Alternate SlyA and H-NS nucleoprotein complexes control hlyE expression in Escherichia coli K-12

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Summary

Haemolysin E is a cytolytic pore-forming toxin found in several Escherichia coli and Salmonella enterica strains. Expression of hlyE is repressed by the global regulator H-NS (histone-like nucleoid structuring protein), but can be activated by the regulator SlyA. Expression of a chromosomal hlyE–lacZ fusion in an E. coli slyA mutant was reduced to 60% of the wild-type level confirming a positive role for SlyA. DNase I footprint analysis revealed the presence of two separate SlyA binding sites, one located upstream, the other downstream of the hlyE transcriptional start site. These sites overlap AT-rich H-NS binding sites. Footprint and gel shift data showed that whereas RNAP, but inhibited binding of H-NS. Accordingly, in vitro transcription analyses showed that addition of SlyA protein relieved H-NS-mediated repression of hlyE. Based on these observations a model for SlyA/H-NS regulation of hlyE expression is proposed in which the relative concentrations of SlyA and H-NS govern the nature of the nucleoprotein complexes formed at PhlyE. When H-NS is dominant RNAP binding is inhibited and hlyE expression is silenced; when SlyA is dominant H-NS binding is inhibited allowing RNAP access to the promoter facilitating hlyE transcription.

Introduction

Cytolytic toxins are major virulence factors secreted by bacterial pathogens. The most extensively studied of these is haemolysin A, a 110 kDa protein belonging to the family of RTX ‘repeat toxins’, which is secreted by a specific type I secretion system and associates with target cell membranes where it is thought to form pores (Felmlee et al., 1985; Bhakdi et al., 1989; Welch et al., 1992). Another unrelated pore-forming toxin, haemolysin E, designated HlyE, CyaA or SheA, is a 34 kDa protein identified in Escherichia coli and Salmonella enterica serovars Typhi and Paratyphi A (Ludwig et al., 1995; 1999; Oscarsson et al., 1996; 2002; del Castillo et al., 1997; Fernandez et al., 1998; Atkins et al., 2000; Wallace et al., 2000; von Rhein et al., 2006). The pore structure of HlyE remains controversial in that separate studies have suggested that it can form either predominantly octomeric or predominantly 13-meric rings that can insert into cell membranes (Eifler et al., 2006; Tzokov et al., 2006). HlyE export is not yet fully understood, although it is known that HlyE accumulates in the periplasm of E. coli independently of type I, II, III, IV and V secretion systems, and its subsequent release is mediated, at least in part, by membrane blebbing (Wai et al., 2003; Wyborn et al., 2004a). It has been shown that HlyE affects Ca2+ signalling in epithelial cells, induces apoptosis in human and mouse macrophages, and causes haemolysis of human, rabbit, sheep and horse erythrocytes (Oscarsson et al., 1999; Dai et al., 2000; Soderblom et al., 2002; 2005).

Several different types of E. coli, including Shiga toxin-producing (O157:H7), enteroinvasive, enteroaggregative, enterotoxigenic and avian strains, have been shown to carry functional copies of the hlyE gene (del Castillo et al., 2000; Ludwig et al., 2004; Wyborn et al., 2004a; Kerenyi et al., 2005; McPeake et al., 2005). In contrast, some other enteropathogenic strains have non-functional hlyE genes containing frameshift mutations, and strains isolated from extraintestinal (e.g. uropathogenic or newborn meningitis-associated) infections harbour non-functional copies of hlyE with chromosomal deletions (Ludwig et al., 2004). It has been suggested that due to interference between the mechanisms regulating production of HlyA and HlyE, there is possible incompatibility between hlyA and hlyE in the E. coli chromosome (Kerenyi et al., 2005). A functional hlyE gene is also present in non-pathogenic E. coli K-12,
Yersinia pseudotuberculosis has been shown to directly
function of the protein, but as yet no such molecular signal
has been identified for SlyA (Kenney, 2002; Wilkinson and Grove, 2006).

SlyA is a member of the family of ‘winged-helix’ transcription factors that includes MarR from E. coli, RovA from Yersinia spp. and PecS from Erwinia chrysanthemi (Reverchon et al., 1994; Sulavik et al., 1995; Revell and Miller, 2000; Heroven et al., 2004; Cathelyn et al., 2006; Ellison and Miller, 2006; Wilkinson and Grove, 2006). SlyA regulates the expression of a large number of S. Typhimurium genes, the majority of which are predicted to encode membrane, periplasmic or secreted proteins, suggesting that a major role of SlyA is to alter the cell envelope during stationary phase and in the intracellular environment of host cells (Spory et al., 2002; Stapleton et al., 2002; Navarre et al., 2005). SlyA is implicated in virulence, survival in mouse macrophages, resistance to oxidative stress and resistance to antimicrobial peptides (Libby et al., 1994; Daniels et al., 1996; Buchmeier et al., 1997; Spory et al., 2002; Shi et al., 2004; Linehan et al., 2005). Analysis of the transcriptome of a S. Typhimurium slyA mutant revealed that many slyA-dependent genes are also controlled by the magnesium-sensitive PhoP/PhoQ regulatory system (Navarre et al., 2005).

SlyA forms a dimer and has a DNA-binding domain containing a DNA-recognition helix flanked by a β-strand wing (Alekshun et al., 2001; Wu et al., 2003; Okada et al., 2007). The DNA-binding motifs are separated by a channel that allows contact with both the minor and major grooves of the DNA helix (Okada et al., 2007). The S. Typhimurium SlyA dimer (32 kDa) recognizes the palindromic DNA consensus sequence TTAGCAAGCTAA (Stapleton et al., 2002). Although the RovA protein in Yersinia pseudotuberculosis has been shown to directly activate transcription by stimulating RNA polymerase (RNAP) in the absence of other accessory factors, it is thought that SlyA-type proteins generally activate transcription indirectly, by binding to DNA and modulating the activity of other factors such as PhoP or the nucleoid-associated protein H-NS (Heroven et al., 2004; Navarre et al., 2005; Tran et al., 2005). Some members of the MarR subfamily interact with small ligands to alter the function of the protein, but as yet no such molecular signal has been identified for SlyA (Kenney, 2002; Wilkinson and Grove, 2006).

H-NS is a 15 kDa protein that acts as a regulator of more than 200 E. coli genes mostly related to adaptation to environmental stress (Drlica and Rouviere-Yaniv, 1987; Hulton et al., 1990; Atlung and Ingmer, 1997). H-NS can bind to DNA and form higher-order oligomeric H-NS complexes in a concentration-dependent manner, changing DNA topology and interfering with the action of other transcription factors (Tupper et al., 1994; Esposito et al., 2002; Rimsky, 2004). It has been shown that H-NS binds to the upstream and downstream regions of the hlyE promoter (PhlyE) in E. coli and is involved in silencing hlyE expression (Westermark et al., 2000). Further investigation showed that the PhlyE contained overlapping high-affinity binding sites for both H-NS and SlyA (Wyborn et al., 2004b). In addition, PhlyE was characterized as a class I promoter that could be activated by the oxygen-responsive regulator FNR and/or the glucose-responsive regulator CRP (Green and Baldwin, 1997; Ralph et al., 1998; Westermark et al., 2000). In this study, using in vivo and in vitro analyses the interactions between H-NS, SlyA and RNAP at the PhlyE were examined to elucidate the mechanism whereby SlyA can antagonize H-NS-mediated repression of hlyE transcription.

Results

Effect of hns and slyA mutations on chromosomal hlyE–lacZ expression in vivo

The level of hlyE expression is increased in an E. coli hns mutant (Westermark et al., 2000; Wyborn et al., 2004b). Expression of hlyE–lacZ carried on a multicopy plasmid is enhanced by introduction of multicopy slyA in a parent strain but not in an hns mutant, suggesting that SlyA has no intrinsic activator role, but that it probably relieves H-NS-mediated repression of hlyE transcription (Wyborn et al., 2004b). To discount the possibility that previous results were affected by plasmid copy number and regulator titration the effects of hns and slyA mutations on expression of a chromosomal hlyE–lacZ fusion were determined (Table 1). Aerobic cultures supplemented with 0.2% glucose were used to minimize the activities of FNR and CRP proteins. At 20°C, β-galactosidase activity of the hns mutant cultures was 1.8-fold higher than of the cultures of the parent strain, confirming the repressive effect of H-NS on hlyE–lacZ expression. Expression of hlyE in a slyA mutant was reduced to 60% of that of the parent, demonstrating a positive effect of slyA in the presence of functional hns gene. In the hns slyA double mutant, hlyE expression was twofold greater than in the parent, and slightly higher than the hns single mutant, suggesting that slyA has a small negative effect on hlyE expression in the absence of H-NS. At 37°C, hlyE–lacZ expression was ~25% lower than that observed at 20°C for the
parental, hns, and hns slyA strains (Table 1). However, expression of hlyE in the slyA mutant was similar at both temperatures. These data support the hypothesis that SlyA relieves H-NS-mediated repression of hlyE expression.

SlyA binds at two separate sites at the hlyE promoter and inhibits H-NS binding

Previous in vitro DNase I protection analysis with PhlyE identified a SlyA footprint from −70 to −30 relative to the hlyE transcription start site, reflecting the presence of two separate palindromic SlyA-recognition sequences in this upstream region, these being SlyA Ia (\(\text{TTATCATAT TAA}^{−50}\)) and SlyA Ib (\(\text{ATAAGATAAAG}^{−39}\)) (consensus-matching bases in bold) (Wyborn et al., 2004b). In this work, using a PhlyE DNA fragment extending from −171 to +222, we have observed another SlyA binding site (named SlyA II) in the region downstream of the transcript start site. In the presence of SlyA, protection of PhlyE was observed from −70 to −30 (corresponding to the previously described SlyA I site) and also in the region downstream of the transcript start site from +34 to +109 (Fig. 1). The crystal structures of E. coli MarR and Enterococcus faecalis SlyA-like proteins show their cross-sections are \(\sim70\) Å, suggesting they would protect \(\sim20\) bp of DNA (Aleksun et al., 2001; Wu et al., 2003). This suggests the presence of two SlyA dimers at the SlyA I site and at least three dimers at the SlyA II site. The downstream site, SlyA II, was protected in the presence of SlyA at concentrations of 0.3 \(\mu\)M and above, whereas the SlyA I site was only partially protected in the presence of 0.6 \(\mu\)M and fully protected with 1.2 \(\mu\)M SlyA (Fig. 1), suggesting that the SlyA protein has a higher affinity for the SlyA II site.

Wyborn et al. (2004b) described two regions of H-NS binding at the PhlyE using 1 \(\mu\)M H-NS which extended...
from −75 to −37 (H-NS I) and from −22 to +11 (H-NS II). At higher concentrations up to 9.6 μM, H-NS was shown to interact with a larger region of PhlyE extending from −137 to +182 (Westermark et al., 2000). Taken together these data suggested that at lower concentrations H-NS may bind at AT-rich specific nucleation sites, and at higher concentrations, multiple H-NS proteins oligomerize to occupy both the upstream and downstream regions of PhlyE. To test the hypothesis that SlyA can modulate the interaction of H-NS with PhlyE, footprinting analysis was performed using combinations of H-NS and SlyA protein.

Using 2.4 μM H-NS, regions of PhlyE protection were observed from −70 to −30 (corresponding to the H-NS I site found by Wyborn et al. (2004b)), −16 to −9, and −7 to +40 (corresponding approximately to the H-NS II site) (Fig. 2, lane 3). The H-NS footprint was also characterized by hypersensitive bases at −30, −17 and −8. With 2 μM SlyA, protection was observed at −70 to −38 (SlyA I) and from +35 to beyond the end of the autoradiographs (SlyA II), with a hypersensitive region from −30 to −9 (Fig. 2, lane 4). In reactions where 0.1 μM H-NS was added to the SlyA:PhlyE complex, both SlyA I and II sites remained protected (Fig. 2, lane 5). On addition of 1.2 μM H-NS, protection of the SlyA I and II sites was abolished, and replaced by weak protection of the H-NS II site, but no apparent protection of H-NS I (Fig. 2, lane 6). Furthermore, the pattern of hypersensitive bases from −30 to −9 remained, suggesting that the addition of 1.2 μM H-NS allowed some SlyA to associate with the complex, and that H-NS was unable to fully interact with PhlyE. In reactions where 2.4 or 3.6 μM H-NS were added, the pattern of protection observed resembled the footprint made by the H-NS protein alone, with protection at both H-NS I and II sites (Fig. 2, lanes 7 and 8).

In reactions where 0.1 μM SlyA was added to PhlyE before addition of 2.5 μM H-NS (Fig. 3, lane 3), the resulting footprint resembled that produced by H-NS alone (Fig. 3, lane 3). When 0.5 μM or 1 μM SlyA was added before H-NS, the pronounced H-NS footprint was absent, and hypersensitive sites were present at −91, −30, −16 (Fig. 3, lanes 6 and 7). Therefore, in the presence of a high level of H-NS, addition of a low level of SlyA did not affect the binding of H-NS at PhlyE, but when the level of SlyA was increased, a unique pattern of protection was obtained which is distinct from, yet shares some features of, the footprints made by H-NS or SlyA alone.

**Fig. 2.** H-NS competes with SlyA for binding at the hlyE promoter. DNase I protection assays at the hlyE promoter in the presence of SlyA and H-NS. Lane M, Maxam–Gilbert G-track; lanes 1 and 2, no protein; lane 3, H-NS (2.4 μM); lane 4, SlyA (2 μM); lanes 5–8, H-NS (0.1, 1.2, 2.4 and 3.6 μM respectively) with fixed amount of SlyA (2 μM). Regions of protection (open boxes) and numbering relative to the hlyE transcript start site are shown.

**Fig. 3.** SlyA remodels a PhlyE:HS nucleoprotein complex. DNase I protection assays of the PhlyE:HS complex in the presence of increasing concentrations of SlyA. Lane M, Maxam–Gilbert G-track; lanes 1 and 2, no protein; lane 3, H-NS (2.4 μM); lane 4, H-NS (2.4 μM); lanes 5–7, SlyA (0.1, 0.5 and 1 μM respectively) with fixed amount of H-NS (2.4 μM). Regions of protection (open boxes) and numbering relative to the hlyE transcript start site are shown.
Overall, the footprinting data suggested that a PhlyE:SlyA complex is formed when SlyA binds at specific sites in the upstream and downstream regions of the promoter, and when present at a high enough concentrations, SlyA prevents the binding of low levels of H-NS at the H-NS I and H-NS II sites. At intermediate concentrations of H-NS, the pattern of protection and hypersensitive bases suggests that the structure of the PhlyE:SlyA complex is altered, but H-NS is unable to fully interact with PhlyE. When H-NS is present at a higher concentration it can displace SlyA from SlyA sites I and II and thus occupy the H-NS I and II sites enabling the formation of higher-order H-NS complexes.

Interaction of SlyA, H-NS and RNAP at the hlyE promoter

It has been suggested that H-NS binding renders PhlyE inaccessible to RNAP (Westermark et al., 2000). Whereas previous work analysed the interaction of either H-NS or SlyA with PhlyE, in this study electrophoretic mobility shift assays (EMSA) were used to examine the interaction of mixtures of H-NS, SlyA and RNAP with PhlyE.

A SlyA:PhlyE complex with significantly retarded mobility was formed on addition of 1.25 μM SlyA to PhlyE DNA (Fig. 4A, lane 2). Addition of up to 0.63 μM H-NS to this complex had no significant effect on the SlyA:PhlyE complex (Fig. 4A, lanes 3–5), but mobility was increased in the presence of 1.25 and 2.5 μM H-NS (Fig. 4A, lanes 6 and 7). This increase in mobility in the presence of H-NS indicates the formation of a SlyA:H-NS:PhlyE complex (note that in comparison with SlyA:PhlyE, the H-NS:PhlyE complex migrates significantly further; Fig. 4B, lane 2). In the reverse experiment, increasing amounts of SlyA were added to a complex of PhlyE and 1.25 μM H-NS. The mobility of H-NS:PhlyE was not affected by addition of up to 0.63 μM SlyA (Fig. 4B, lanes 2–5), but was significantly altered after addition of 1.25 μM or 2.5 μM SlyA (Fig. 4B, lanes 6 and 7). The simplest explanation for these data is that initial formation of a SlyA:PhlyE complex prevents binding of H-NS when H-NS is present at low concentrations. When a critical concentration of H-NS is reached, a SlyA:H-NS:PhlyE complex forms with an altered structure, as indicated by the changes in the DNase I footprint and EMSA mobility. The DNase I footprints (Fig. 2, lane 8) indicate that H-NS can ultimately displace SlyA. However, when the SlyA concentration increases relative to that of H-NS, the H-NS:PhlyE complex is replaced by a SlyA:PhlyE complex via a SlyA:H-NS:PhlyE intermediate. The switches between these different nucleoprotein complexes appear to occur over narrow concentration ranges, suggesting that at critical thresholds a ‘see-saw’ mechanism operates in which SlyA antagonizes H-NS interaction and H-NS antagonizes SlyA binding at PhlyE.

Fig. 4. Interaction of SlyA, H-NS and RNAP with hlyE promoter DNA. Electrophoretic mobility shift assays were carried out in 4% acrylamide/0.1 × TBE gels using 20 ng of a radiolabelled 244 bp DNA fragment (–171 to +73 relative to the hlyE transcript start) combined with varying amounts of protein: (A) fixed amount (1.25 μM) of SlyA with increasing level of H-NS; (B) fixed amount of H-NS (1.25 μM) with increasing level of SlyA; (C) 1 unit of RNAP with increasing level of SlyA; (D) 1 unit of RNAP with increasing level of H-NS. Protein concentrations are shown in μM. F, free DNA; S, SlyA:DNA complex; H, H-NS:DNA complex; SH, SlyA:H-NS:DNA complex; RP, RNAP:DNA complex.

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The effect of adding SlyA or H-NS to PhlyE in the presence of RNAP was then tested. Addition of up to 2.5 μM SlyA did not affect the mobility of the RNAP:PhlyE complex (Fig. 4C, lanes 2–7). Although it is not known at this stage whether both RNAP and SlyA are bound at PhlyE simultaneously, the mobility of a mixture of PhlyE, RNAP and SlyA was lower than SlyA:PhlyE alone (Fig. 4C, lanes 7 and 8), indicating that addition of SlyA did not interfere with the binding of RNAP to PhlyE. However, the RNAP:PhlyE complex was severely affected by the addition of H-NS (Fig. 4D). As the H-NS concentration was increased, the proportion of RNAP:PhlyE complex was reduced and the proportion of H-NS:PhlyE was increased (Fig. 4D, lanes 2–7), suggesting that H-NS replaces RNAP at PhlyE in a concentration-dependent manner. Thus, the EMSA data supported the hypothesis that H-NS negatively regulates PhlyE by promoter occlusion. In contrast, SlyA did not prevent RNAP binding at PhlyE, but did inhibit H-NS binding.

SlyA relieves H-NS-dependent repression of hlyE transcription in vitro

The footprinting and EMSA data show that different nucleoprotein complexes are formed at PhlyE depending on the relative concentrations of H-NS and SlyA through competition for overlapping binding sites upstream and downstream of the hlyE transcript start site. This suggested a regulatory mechanism whereby increased intracellular concentrations of SlyA remodel H-NS binding at PhlyE to relieve H-NS-mediated repression by allowing RNAP to access the promoter. Plasmid pGS1886 contains the PhlyE and the 5′-end of the hlyE gene extending from –474 to +222 relative to the transcript start site. It was predicted that addition of H-NS would repress production of hlyE mRNA in an in vitro transcription reaction using pGS1886, and that addition of SlyA would modulate the effect of H-NS and allow hlyE transcription. At 25°C RNAP-induced expression of a hlyE transcript of 222 nt (Fig. 5A, lane 1) was abolished when 0.5 μM H-NS was added to the reaction (Fig. 5A, lane 5). In a mixture of RNAP and 0.5 μM H-NS supplemented with 1 μM or 12 μM SlyA, the hlyE transcript was still repressed at 25°C (Fig. 5A, lanes 7 and 8). However, at 37°C, addition of 1 μM or 12 μM SlyA partially relieved the repression caused by the presence of 0.5 μM H-NS (Fig. 5B, compare lanes 5 and 6 with lanes 7 and 8). Although reproducible, the enhancement in the abundance of the hlyE transcript when SlyA was added to the PhlyE:H-NS complex was somewhat disappointing under the conditions used for the experiments in Fig. 5A and B. Using an equivalent supercoiled template in place of the linearized DNA used for the experiments in Fig. 5A and B failed to improve SlyA-mediated inhibition of SlyA repression under the same conditions (not shown). However, a greater effect was observed when a PCR product (–474 to
+222 relative to the hlyE transcript start) that contained both P_{hlyE} and the promoter of the divergently transcribed umuD gene was used in the in vitro transcription reactions. Addition of 2 μM H-NS repressed both hlyE and umuD transcription (Fig. 5C). Therefore, 2 μM H-NS was used in SlyA titration experiments. At low SlyA:H-NS ratios both P_{hlyE} and P_{umuD} were still repressed (Fig. 5D, lanes 2 and 3). However, at a higher concentration of SlyA, transcription of hlyE was specifically restored (Fig. 5D, lane 4). In contrast to P_{hlyE}, umuD was repressed whenever H-NS was present, showing that SlyA specifically antagonizes H-NS repression of P_{hlyE}.

**Discussion**

Although it has been previously demonstrated that the introduction of slyA on a multicopy plasmid confers a haemolytic phenotype on *E. coli* by enhancing expression of the haemolysin E gene, it is possible that this could be an artefactual effect of SlyA overexpression that is not normally seen in wild-type bacteria (Ludwig *et al*., 1995).

However, in this study, *E. coli* mutants with lesions in chromosomal slyA showed significantly less expression of a chromosomal hlyE-lacZ fusion in aerobic, glucose-supplemented cultures, suggesting that, at least in these conditions, SlyA is involved in upregulation of hlyE expression in the *E. coli*.

It has previously been shown that H-NS binds to P_{hlyE} in regions upstream (H-NS I) and downstream (H-NS II) of the transcript start site, and that a SlyA binding site overlaps H-NS I (Westermark *et al*., 2000; Wyborn *et al*., 2004b). In addition, both FNR and CRP contribute to regulation of hlyE expression (Green and Baldwin, 1997; Ralph *et al*., 1998; Westermark *et al*., 2000). FNR and CRP are ~50 kDa proteins that recognize related inverted repeat sequences (Scott *et al*., 2000). A sequence in P_{PhyE} (–68TTTGATATTTATCATA–58) more closely resembles the FNR consensus (NTTGATNNNNNTCAAN) than the CRP consensus (TGTGANNNNNTCACA), and *in vivo* transcription studies revealed that although FNR occupies P_{PhyE} more frequently than CRP, the latter is a more efficient activator of hlyE expression (Wyborn *et al*., 2004b). These data suggested that hlyE expression is activated by FNR and CRP, repressed by H-NS, and that H-NS-mediated transcriptional silencing is relieved by SlyA. In this study *in vivo* and *in vitro* analyses show that SlyA competes with H-NS for binding sites located upstream and downstream of the hlyE transcriptional start site, revealing that SlyA can directly antagonize H-NS-mediated silencing of hlyE expression.

Previous DNase I protection assays using a shorter region of P_{PhyE} than used here identified a SlyA footprint extending from –70 to –30 relative to the hlyE transcript start site (Wyborn *et al*., 2004b). Within this region are two potential 12 bp SlyA-recognition motifs, SlyA_{Ia} (–61TTATCATATTAA–50), and SlyA_{Ib} (–50ATAGAATTAAG–39) (consensus-matching bases in bold). In SlyA_{Ia}, 8 out of 12 base pairs match the SlyA-recognition consensus (TTAGCAAGCTAA) compared with only 6 out of 12 base pairs in SlyA_{Ib}. In this study we identified a second region of P_{PhyE} extending from +34 to +109 that is protected by SlyA. In this region, SlyA II contains potential SlyA-recognition motifs SlyA_{Ia} (–47TTATATTTATCATA–58, consensus match 7/12), SlyA_{Ib} (–56TTAAAGGCGCAA–67, 7/12), SlyA_{Ic} (–76TTATGACTGAAA–82, 6/12) and SlyA_{Id} (–86GTGGCA GATAAA–96, 6/12).

The downstream position of the SlyA II site is an unusual location for typical bacterial transcriptional activators. However, it has been shown that some other S. Typhimurium genes that are positively regulated by SlyA contain SlyA binding sites downstream from the transcript start site, including pagC and ugtL (Shi *et al*., 2004; Navarre *et al*., 2005). Consistent with our observations at PhylE, it has been suggested that SlyA binding to such sites remodels the local nucleoprotein structure, counteracting the effect of negative regulators such as H-NS, and clearing the way for classical transcriptional activators such as PhoP, or as in the case of hlyE, FNR and CRP (Ellison and Miller, 2006).

PhyE is AT-rich and previous bending predictions have shown intrinsic curvature and sharp bends in the hlyE gene (Westermark *et al*., 2000). Our analysis using the Model.it server gave similar results, showing the hlyE translational start site (+73 relative to the transcription start) is approximately located at an apex of curvature in the region (Fig. 6B). PhyE is thus suitable for interaction with H-NS, which is known to bind preferentially to AT-rich curved DNA (Bracco *et al*., 1989). In addition, H-NS binding further increases DNA curvature (Rimsky, 2004).

In contrast to the H-NS I and H-NS II binding sites upstream and downstream of the transcript start site, there is no H-NS protection in the region of the GC-rich spacer between the –35 and –10 elements (Fig. 6A). A similar H-NS protection pattern has been seen in the promoter of the glycine-betaine transport operon proU (Lucht and Bremer, 1994).

Previous investigations highlighted the importance of the –35 to –10 region of PhyE for derepression by SlyA (Ludwig *et al*., 1999). It contains the unusual –10 element TATGAAT, and it is likely that affinity of a RNAP sigma factor for this heptamer is less than that of a typical TATAAT hexamer. Furthermore, a reduction in the GC content of the GC-rich spacer diminished the capability of SlyA to activate hlyE expression (Ludwig *et al*., 1999). The observations reported here suggest that lowering the GC content of the spacer could increase DNA curvature and H-NS affinity in this region, increasing the stability of the DNA:H-NS complex, and preventing derepression by SlyA.

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The experimental data reported here are consistent with gene silencing models in which the curved DNA to which H-NS initially binds is located upstream or downstream of the promoter that is to be repressed (Rimsky, 2004). It was recently suggested that H-NS dimers form DNA:H-NS:DNA bridges that trap RNAP at promoters (Dorman, 2004; 2007; Dame et al., 2005; 2006). In this study it is shown that transcription of hlyE in vitro is repressed by the addition of H-NS alone and it is likely that this is caused by H-NS dimers initially bridging between the H-NS I and H-NS II sites. Therefore, it is suggested that H-NS first binds at AT-rich regions up- and downstream of the hlyE transcript start site (Wyborn et al., 2004b; Fig. 7, A). This binding increases the curvature of DNA allowing formation of a DNA:H-NS:DNA complex. The zone of H-NS protection then extends by oligomerization, ‘zipping-up’ the up- and downstream regions of PhlyE, and silencing transcription by trapping or excluding RNAP (Fig. 7, B). A recent investigation of DNA:H-NS interaction kinetics showed that a DNA:H-NS:DNA complex can be unzipped by a force of ~7 pN at a rate of 70 bp s⁻¹, while RNAP can exert forces of up to 25 pN (Wang et al., 1998; Dame et al., 2006). The H-NS:DNA interaction can therefore be easily overcome in the right conditions, which SlyA may help to establish by allowing dynamic re-organization of promoter complexes in response to different environmental stimuli. The footprinting analysis shows that binding sites for SlyA overlap the AT-rich H-NS-binding sequences and that SlyA affects the ability of H-NS to bind to PhlyE, probably by binding first to the higher-affinity SlyA II site to form an intermediate complex SlyA:H-NS:PhlyE in which the H-NS dimer interactions bridging H-NS II and H-NS I are disrupted (Fig. 7, C and D). This SlyA-remodelled nucleoprotein complex is capable of accommodating RNAP allowing hlyE expression (Fig. 7, E and F). When the intracellular concentration of SlyA decreases, or the concentration of H-NS increases, H-NS is able to displace both SlyA and RNAP silencing hlyE expression (Fig. 7, A and B). Thus, in the proposed regulatory model the relative concentrations of SlyA and H-NS determine the type of nucleoprotein complex formed at PhlyE and when SlyA is dominant it inhibits binding of H-NS and, in combination with the GC-rich spacer region, maintains the topology of the promoter in a conformation that enables RNAP to begin hlyE transcription. While SlyA-mediated antagonism of H-NS-mediated silencing has been suggested previously it is now shown that H-NS can interfere with SlyA binding. Thus, SlyA and H-NS...
operate a ‘see-saw’ mechanism, switching between SlyA- and H-NS-based nucleoprotein complexes to either promote (SlyA) or silence (H-NS) hlyE expression. This mechanism has a number of parallels with the recently reported H-NS antagonism by VirB at the Shigella flexneri iscB promoter (Turner and Dorman, 2007). Like SlyA at P_hlyE, VirB has no positive effect on iscB expression in the absence of H-NS, but VirB alters the structure of PiscB in the region occupied by H-NS and this requires a DNA sequence located downstream of the iscB transcript start (Turner and Dorman, 2007).

Another related mechanism to that proposed here has been described for the virF virulence gene promoter of S. flexneri, where interaction between H-NS proteins bound to two segments of AT-rich DNA facilitates promoter looping and repression of virF transcription (Falconi et al., 1998). However, the DNA:H-NS interaction is also dependent on environmental conditions including temperature, which alters the degree of DNA curvature in the virF promoter leading to the displacement of H-NS due to a shift in the location of the centre of the bend (Prosseda et al., 2004). In vivo and in vitro transcription assays suggest that an increase in temperature alone cannot relieve repression of hlyE by H-NS (Table 1, Fig. 5). However, SlyA counteracts H-NS-mediated repression results in derepression of hlyE transcription in vitro at 37°C, but not at 25°C. This may be due to increased stability of H-NS:P_hlyE complex at lower temperatures. Although derepression by SlyA in vitro is temperature-dependent this did not appear to be the case in vivo (Table 1), suggesting there are additional factors at play in vivo.

SlyA is closely related to the Y. pseudotuberculosis regulator RovA, which regulates expression of the virulence factor invasin. The inv promoter has overlapping binding sites for RovA and H-NS, and RovA functions as an antirepressor of inv expression when H-NS is present (Heroven et al., 2004; Tran et al., 2005). RovA was also shown to activate inv transcription by direct contact with RNAP in the absence of H-NS. Although it is possible that it may activate some promoters directly, the addition of SlyA to RNAP did not increase the amount of hlyE transcription in vitro (data not shown). The level of expression of a chromosomal hlyE–lacZ fusion in an E. coli hns slyA double mutant was slightly greater than in an hns single mutant, suggesting that SlyA may have a small negative effect on hlyE transcription.
effect on hlyE transcription in vivo in the absence of H-NS, and active CRP and FNR (Table 1). A negative effect of SlyA on hlyE expression may be a consequence of the proximity of the promoter −35 element and the SlyA lb recognition site (Fig. 6A). It has been shown that relief of H-NS-mediated hlyE silencing by SlyA is much less efficient in the absence of CRP or the hlyE promoter normally needs CRP or FNR to be activated to higher levels (Westermark et al., 2000; Wyborn et al., 2004b). This is consistent with our observation that in the absence of CRP and FNR, addition of SlyA only leads to partial derepression of the hlyE promoter in vitro.

Virulence factor production by bacteria is an energy-expensive process that is also likely to alert the defence mechanisms of host cells, and it is therefore vital that bacteria can tightly repress virulence genes, but also that the repression can be quickly reversed in response to appropriate conditions. Many bacterial virulence factors are acquired by horizontal gene transfer and it has been suggested that gene silencing by H-NS and H-NS-like proteins allows the newly acquired genes to be integrated into regulatory networks with little effect on the fitness of the bacterium (Doyle et al., 2007). However, to function as virulence factors the genes must be expressed when it is advantageous to do so. Consequently, the ability of SlyA to act as an H-NS antagonist is likely to be important for bacterial pathogenesis and evolution.

### Experimental procedures

#### Bacterial strains, plasmids and microbiological methods

Relevant characteristics of bacterial strains, plasmids and oligonucleotides used are given in Table 2. The slyA::Tn5 mutant FB21922, containing a transposon insertion at position +198 from the slyA start codon, was obtained from the Blattner laboratory (Kang et al., 2004). The mutation was transferred to E. coli M182 via P1 vir-mediated transduction to create strain JRG5358, and also into hns mutant strain JRG4864 to produce a hns slyA double mutant. The hlyE–lacZ fusion from plasmid pGS1629 was transduced with phage λ into E. coli M182 and the slyA, hns and hns slyA mutants to produce single-copy chromosomal hlyE–lacZ reporter strains JRG5580, 5581, 5582 and 5583. Bacteria were grown in L broth (tryptone 10 g l−1; yeast extract 5 g l−1; NaCl 10 g l−1) at 20°C, 25°C or 37°C. Media were supplemented with ampicillin (Ap, 100 μg ml−1), tetracycline (Tet, 35 μg ml−1) kanamycin (Kn, 20 μg ml−1) or chloramphenicol (Cm, 20 μg ml−1) as appropriate. For β-galactosidase measurements, cultures were grown in 250 ml universal bottles containing either 10 ml (aerobic) or 250 ml (anaerobic) of

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**Table 2.** Bacterial strains, plasmids and oligonucleotides used in this study.

| Strain or plasmid | Genotype or relevant characteristics | Source or reference |
|-------------------|-------------------------------------|---------------------|
| **E. coli** | | |
| DH5x | Δlac | Sambrook and Russell (2001) |
| M182 | Δlac | Busby et al. (1983) |
| BL21/DE3 | Protease-deficient strain used for protein expression | Studier (1975) |
| FB21922 | M1655 slyA::Tn5 mutant; KnR | Kang et al. (2004) |
| JRG4864 | M182 Δhns; CmR | Wyborn et al. (2004b) |
| JRG5329 | M182 Δhns slyA::Tn5; CmR KnR | This work |
| JRG5358 | M182 slyA::Tn5; KanR | This work |
| JRG5580 | M182 hlyE–lacZ single-copy chromosomal reporter fusion; ApR | This work |
| JRG5581 | M182 hlyE–lacZ slyA::Tn5; ApR KnR | This work |
| JRG5582 | M182 hlyE–lacZ Δhns; ApR CmR | This work |
| JRG5583 | M182 hlyE–lacZ slyA::Tn5 Δhns; ApR CmR KnR | This work |
| **Plasmid** | | |
| pGEX-KG | ApR GST fusion expression vector | Guan and Dixon (1991) |
| pRLG770 | ApR transcription plasmid with internal RNAI control gene | Ross et al. (1990) |
| pRW50 | Low-copy-number TetR lac reporter vector | Lodge et al. (1990) |
| pUC18 | ApR high-copy-number cloning vector | Vieira and Messing (1991) |
| pUC118 | ApR high-copy-number cloning vector | Vieira and Messing (1991) |
| pGS1482 | GST–SlyA overexpression plasmid | Stapleton et al. (2002) |
| pGS1629 | hlyE–lacZ reporter plasmid | Wyborn et al. (2004b) |
| pGS1875 | GST–H-NS overexpression plasmid | This work |
| pGS1886 | 696 bp hlyE promoter fragment in pRLG770 for in vitro transcription | This work |
| pGS2079 | 242 bp hlyE promoter fragment in pRLG770 for DNA footprinting | This work |
| pGS2135 | 393 bp hlyE promoter fragment in pUC18 for DNA footprinting | This work |
| **Oligonucleotide a** | | |
| S025 | GTTTGGATCCATGAGCGAAGACCTAAGAATACTG | |
| S026 | ACAAAAGCTTATTGCTTATGCGAAGAATCG | |
| S0101 | CACCTGAAATTTCAGCTTGGCACAGAAG | |
| S0102 | AAGAAAGCTTACCTTTTATGTTTC | |
| S0104 | CAGCGTGCTCACCAGCGCGACG | |
| S0106 | TTCTAAGCTTATCATGTCGCTT | |

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a. Engineered restriction sites in oligonucleotides are italicized and positions of start and stop codons of H-NS are shown in bold.
Plasmids pGS1886 was digested with HindIII and 20 ng of the linearized plasmid DNA was incubated for 5 min at 37°C in a 21 μl reaction volume containing 40 mM Tris-acetate pH 7.9, 10 mM MgCl2, 1 mM DTT, 100 mM KCl, 100 μg ml−1 BSA and varying amounts of SlyA and H-NS as indicated. Single-round transcription reactions were incubated for 15 min at 37°C in the presence of 4 μl of a solution containing unlabelled and labelled nucleotide triphosphates [UTP at 50 μM; ATP, CTP and GTP at 1.2 mM; and 5 μCi 32P-labelled UTP (800 Ci mmol−1, Perkin Elmer)], 0.5 μg of heparin and 1 unit of RNAP holoenzyme (Epicentre). Reactions were terminated by adding 18 μl of Stop/Loading dye solution (95% formamide, 20 mM EDTA pH 8, 0.05% bromophenol blue, 0.05% xylene cyanol), and 15 μl of each reaction was loaded on a 4% acrylamide, 1× TBE gel, dried and analysed by autoradiography.

**DNase I footprinting**

Plasmid pGS2079 or pGS2135 was digested with HindIII and the recessed 3′-OH terminus was filled in using Klenow fragment and 20 μCi 32P-labelled dATP as described above. The DNA was then digested with EcoRI to produce a radiolabelled 244 bp or 393 bp hlyE promoter fragments. In separate reactions the order of restriction digestion was reversed to label the fragment on the other strand. Following purification by agarose gel extraction 250 ng of DNA fragment was incubated at room temperature in a 50 μl final volume containing varying amounts of SlyA and H-NS as indicated and 25 μl of DNA binding buffer from the Promega Core Footprinting System. Following the formation of protein:DNA complexes 50 μl of a solution of 5 mM CaCl2 and 10 mM MgCl2 and 3 μl of 1 U μl−1 RQ1 DNase I were added. Reactions were terminated with Promega Stop Solution after 2 min, extracted with phenol:chloroform, precipitated with ethanol and vacuum dried. A Maxam–Gilbert G-track reaction was used as a calibration as described previously (Maxam and Gilbert, 1980). Dried reactions were re-suspended in 10 μl of loading buffer (80% formamide, 0.1% bromophenol blue, 0.1% xylene cyanol, 10% glycerol, 8 mM EDTA pH 8) for electrophoretic fractionation on a 6% polyacrylamide, 7 M urea gel in 1× TBE buffer, and subjected to autoradiographic analysis.

**DNA curvature prediction**

The curvature of the hlyE promoter was analysed using the web-based Model.it prediction program at http://hydra.icgeb.trieste.it/dna/model_it.html (Vlahoviček et al., 2003) and the resulting helix co-ordinates were displayed and visualized using RASMOL (Sayle and Milner-White, 1995).

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