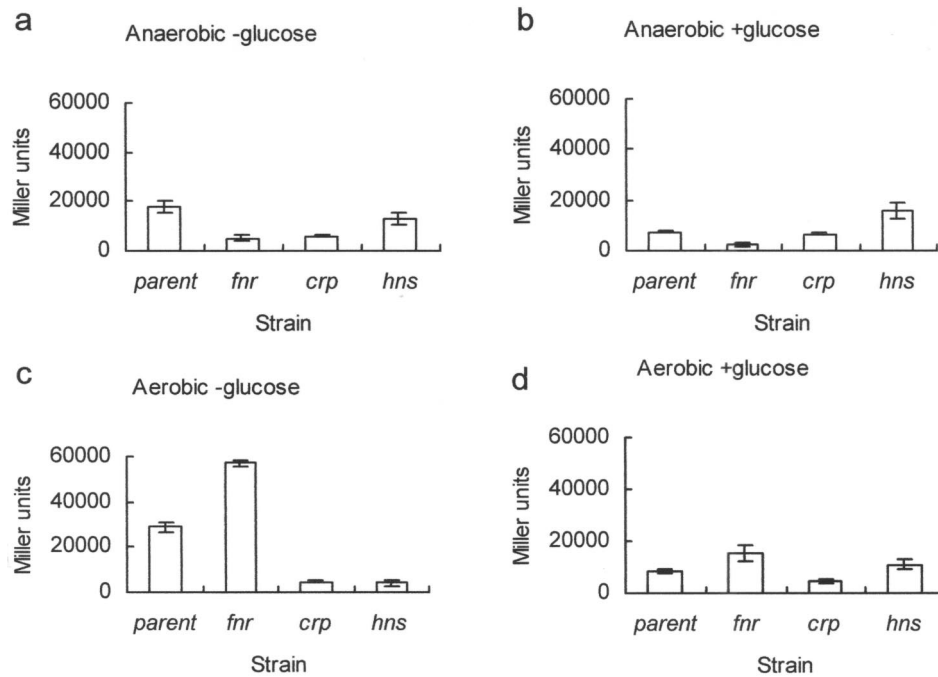
FIG. 1. Effects of FNR, CRP, and H-NS on in vivo transcription of *hlyE*. All cultures were grown in L-broth at 37°C for ~16 h under the indicated conditions. The activity of the *hlyE* promoter was estimated by measuring the β-galactosidase activity associated with each culture carrying the *hlyE::lacZ* plasmid pGS1629. Strains: parental, M182; *fnr*, JRG4747; *crp*, JRG4830; *hns*, JRG4864 (Table 1). Error bars indicate 2 standard deviations from the means ($n = 3$).

hlyE expression was reduced in cultures of the parental strain containing glucose compared to those without glucose (Fig. 1b). Moreover, while *hlyE* expression was reduced in the *fnr* strain compared to that in the parent strain in the presence of glucose, it was, as expected, unaffected in the *crp* mutant (Fig. 1b). The transcription of *hlyE* in the *hns* strain under anaerobic conditions in the presence of glucose is presumably driven by FNR alone. Under these conditions, *hlyE* expression was greater for the *hns* strain than for the corresponding parental cultures, suggesting that H-NS has a negative effect on FNR-driven *hlyE* expression (Fig. 1b). Thus, we concluded that CRP, FNR, and H-NS all contribute towards the regulation of *hlyE* expression.

Similar experiments were done with aerobic cultures. For these conditions, we predicted that the glucose-responsive effects of CRP would be retained, whereas the positive regulation by FNR would be abolished. The prediction for the CRP response was confirmed by the data (Fig. 1c and d). However, the data for the parental and *fnr* cultures suggested that even under aerobic growth conditions, some active FNR was present in the bacteria, and that even this small amount of active protein (relative to anaerobic conditions) was sufficient to interfere with CRP-mediated *hlyE* expression (Fig. 1c). Thus, these data suggest that under such conditions, FNR and CRP are in competition for binding at P*hlyE*, and that the simplest explanation of these observations is that FNR is more efficiently bound than CRP at P*hlyE* but is not as effective as CRP in activating *hlyE* transcription. Such an interpretation is consistent with the sequence of the FNR/CRP box in P*hlyE*, which lacks the discriminatory G-C base pairs that promote CRP specificity, and with the observation that, unlike CRP, FNR is

a poor activator of class I promoters such as P*hlyE* (reviewed by Green et al. in reference 12). The effect of the *hns* mutation on *hlyE* expression in aerobic cultures was also interesting (Fig. 1c and d). In the absence of glucose, H-NS appeared to have a positive effect on *hlyE* expression (Fig. 1c). However, in the presence of glucose, H-NS appeared to have little effect on *hlyE* expression (Fig. 1d). Thus, under aerobic conditions, H-NS has a positive influence on CRP-mediated *hlyE* expression. This positive effect of H-NS was not observed under anaerobic conditions, presumably because FNR and not CRP occupies P*hlyE* under these conditions.

To further investigate the effects of the regulators on *hlyE* expression, we monitored the activity of the *hlyE::lacZ* fusion in pGS1629 in strains carrying multiple relevant mutations. Under anaerobic conditions in the absence of glucose, *hlyE* expression in an *fnr crp hns* triple mutant was low compared to that of the parental strain (Fig. 2a). Restoring *hns* did not affect *hlyE* expression. Similarly, restoring CRP did not enhance *hlyE* expression, despite the absence of glucose (Fig. 2a). However, the restoration of *fnr* produced a significant increase in *hlyE* expression under these conditions (Fig. 2a). This suggests that during anaerobic growth in liquid cultures in the absence of glucose, FNR is the major regulator of *hlyE* expression, and that CRP-mediated *hlyE* expression requires the presence of H-NS. The pattern of expression obtained in anaerobic cultures in the presence of glucose was similar to that obtained in the absence of glucose, except that *hlyE* expression in the *fnr crp hns* triple mutant and the *crp hns* double mutant was significantly higher than that for the corresponding cultures that lacked glucose (Fig. 2b). The *hlyE* expression patterns for equivalent aerobic cultures in the presence
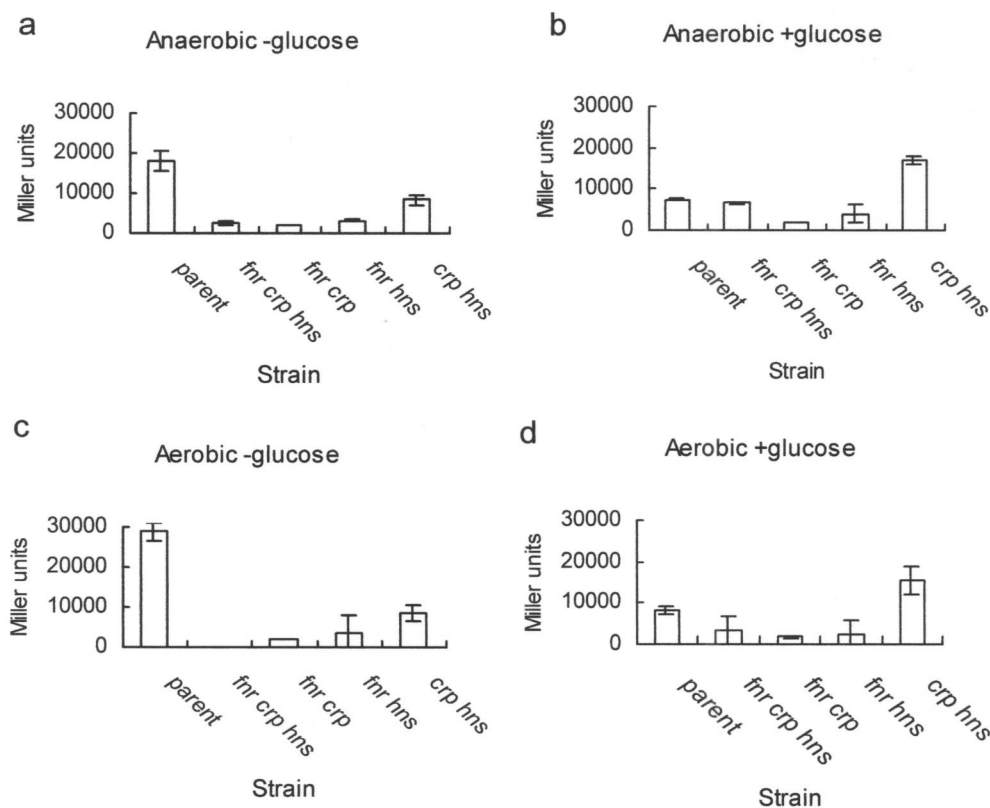
FIG. 2. Effects of FNR, CRP, and H-NS on in vivo transcription of hlyE. All cultures were grown in L-broth at 37°C for ~16 h under the indicated conditions. The activity of the hlyE promoter was estimated by measuring the β-galactosidase activity associated with each culture carrying the hlyE::lacZ plasmid pGS1629. Strains: parental, M182; fnr crp hns, JRG4866; fnr crp, JRG2631; fnr hns, JRG4865; crp hns, JRG4732. Error bars indicate 2 standard deviations from the means (n = 3).

and absence of glucose were similar to those obtained under anaerobic conditions (Fig. 2c and d).

In summary, the data presented here suggest that FNR and CRP are positive regulators of hlyE expression in liquid culture in response to oxygen and glucose starvation, respectively. This is in agreement with observations made with cultures grown on a solid medium (36). However, rather than the approximately eightfold enhancement in hlyE expression observed in an hns mutant on solid medium (36), in liquid culture H-NS has a positive effect on CRP-driven hlyE expression, a negative effect on FNR-driven hlyE expression, and little intrinsic regulatory activity in the absence of FNR and CRP.

H-NS binds at two regions of the hlyE promoter with high affinity. Footprinting studies have shown that FNR and CRP activate hlyE expression from the same site centered −61.5 bp upstream of the SlyA-associated transcription start site (10, 36). A further footprinting analysis indicated that H-NS protects a large region of the hlyE promoter, extending from −137 to +172 (relative to the transcription start site) (36). To investigate how much of this extensive H-NS protection is required for the observed regulation of hlyE expression, we used two approaches as follows.

Firstly, a 696-bp region of the hlyE promoter region was amplified by PCR (Fig. 3a). Gel shift assays revealed that H-NS retarded the mobility of this fragment upon electrophoretic separation in Tris-borate-EDTA-buffered polyacryl-amide gels (Fig. 3b). In the presence of 1 μM H-NS, retardation of some of the DNA target was observed as a smear in the gel. Upon the addition of further H-NS (2 μM), the free DNA was replaced entirely by a retarded smear. Only at 4 μM H-NS was a distinct retarded complex observed (Fig. 3b). The amplified fragment contains restriction sites for SspI, BsaMI, and DraI, such that a triple digest yields four fragments (Fig. 3a). Gel shift assays using a mixture of hlyE DNA fragments as the target DNA revealed that H-NS interacts with all four PhlyE fragments to some extent. However, the 147-bp DraI-BsaMI fragment was bound with the highest affinity, with 50% of this fragment retarded at H-NS concentrations of ~3 to 4 μM (Fig. 3c and d). Thus, this region of PhlyE (−91 to +56) contains both the FNR/CRP box (−61.5) and the highest affinity H-NS site(s).

Secondly, for testing of whether the region of PhlyE containing the high-affinity H-NS site(s) was sufficient to account for the pattern of hlyE regulation observed in vivo, another hlyE::lacZ reporter plasmid was used (Table 1) (10). This second gene fusion (pGS1065) contains a minimal hlyE promoter beginning 18 bp downstream of the BsaMI site and ending 25 bp downstream of the DraI site and thus extends from −97 to +61 relative to the transcript start site in the low-copy-number (two to five copies per cell) vector pRW50 (Table 1). Cultures of strains M182 and M182 hns transformed with pGS1065 were grown under anaerobic conditions in the presence and absence
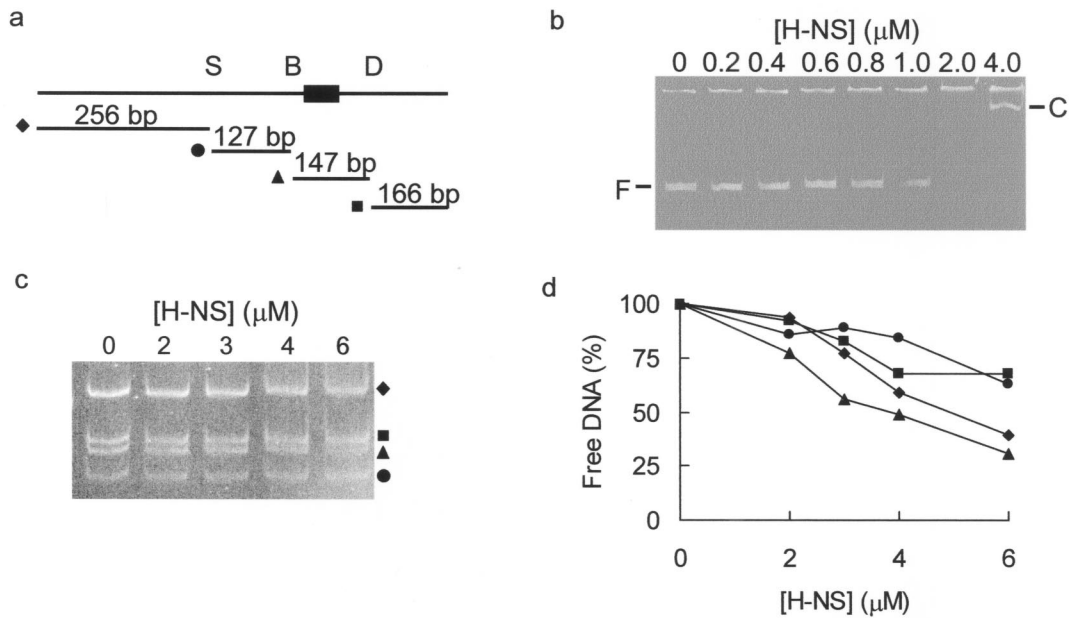
FIG. 3. Identification of high-affinity H-NS sites within P*hlyE*. (a) Schematic representation of the 696-bp region encompassing all of the previously recognized regions of H-NS protection (36). The locations of relevant restriction sites (S, *Ssp*I; B, *Bsa*MI; and D, *Dra*I) and the sizes of the fragments released after triple digestion are indicated. The location of the FNR/CRP box is shown by the filled rectangle. (b) Interaction of H-NS with the 696-bp P*hlyE* fragment. The concentration of HlyE is indicated above each lane. The positions of the retarded complexes (C) and free P*hlyE* DNA (F) are marked. (c) The region of P*hlyE* containing the FNR/CRP box has the highest affinity for H-NS. The concentrations of H-NS used are indicated above each lane, and the targets were purified restriction fragments released from the 696-bp P*hlyE* fragment by digestion with *Ssp*I, *Bsa*MI, and *Dra*I. The symbols relate to the restriction fragments, as indicated in panel a. (d) Quantification of H-NS binding to different regions of P*hlyE*. The amount of each P*hlyE* fragment remaining unretarded in panel c was estimated by quantitative densitometry and plotted against the concentration of H-NS in the assay. The symbols relate to the restriction fragments, as indicated in panel a.

of glucose at 37°C for ~16 h. The transcriptional activity in vivo, as estimated by the measurement of β-galactosidase activity, indicated that this (pGS1065) low-copy-number reporter containing a minimal *hlyE* promoter responded in the same way as the longer medium-copy-number *hlyE*::*lacZ* fusion (pGS1629) to the presence or absence of H-NS (Table 2). Thus, in the presence of glucose, *hlyE* expression was enhanced in an *hns* mutant (365 Miller units) relative to the parent (271 Miller units) (compare with Fig. 1b). In the absence of glucose, *hlyE* expression was slightly lower in the *hns* mutant (399 Miller units) than in the parent (420 Miller units) (compare with Fig. 1a). Furthermore, there were no significant H-NS-related changes in reporter plasmid copy number (not shown), as judged by the method of Taylor and Brose (33). Thus, it was concluded that the *Bsa*MI-*Dra*I fragment of P*hlyE* contains all of the significant regulatory elements that control *hlyE* expression under the growth conditions used here and that the effects on *hlyE* expression observed with pGS1629 were not significantly affected by the copy number of the reporter plasmids.

Identification of the high-affinity H-NS sites in P*hlyE*. For determination of the number of H-NS sites within the minimal P*hlyE* sequence from pGS1065, further gel shift and footprinting assays were undertaken. The gel shifts showed that H-NS bound to P*hlyE* at concentrations close to those observed for other H-NS-regulated genes (~1 μM) (3) and that two distinct retarded complexes were formed (Fig. 4a). The locations of the H-NS binding sites were determined by DNase I footprinting (Fig. 4b). Two protected regions were detected: H-NS I, consisting of an A-T-rich region (20 A-T/24 bp) stretching from

−75 to −37 which overlaps the FNR/CRP box centered at −61.5; and H-NS II, between −22 and +11, overlapping the −10 element of P*hlyE*. The H-NS II site contains a hypersensitive base at position −17 that is separated by several unprotected bases (−16 to +1) from another protected A-T-rich region (8 A-T/10 bp) (Fig. 4b). Based upon the size and properties of H-NS and the extent of the DNase I footprint, it is likely that several H-NS molecules bind P*hlyE*.

TABLE 2. Effect of SlyA and H-NS on transcription from a minimal hlyE promoter in vivo

Strain[a]	Data without glucose		Data with glucose	
	Amt of β-galactosidase (Miller units)[b]	Ratio[c]	Amt of β-galactosidase (Miller units)[b]	Ratio[c]
Parent + vector	420 ± 11	1.46	271 ± 6	1.14
Parent + SlyA	614 ± 10		310 ± 21	
hns + vector	399 ± 10	0.91	365 ± 15	0.82
hns + SlyA	365 ± 17		299 ± 38	
crp fnr + vector	257 ± 6	0.87	152 ± 2	1.11
crp fnr + SlyA	223 ± 7		169 ± 2	

[a] Strains containing the low-copy-number minimal *hlyE*::*lacZ* promoter fusion plasmid (pGS1065) were grown under anaerobic conditions for 16 h at 37°C in L-broth with or without glucose (0.2% [wt/vol]) as indicated. The strains used were as follows: parent, M182(pGS1065); *hns*, JRG4864(pGS1065); *crp fnr*, JRG3212(pGS1065) (Table 1). SlyA indicates the presence of multicopy *slyA* (pGS1657), and vector indicates the presence of the vector (pBluescript).

[b] Means (*n* = 3) and standard deviations are given.

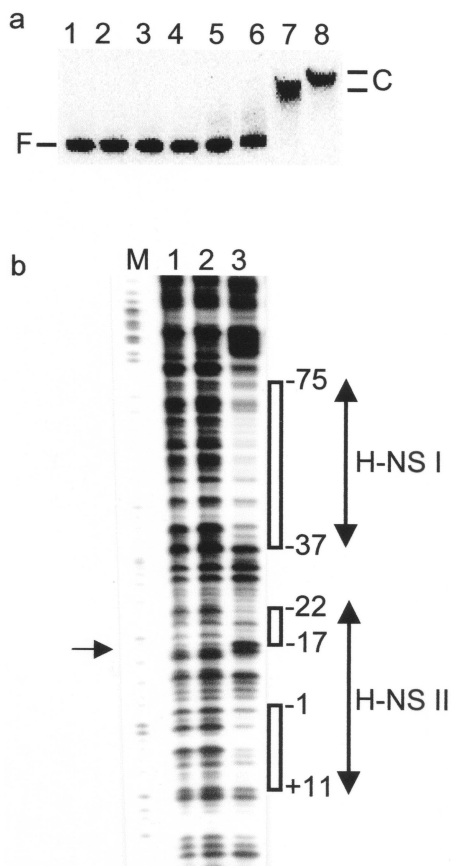[c] Ratio of β-galactosidase activities obtained with multicopy *slyA* and vector transformants.

FIG. 4. Location of H-NS binding sites in P*hlyE*. (a) H-NS binds at P*hlyE*. Lane 1, no protein; lanes 2 to 8, H-NS (0.04, 0.08, 0.12, 0.25, 0.5, 1.0, and 2.0 μM, respectively). The positions of the retarded complexes (C) and free P*hlyE* DNA (F) are indicated. (b) DNase I footprints of H-NS at P*hlyE*. Lane M, Maxam and Gilbert G track; lanes 1 and 2, no protein; lane 3, H-NS (1 μM). The regions of H-NS protection (open boxes) and the hypersensitive base (arrow at the left) are indicated. All numbering is relative to the SlyA-associated transcription start site (36).

Effects of H-NS on SlyA-promoted *hlyE* expression. Overproduction of the *E. coli* or *S. enterica* SlyA protein confers a hemolytic phenotype on *E. coli* K-12 by enhancing *hlyE* expression (16, 17, 21). To test the effects of H-NS on SlyA-driven *hlyE* expression, we measured β-galactosidase activities from cultures of isogenic parental and *hns* strains carrying the *hlyE::lacZ* plasmid pGS1065 and either a multicopy *S. enterica* SlyA expression plasmid (pGS1657; Table 1) or, as a control, the vector (pBluescript). As expected, irrespective of the addition of glucose, the presence of multicopy *slyA* increased *hlyE* expression in cultures of the parental strain, although the enhancement was greater for cultures lacking the glucose supplement (Table 2). In contrast, in the absence of *hns*, SlyA overproduction did not enhance *hlyE* expression (Table 2). This pattern of expression was supported by qualitative studies in which the same strains were grown on blood agar plates. These revealed that multicopy *slyA* enhanced hemolytic activity associated with the parental strain but not with the *hns* strain (not shown). These results confirm and extend the observations of Westermark et al. (36) and suggest that, either directly or indirectly, SlyA activates *hlyE* expression by modu-

lating H-NS binding and ultimately by counteracting the negative effects of H-NS. This suggestion was supported by the observations that multicopy *slyA* did not enhance *hlyE* expression in a *crp fnr* double mutant (Table 2) and that SlyA did not promote open complex formation at P*hlyE* in vitro (not shown). Thus, it was concluded that SlyA, like H-NS, has no intrinsic activator role at P*hlyE* in the absence of FNR and CRP.

Interaction of SlyA with the *E. coli hlyE* promoter. The pattern of regulation reported above suggests that SlyA, like H-NS, interacts directly with the *E. coli hlyE* promoter. Therefore, gel shift assays were used to investigate the interaction of purified SlyA with P*hlyE*. These experiments revealed that the addition of 0.16 to 0.31 μM SlyA was sufficient to retard the mobility of P*hlyE* (Fig. 5a).

A consensus SlyA DNA target was recently suggested to exist (32), and an inspection of the P*hlyE* sequence revealed at least two possible matches. Therefore, the location of the SlyA binding site(s) within P*hlyE* was investigated by DNase I footprinting (Fig. 5b). A protected region, −70 to −38 relative to the previously determined SlyA-associated transcript start site (17, 36), was observed in the presence of 2 μM SlyA. This region of protection was extended further downstream to −31 when the concentration of SlyA was increased to 4 μM (Fig. 5b). Thus, SlyA protects a region of P*hlyE* similar to the H-NS I region (−75 to −37; Fig. 4b). The cross sections of the *E. coli* MarR and *E. faecalis* SlyA-like protein dimers are ~70 Å (1, 37) and therefore would be expected to protect ~20 bp of DNA. Thus, the protection observed here (up to 40 bp) suggests that two SlyA dimers are bound at P*hlyE*. An inspection of the DNA sequence of the protected region revealed two related sequences with partial dyad symmetry that re-
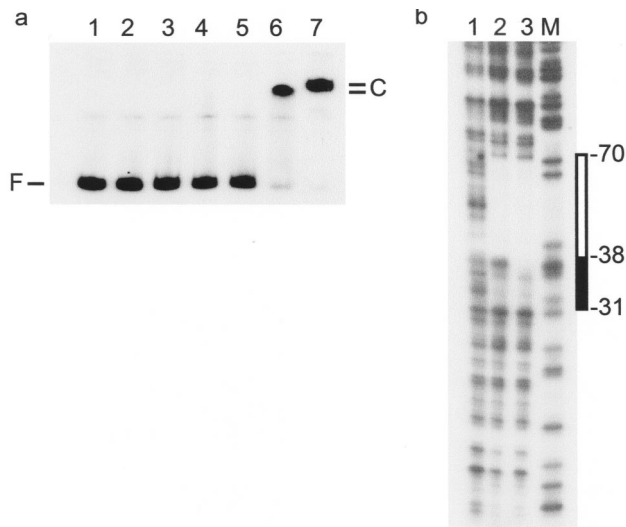

FIG. 5. Interaction of SlyA with the *hlyE* promoter. (a) SlyA retards the mobility of P*hlyE* in band shift assays. SlyA concentrations: lane 1, 0 μM; lane 2, 0.06 μM; lane 3, 0.08 μM; lane 4, 0.1 μM; lane 5, 0.16 μM; lane 6, 0.31 μM; lane 7, 0.63 μM. The positions of the free DNA (F) and the SlyA-DNA complexes (C) are indicated. (b) DNase I footprint of SlyA at P*hlyE*. Lane 1, no protein; lane 2, 2.0 μM SlyA; lane 3, 4.0 μM SlyA; lane M, Maxam and Gilbert G track. The region of SlyA protection is indicated to the right (box). At the higher SlyA concentration, protection was extended, as indicated by the filled box.

semble the proposed SlyA binding consensus; they are SlyA I ($^{-61}$**TTATCA**TA**TTAA**$^{-50}$, with 8 of 12 bases (bold) matching the SlyA binding site consensus **TTAGCAAGCTAA**) and SlyA II ($^{-50}$**ATAG**AAA**TAAAG**$^{-39}$, with 6 of 12 bases matching the consensus). The putative presence of two SlyA dimers at P*hlyE* was consistent with the two distinct complexes with retarded mobilities in the gel shift assays (Fig. 5a). The site centered at −55.5 is the better match to the SlyA binding site consensus and may be occupied in preference to the site centered at −44.5, which accordingly is less similar to the consensus.

DISCUSSION

The starting point for the work described here was the observation that lesions in *hns* or the overproduction of *S. enterica* or *E. coli* SlyA confers a hemolytic phenotype on *E. coli* K-12 by enhancing the expression of the pore-forming toxin HlyE (9, 16, 17, 21). Here we have shown that H-NS and SlyA interact directly with the *hlyE* promoter. Both proteins occupy a common region of P*hlyE* that overlaps the binding site for the global transcription factors FNR and CRP, which are known to activate *hlyE* expression (10, 36). In vivo and in vitro evidence suggests that SlyA activates *hlyE* expression by antagonizing H-NS-mediated repression.

The in vivo transcriptional evidence presented here suggests that FNR occupies the *hlyE* promoter more frequently than CRP even under aerobic conditions. This is evident from enhanced *hlyE::lacZ* expression in aerobic cultures (compare parent, *fnr*, and *crp* strains in Fig. 1c and d). These observations may be explained by a mechanism in which FNR recognizes P*hlyE* efficiently but acts only as a relatively poor activator of *hlyE* transcription (10, 11), whereas, conversely, the recognition of P*hlyE* by CRP is poor, but once CRP is bound, CRP-mediated activation of *hlyE* expression is efficient. This reciprocity of binding site recognition and transcriptional efficiency provides a mechanism for the moderate upregulation of *hlyE* expression in response to two distinct environmental signals (oxygen and glucose starvation) rather than, for example, the much larger degree of upregulation when FNR and CRP act synergistically by binding at different sites within the *ansB* promoter (28). In principle, any pair of transcription factors that recognize similar DNA sequences but have different transcriptional efficiencies could adopt this strategy.

Under some of the conditions studied here, the effects of an *hns* lesion on *hlyE::lacZ* expression in liquid cultures were different from those reported by Westermark et al. for bacteria grown on a solid medium (36). This is perhaps not surprising considering the significant physiological differences between cultures grown in liquid and on solid media. In the previous report (36), H-NS acted as a strong repressor of *hlyE* expression, whereas here H-NS appears to have a positive effect on *hlyE* expression in the absence of glucose but an attenuated or negative effect on *hlyE* expression in the presence of glucose (Fig. 1). This suggests that, in general, H-NS inhibits FNR-driven *hlyE* expression (Fig. 1) but enhances CRP-driven expression in liquid cultures (compare Fig. 1b and c). Moreover, any enhancement of *hlyE::lacZ* expression in the *hns* strain observed here did not approach the eightfold increase observed on solid medium (36). Thus, it appears that H-NS modulates P*hlyE* activity both positively and negatively in response

FIG. 6. Schematic representation of the *hlyE* promoter region. The locations of the FNR/CRP box and SlyA sites are shown. The high-affinity H-NS binding regions observed in DNase I footprints of P*hlyE* are also shown. The transcript start site (+1) is marked. The region of the H-NS I site that extends downstream of the FNR/CRP box is indicated by a thick arrow, and the part overlapping the FNR/CRP box is indicated by a thin arrow.

to the prevailing growth and/or environmental conditions, such as growth in liquid and on solid media. This may be a reflection of the formation of different H-NS–P*hlyE* complexes in response to environmental signals. This idea is supported by the identification of two regions within the larger previously described H-NS–P*hlyE* complex (36) that bind H-NS with high affinities. The first region (H-NS I) overlaps with, and extends downstream from, the FNR/CRP site (Fig. 6). The extent of protection associated with the H-NS I site (38 bp) suggests that more than one H-NS dimer is bound, and the formation of different subcomplexes could potentially act to modulate FNR/CRP-driven transcription activation either positively (occupation of the downstream portion of H-NS I; thick arrow in Fig. 6) or negatively (occupation of sequences overlapping the FNR/CRP-binding site; thin arrow in Fig. 6). While H-NS usually acts as a negative regulator of gene expression, *hlyE* is not the only example of a CRP-regulated gene whose expression is positively regulated by H-NS; for example, expression of both the *malT* and *csiD* genes in *E. coli* is stimulated by H-NS (8, 14). Thus, it would appear that a CRP family transcription factor plus H-NS is a versatile combination, providing the means to generate a range of regulatory effects.

The second region of H-NS protection (H-NS II) overlaps the basic promoter elements, and H-NS bound at this region would be expected to repress *hlyE* expression by promoter occlusion. The findings that SlyA does not enhance *hlyE* expression in *hns* cultures grown in liquid medium under anaerobic conditions and that SlyA appears unable to activate *hlyE* expression in the absence of FNR and CRP and the determination of the location of the region of P*hlyE* occupied by SlyA (Fig. 6) suggest that SlyA activates *hlyE* expression by antagonizing the negative action of H-NS. Previous studies have indicated that the intracellular levels of H-NS change with the growth phase. However, whereas one proteomics-based study suggested a fivefold increase in H-NS in stationary-phase cultures (31), a more recent immunological analysis suggested a twofold decrease (4). In addition, we have shown that intracellular H-NS levels increase as growth temperature decreases, as evidenced by an approximately twofold increase in H-NS immunoblot signal intensity from cultures grown at 20°C compared to those grown at 37°C (N. R. Wyborn and J. Green, unpublished data). Thus, it appears that intracellular H-NS levels are influenced by the environment. Therefore, we suggest that when environmental conditions dictate a low intra-

cellular concentration of H-NS, the H-NS I region of P*hlyE* is occupied to modulate the activity of the upstream activator (FNR or CRP). As the intracellular levels of H-NS increase, H-NS II is occupied and *hlyE* expression is downregulated by promoter occlusion. The footprinting studies described by Westermark et al. (36) indicated that further increases in H-NS levels result in the formation of higher order complexes that occupy P*hlyE* from the −10 element to position +172 and from just upstream of the −35 element to position −137. Thus, it appears that these higher order complexes extend from the primary sites of interaction (H-NS I and H-NS II) identified here. Such higher order complexes, which effectively silence *hlyE* expression, appear to have directionality in that they extend upstream from H-NS I (to as far as position −137) to occlude the FNR/CRP site and downstream from H-NS II (to as far as +172) to occlude the basic promoter elements. This leaves the region between the two primary sites of interaction unoccupied. Thus, we suggest that the formation of different H-NS–P*hlyE* complexes offers the opportunity for H-NS, in combination with FNR and CRP, to control *hlyE* expression both positively and negatively. This type of behavior, in which specific patterns of protection are replaced by general protection as the concentration of H-NS increases, has been observed for footprints of *gal* promoter variants (25). It was also shown previously that this general protection was dependent on the polymerization of H-NS on the DNA and that this was more likely if there was an initial nucleation event on the DNA at specific sites at low H-NS concentrations (25). It appears that the H-NS I and H-NS II regions of P*hlyE* are such nucleation sites. By competing with H-NS for the region of P*hlyE* downstream of the FNR/CRP site, SlyA may prevent the formation of the negatively acting H-NS–P*hlyE* complexes by blocking a primary interaction between H-NS and P*hlyE*, allowing FNR and CRP to continue to activate *hlyE* expression. Presumably, the action of the *E. coli* SlyA protein will prove to be similar to that of the *Salmonella* protein studied here, given that their primary structures are 89% identical and that the overproduction of *E. coli* SlyA also enhances *hlyE* expression (16, 21).

In summary, we have shown that the H-NS-mediated regulation of *hlyE* expression in *E. coli* K-12 is more complex than was previously suggested because H-NS can contribute positively as well as negatively to *hlyE* expression. This range of regulatory activity is probably associated with the progressive formation of higher order H-NS–P*hlyE* complexes that extend from two primary sites of interaction to ultimately bring about silencing of the *hlyE* gene. The H-NS and SlyA footprints suggest that H-NS regulation is overcome by the overproduction of SlyA, which by binding at a site that overlaps one of the primary H-NS sites (H-NS I), prevents the formation of repressive H-NS–P*hlyE* complexes. Further detailed in vitro analyses will be required to fully analyze the complex relationships between H-NS, SlyA, FNR, and CRP and their consequences for *hlyE* expression.

ACKNOWLEDGMENTS

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