SlyA and H-NS Regulate Transcription of the *Escherichia coli* K5 Capsule Gene Cluster, and Expression of slyA in *Escherichia coli* Is Temperature-dependent, Positively Autoregulated, and Independent of H-NS*5

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In this paper, we present the first evidence of a role for the transcriptional regulator SlyA in the regulation of transcription of the *Escherichia coli* K5 capsule gene cluster and demonstrate, using a combination of reporter gene fusions, DNase I footprinting, and electrophoretic mobility shift assays, the dependence of transcription on the functional interplay between H-NS and SlyA. Both SlyA and H-NS bind to multiple overlapping sites within the promoter in *vitro*, but their binding is not mutually exclusive, resulting in a remodeled nucleoprotein complex. In addition, we show that expression of the *E. coli* slyA gene is temperature-regulated, positively autoregulated, and independent of H-NS.

Extraintestinal isolates of *Escherichia coli* associated with disease often express high molecular weight acidic polysaccharide capsules, or K antigens, on their cell surface. Over 80 such K antigens have been identified, and these have been divided into four principal groups based on serological, biochemical, and genetic data (1). The K5 polysaccharide is commonly associated with strains causing urinary tract infections and belongs to Group 2. These capsules resemble those possessed by *Neisseria meningitidis* and *Haemophilus influenzae* and are temperature-regulated, being expressed at 37 °C but not at 20 °C (2). Temperature regulation of virulence factors is a common feature of bacterial pathogens, including *Shigella*, *Yersinia*, and *Salmonella* spp., and ensures that such factors are only expressed in appropriate environments (3).

The K5 capsule gene cluster is composed of three principal regions. Region 2 is serotype-specific and contains the genes (*kftABCD*) responsible for synthesis of the polysaccharide. Regions 1 (*kpsFEDUCS*) and 3 (*kpsMT*) are conserved throughout Group 2 and consist of genes responsible for transport and assembly of the polysaccharide capsule (Fig. 1). Transcription is driven by two convergent temperature-regulated promoters located upstream of regions 1 and 3 (4, 5). The region 3 promoter is located 741 base pairs 5′ of the first of two genes in region 3, *kpsM*, and transcription proceeds through region 2 with the aid of the transcription antitermination factor, RfaH (Fig. 1) (5, 6). RfaH function is dependent on a short sequence present in the region 3 mRNA known as the JUMPStart sequence (7), and it is also involved in the expression of *E. coli* Group 1 capsules (8).

The region 1 promoter (PR1) is located 225 base pairs 5′ of the *kpsF* gene (4). Transcription from this promoter proceeds past an intragenic Rho-dependent terminator in *kpsF* to yield an 8-kb polycistronic region 1 transcript that is later processed to yield a separate, *kpsS*-specific transcript (Fig. 1). Unlike the region 3 promoter, there is no dependence upon RfaH for transcription elongation at PR1 (4). The IHF protein is required for maximum transcription from PR1 at 37 °C and binds to a single site located 130 bp 3′ to the transcription start point at PR1 (Fig. 1) (9). Two additional regulators, H-NS and BipA, play an unusual dual role in the temperature regulation of transcription at this promoter; both are required for maximum transcription at 37 °C, yet both also contribute to repression of transcription at 20 °C (9). BipA is a ribosome-binding GTPase that is able to regulate gene expression at the level of translation (10). It is required for the growth of *E. coli* at low temperature (11) and regulates the expression of multiple pathogenicity islands in this bacterium (12). Thus, the role of this protein in regulating transcription from PR1 is probably indirect.

H-NS is a global regulator able to influence the expression of ~5% of all genes in *E. coli* (13) and whose direct influence on transcription is almost universally negative, often regulating gene expression in response to environmental conditions including temperature and osmolarity (14). Examples of direct activation of gene expression by H-NS are rare. In the case of the maltose regulon, H-NS has been shown to stimulate MalT translation (15), and H-NS plays a positive role in the activation of the *tra* gene of pRK100 (16). H-NS binds to no specific target sequence in DNA but instead recognizes intrinsic DNA curva-

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3 The abbreviations used are: PR1, region 1 promoter; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; EMSA, electrophoretic mobility shift assay.
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Recently, the principal role of H-NS has been proposed to be one of “xenogenic silencing,” preventing the expression of genes encoded on horizontally acquired DNA by recognizing abnormally high AT content, thus preventing any reduction in fitness that might be conferred by the invading DNA (18). Antirepressors, an example of which is the SlyA protein, can then alleviate such repression. SlyA was originally identified as a Salmonella typhimurium gene product able to induce a hemolytic phenotype upon E. coli K-12 (19, 20). It has been shown to be essential for the virulence of S. typhimurium in mice, resistance to the oxidative burst, and survival within murine macrophages (19, 21, 22). SlyA has since been characterized as a regulator of a variety of virulence factors in S. typhimurium, the majority of which occur in either the cell membrane or periplasm or are surface-exposed (23). This led to the proposal that one of the principal functions of SlyA is to effect alterations in the bacterial cell surface with the ultimate aim of protecting it from host defense mechanisms (23). In addition, the SlyA homologue of Yersinia spp., RovA, has been shown to contribute to virulence in Yersinia, including a role in bubonic plague (24, 25). RovA regulates the transcription of the outer membrane protein invasin in Y. pseudotuberculosis and Y. enterocolitica (26, 27) through H-NS antirepression (28, 29).

Here, we demonstrate the involvement of SlyA in the regulation of K5 capsule gene expression and show that H-NS and SlyA interact to promote transcription from PR1, in a novel manner that is different from that previously proposed for H-NS antirepression. In addition, we demonstrate that expression of the E. coli slyA gene is temperature-regulated, positively autoregulated, and independent of H-NS.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Plasmids, and DNA Fragments—The strains and plasmids used in this study are listed in Table 1. Bacteria were routinely grown in LB medium at 37 or 20 °C, as indicated, and supplemented with antibiotics as appropriate at the following concentrations: 50 μg ml⁻¹ kanamycin, 50 μg ml⁻¹ streptomycin, 12.5 μg ml⁻¹ tetracycline, and 100 μg ml⁻¹ ampicillin. X-gal was used at a concentration of 40 μg ml⁻¹. Strain HA1 was constructed as follows. A PCR-amplified fragment of PR1 stretching 870 bp 5’ to the start codon of kpsF was introduced into the lacZ reporter plasmid pRS415 (30) to generate plasmid pHAl. This plasmid was transformed into E. coli P90C, and the resulting transformants were infected with phage AR5415 (30). Phage purified from the resulting plaques were used to reinflect fresh P90C, and λ-lysogens were identified as blue colonies on LB-X-gal medium. Correct insertion of the PR1-lacZ fusion at the λatt site in P90C was confirmed by colony PCR, and normal regulation of the promoter (9) was confirmed by performing β-galactosidase assays on cultures grown at 37 and 20 °C.

β-Galactosidase Assays—Overnight cultures were diluted 1:100 into fresh, prewarmed LB plus antibiotics, and grown to an A₆₀₀ of ~0.4 – 0.6. Assays were performed as described (31).

Protein Purification—The hns gene was cloned into plasmid pET22b to generate plasmid pET22b-H-NS expressing a C-terminally His₆-tagged H-NS protein. The protein was overexpressed in E. coli strain BL-21 and purified essentially as described (32). Purification of H-NS was confirmed by SDS-PAGE (see supplemental Fig. S1). SlyA was purified as follows. The slyA structural gene was cloned into plasmid pGEX-6P-1 and expressed in strain E. coli BL-21, yielding a glutathione S-transferase-SlyA fusion protein. An overnight culture of BL-21pGST-SlyA was diluted 1:100 into fresh ampicillin-containing LB medium and grown at 37 °C with vigorous shaking to A₆₀₀ of ~0.5. Expression of the glutathione S-transferase-SlyA fusion was induced by adding isopropyl-β-D-thiogalactopyranoside to a final concentration of 1 mM. Cells were incubated for a further 3 h at 25 °C before centrifugation at 4 °C. Cells were resuspended in 8 ml of ice-cold phosphate-buffered saline, 20% glycerol and passed twice through a French pressure cell. Clarified cell-free extracts were adsorbed onto a 1-ml Glutathione-Sepharose column (Amersham Biosciences) equilibrated with phosphate-buffered saline. The column was washed with 20 ml of phosphate-buffered saline and equilibrated with 10 ml of Pre-Scission protease buffer (50 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, pH 8). The SlyA protein was cleaved from glutathione S-transferase by overnight digestion with 5 units of Pre-Scission protease (Amersham Biosciences) at 4 °C, and eluted in Pre-Scission protease buffer. In both cases, protein concentration was determined using the Bio-Rad protein assay using bovine serum albumin standards. Purification of SlyA was confirmed by SDS-PAGE (see supplemental Fig. S1).

EMSA—V₁, V₂, and V₃ DNA fragments (10, 20, and 35 nM, respectively) were incubated with varying concentrations of purified SlyA, H-NS, or both, as indicated. A 200-bp fragment spanning the multiple cloning site of pBluescript (Stratagene) was amplified using M13 Forward primer (5’-TGTAAAACGACGGCCAGT-3’) and M13 Reverse primer (5’-GGGAAAAACGTATGACGACCAGT-3’) and used as a negative control at a final concentration of 18 nM. The reaction buffer contained 10 mM Tris-HCl (pH 9), 50 mM KCl, and 0.1% Triton X-100 in a final reaction volume of 10 μl. Samples were incubated at either 37 or 20 °C for 10 min before being mixed with 5× loading dye (Bioline) and resolved on 5% polyacrylamide gels containing 2% glycerol. Electrophoresis was performed in 1× TBE at 12 V cm⁻¹ before staining in 0.5 μg ml⁻¹ ethidium bromide for 15 min.

DNase I Footprinting—5 μg of plasmid pDC2B was digested with SpeI and HindIII, and the promoter fragment separated from the vector backbone on a 0.7% agarose gel, followed by purification using a Qiagen gel extraction kit according to the manufacturer’s instructions. The purified fragment was labeled with Klenow enzyme and 20 μCi of [α⁻³²P]dATP for 20 min at room temperature. Labeled DNA was purified using a Qiagen PCR cleanup kit, and a final concentration of 5 nM promoter fragment was incubated with varying concentrations of SlyA, H-NS, or both, in a buffer containing 10 mM Tris-HCl (pH 9), 50 mM KCl, and 0.1% Triton X-100, in a total volume of 50 μl. After a 10-min incubation, 50 μl of a 10 mM MgCl₂, 5 mM CaCl₂ solution was added to each sample before digesting with 0.14 units of DNase I for 3 min at room temperature. Digestion reactions were terminated by the addition of 90 μl of stop solution (200 mM NaCl, 30 mM EDTA, and 1% SDS) prewarmed to
37 °C. Digestion products were extracted with phenol-chloroform and ethanol-precipitated, followed by resuspension in 10 μl of formamide loading dye (1:2, 0.1 M NaOH/formamide, 0.1% xylene cyanol, 0.1% bromphenol blue). Resuspended samples were denatured by heating to 90 °C for 3 min followed by immersion in ice for 2 min, and 3 μl of each sample was loaded onto a 6% denaturing polyacrylamide sequencing gel for electrophoretic fractionation. Footprinting reactions were calibrated with Maxam and Gilbert G-tracks (33).

RNA Extraction—For RNA extraction, E. coli strains were cultivated in LB medium overnight at 37 °C before being inoculated into fresh LB medium (1:100) and grown at 37 °C with shaking at 200 rpm. When the A600 reached 0.5, RNA was extracted from 24 ml of culture using the RNEasy Protect Bacteria Mini Kit (Qiagen). RNA was quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop), where an A260 of 1.0 equals 40 μg ml⁻¹.

Quantitative Reverse Transcription-PCR—An aliquot containing 100 ng of RNA was reverse-transcribed with Euroscript RT (Eurogentec) according to the manufacturer’s instructions using specific reverse primers. Reverse-transcribed RNA samples were quantified using Eurogentec SYBR Green (Eurogentec) on the ABI Prism 7000 (Applied Biosystems), and the relative amounts of cDNA were determined using ABI Prism SDS software (Applied Biosystems). Relative standard curves were prepared from serially diluted PCR products, and 16S rRNA was used as a control. The primer sequences were KpsEF (5’-GTGAAAGTCTGCCGTATCCGTG-3’), KpsER (5’-CCACAGATGATGATTTCGCAC-3’), 16SrRNAF (5’-CAGCTCAGTGTGAGATGT-3’), and 16SrRNAR (5’-CAGGTCTAAAGGGCAGGAT-3’).

TABLE 1

| Strain/Plasmid | Relevant genotype | Source/Reference |
|----------------|-------------------|-----------------|
| 12860dlysA::amp | EIEC O124, slyA::amp | Ref. 36 |
| BL-21 | F– | This study |
| HA1 | HA1 × P1(MC41000) | This study |
| HA1hns::kan | HA1 × P1(MG1655slyA::kan) | This study |
| HA1slyA::kan | hns::kan | Ref. 9 |
| MC41000 | K-12 slyA::kan | J. Green |
| MG1655slyA::kan | P1(K-12)K5 | Ref. 6 |
| MS101 | MS101 × P1(12860dlysA::amp) | This study |
| P9OC | F– ara 3(lacpro) thi-1 | Ref. 30 |
| P90C | P90C × P1(MC41000) | This study |
| P90ClysA::kan | P90C × P1(MG1655slyA::kan) | This study |

* Strain construction via P1 transduction.

3.6

TABLE 2

SlyA is able to activate transcription from PR1 at 37 °C

Shown is β-galactosidase activity of HA1 and derivatives bearing either plasmid pBluescript or pRec5. β-Galactosidase activity is given in Miller units ± S.E. Data are the means of three independent experiments performed in triplicate.

| Strain | β-Galactosidase activity |
|--------|--------------------------|
|        | Miller units |
| HA1    | 51.1 ± 3.6 |
| HA1 pBluescript | 45.7 ± 3.9 |
| HA1 pRec5 | 120.7 ± 8.1 |

RESULTS

Identification of SlyA as an Activator of Capsule Gene Transcription—To identify putative regulators of capsule gene transcription, a gene library of strain P90C was constructed by cloning a partial Sau3A digest of P90C chromosomal DNA into the multicopy vector pBluescript SK+. The library was introduced into strain HA1 that has a chromosomal PR1-lacZ fusion (Table 1), and clones harboring potential activators of capsule gene transcription were identified as dark blue colonies on media supplemented with X-gal. Analysis of the DNA sequences of candidate clones revealed the common presence of the transcriptional regulator slyA. One such clone, pRec5, was used in β-galactosidase assays, which showed that multicopy slyA enhanced in vivo transcription from PR1 (Table 2). To confirm that SlyA was involved in the regulation of capsule gene transcription, the slyA::kan mutation from strain MG1655slyA::kan was moved to HA1 by P1 transduction. Inactivation of slyA resulted in a 6-fold reduction in transcription from PR1 in β-galactosidase assays (Table 3). Complementation of the slyA::kan mutation with plasmid pACYC-SlyA confirmed that the slyA gene product was able to stimulate transcription at PR1, and significant activation of PR1 transcription was also observed in the HA1 parent strain containing plasmid...
TABLE 3

Transcription from PR1 is dependent upon SlyA and H-NS

Shown is β-galactosidase activity of HA1, HA1\(slyA::kan\), HA1\(hns::kan\), and derivatives bearing either plasmid pACYC184 or pACYC-SlyA. β-Galactosidase activity is given in Miller units ± S.E. Data are the means of three independent experiments performed in triplicate.

| Strain            | β-Galactosidase activity (Miller units) |
|-------------------|----------------------------------------|
|                   | 37 °C                                  | 20 °C       |
| HA1               | 61.3 ± 1                               | 10.1 ± 0.3  |
| HA1 pACYC184      | 59.9 ± 1.3                             | 10.0 ± 0.5  |
| HA1 pACYC-SlyA    | 155.3 ± 2.9                            | 118.2 ± 5.5 |
| HA1slyA::kan      | 10.7 ± 0.7                             | 5.2 ± 0.4   |
| HA1slyA::kan pACYC184 | 17.2 ± 3.5                           | 3.8 ± 0.4   |
| HA1slyA::kan pACYC-SlyA | 90.6 ± 3.9                          | 65.4 ± 2.3  |
| HA1hns::kan       | 29.7 ± 0.6                             | 24.0 ± 1    |
| HA1hns::kan pACYC184 | 32.3 ± 1.1                          | 27.2 ± 0.4  |
| HA1hns::kan pACYC-SlyA | 44.7 ± 0.8                        | 41.8 ± 0.3  |

pACYC-SlyA (Table 3). Additional evidence for the role of SlyA in capsule gene expression was obtained by directly measuring the transcription of \(kpsE\), the second gene in the capsule operon (Fig. 1), in the K5 strain MS101 and its \(slyA::amp\) derivative (Table 1). The presence of multicopy \(slyA\) in MS101 resulted in a 2.4-fold increase in yield of \(kpsE\) transcript, whereas inactivation of the \(slyA\) gene resulted in an ~10-fold reduction of \(kpsE\) transcription relative to the wild-type (Table 4). Taken together, these data suggest that SlyA is required for and can promote transcription from PR1.

Interaction of SlyA with PR1—To demonstrate that SlyA interacts directly with PR1, competitive electrophoretic mobility shift assays (EMSAs) were performed with increasing concentrations of purified SlyA protein and three PCR-generated promoter fragments spanning a region from 316 bp 5’ of the transcription start point to 218 bp 3’ of the transcription start point (Fig. 1). At 37 °C, SlyA specifically bound to the \(V_1\) fragment, spanning a region 5’ to the transcription start point, with 0.5–0.6 μM SlyA being sufficient to almost completely retard the mobility of the \(V_1\) fragment (Fig. 2A). At these concentrations, no binding was detectable to the \(V_2\), \(V_3\), or negative control fragments (Fig. 2A). This indicates that SlyA binds specifically to the \(V_1\) region of PR1. The \(K_d\) value for SlyA binding to \(V_1\) was determined to be 0.43 μM (data not shown). Comparison of the binding properties of SlyA at the capsule permissive temperature (Fig. 2A) with those performed at 20 °C (data not shown) revealed no significant difference in the DNA binding capability of SlyA at either temperature. The locations of the specific SlyA binding sites in the \(V_1\) fragment were determined using DNase I footprinting. This revealed two extended regions of DNase I protection (Fig. 2B), designated SlyA-I and SlyA-II, the second of which can be seen in more detail in Fig. 2C. Some binding to the SlyA-I site was still apparent in the presence of the nonspecific competitor heparin (100 μg ml⁻¹), whereas the addition of heparin eliminated binding at the SlyA-II site, suggesting that this may be a lower affinity binding site compared with SlyA-I (Fig. 2B).

Role of H-NS in the Regulation of PR1—Previous studies have shown that H-NS plays an unusual role in regulating Group 2 capsule gene expression, appearing to maximize transcription from PR1 at 37 °C while repressing transcription at 20 °C (9). The nucleotide sequence 5’ to the transcription start point of PR1 (to position –320) is 75% AT, and analysis of this region using Bend.It software (available on the World Wide Web) revealed four stretches of pronounced DNA curvature, each predicted to possess at least 8° curvature/10.5-bp helical turn of the DNA. These regions all corresponded to runs of A and T, the first three of which fell within the two characterized SlyA binding sites. Given that H-NS has a preference for binding intrinsically curved AT-rich DNA, we investigated whether H-NS acted directly or indirectly on the promoter using EMSA and DNase I footprinting analysis. At concentrations of 0.3–0.4 μM, H-NS was able to bind specifically to the promoter upstream fragment (\(V_1\)) at 37 °C (Fig. 3A) with no detectable binding to fragments \(V_2\) and \(V_3\) at these H-NS concentrations (Fig. 3A). The \(K_d\) value for H-NS binding to \(V_1\) was determined to be 0.36 μM (data not shown). There was no difference in the binding of HNS to the \(V_1\) fragment at 20 °C (data not shown). H-NS protected extensive regions of PR1 in DNase I footprinting assays (Fig. 3B). The H-NS-I binding site extended outwards from a core region located between positions –187 and –208 upon increasing H-NS concentration and extended 5’ at least as far as –285 and thus overlapped the SlyA-I binding site (data not shown). The H-NS-III site extended across the transcript start point (Fig. 3B).

Functional Interplay between H-NS and SlyA at PR1—The overlapping pattern of the binding sites was similar to that observed at other promoters regulated by SlyA and its \(Yersinia\) spp. homologue RovA, at which SlyA has been postulated to alleviate H-NS-mediated repression through competition for binding sites (28, 34). However, the interplay between H-NS
and SlyA at PR1 is clearly more complex, because despite being able to bind to the promoter, H-NS has no apparent repressive effect on transcription at 37 °C. In order to determine the func-
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![Diagram](image)

FIGURE 4. Competitive EMSA analysis of PR1. Shown is the effect of increasing SlyA concentration on the mobility of the V1 fragment, at 20 °C in the presence of 0.5 μM H-NS. The H-NS·V1 complex is denoted by stars, the SlyA·V1 complexes are denoted by circles, and the H-NS·V1·SlyA complex is denoted by squares.

The functional significance of this interplay in *in vivo*, β-galactosidase assays were performed on cultures of HA1 or its *hns::kan* derivative bearing single copy or multicopy SlyA. The results show that in the absence of H-NS at 37 °C, transcription from PR1 was reduced ~50% relative to the wild-type (Table 3), confirming a positive role for H-NS in transcription from PR1 at 37 °C. Multicopy slyA (pACYC-SlyA) enhanced transcription from PR1 2.5-fold but in an H-NS-dependent manner; the level of transcription in the *hns* mutant grown under the same conditions increased only 1.5-fold and failed to reach the level observed in HA1. At the capsule nonpermissive temperature, the loss of H-NS resulted in a 2.5-fold increase in transcription from PR1, confirming a role for H-NS in the repression of capsule gene transcription at 20 °C. In the presence of multicopy slyA, transcription in the wild-type strain increased ~12-fold at 20 °C. This effect was unexpected and not observed in the *hns* mutant under the same conditions, where only a modest (1.5-fold) increase in transcription was observed (Table 3). Therefore, it can be seen that the presence of H-NS was required for maximal SlyA promotion of transcription from PR1, indicating a functional interaction between H-NS and SlyA in the regulation of transcription from PR1.

To investigate the relationship between H-NS and SlyA, competitive EMSA and DNase I footprinting analysis was performed in the presence of both proteins. EMSA analysis at 20 °C revealed that a concentration of 0.5 μM H-NS was able to form a stable H-NS·V1 complex, whereas 0.42 μM SlyA formed a less stable complex with a smeared appearance on the EMSA (Fig. 4, lanes 2 and 3). Co-incubation of the V1 fragment with both H-NS and SlyA at these concentrations reduced the amount of H-NS·V1 complex with the appearance of two new H-NS·V1·SlyA complexes distinct from those generated by either H-NS or SlyA alone (Fig. 4, lane 4). The lack of any smeared complexes might indicate that H-NS is stabilizing the binding of SlyA to V1. Increasing the concentration of SlyA to 0.55 μM in the presence of 0.5 μM H-NS resulted in a further reduction in intensity of the H-NS·V1 complex with the simultaneous appearance of a new less mobile H-NS·V1·SlyA complex that was not detectable at this concentration of SlyA in the absence of H-NS (Fig. 4, cf. lanes 5 and 6). In the presence of H-NS, 0.72 μM SlyA yielded a single low mobility H-NS·V1·SlyA complex distinct from that formed in the absence of H-NS (Fig. 4, cf. lanes 7 and 8). The same pattern of complex formation was seen at 37 °C (data not shown). These EMSA data indicate that SlyA and H-NS are able to bind simultaneously to this promoter and that the predominant nucleoprotein complex formed at this promoter is dependent upon the relative concentrations of SlyA and H-NS present in the cell.

Competitive DNase I footprinting was used in a more detailed investigation of the complexes observed in the EMSA analysis. Individually, SlyA and H-NS occupied the regions of PR1 described above (Fig. 5, lanes 2 and 3). When H-NS was present at 0.3 μM, SlyA bound at the SlyA-I site, as indicated by the characteristic SlyA-induced hypersensitive site at −208 (Fig. 5, cf. lanes 3 and 4). However, upon increasing the concentration of H-NS, a unique footprint was formed at the SlyA-I site. The intensity of the SlyA-induced hypersensitive site at −208 was reduced with increasing concentrations of H-NS, with the simultaneous formation of a second hypersensitive site in close proximity to −208 (Fig. 5, lanes 4–6). In contrast, in the presence of 0.3 μM H-NS, there was the loss of the SlyA footprint at the SlyA-II site with the formation of a unique footprint near position −109 that was different from that induced by SlyA or H-NS binding alone (Fig. 5, lanes 4–6). Two DNase-hypersensitive sites characteristic of H-NS binding in this region persisted in the presence of SlyA, accompanied by the appearance of several hypersensitive sites (near positions +15, −78, and −138) unique to the presence of both H-NS and SlyA (Fig. 5). This is indicative of significant changes in the local promoter architecture and, together with the competitive EMSA data (Fig. 4), indicates that H-NS binding to this region of the DNA persists in the presence of SlyA, resulting in a structurally altered nucleoprotein complex at PR1.

Regulation of SlyA—To characterize the regulation of the *E. coli* slyA gene, β-galactosidase assays were performed throughout the growth cycle of P90CpDC6 grown at 37 °C. Plasmid pDC6 carried a translational fusion of the slyA promoter region (PsyA) and the first 27 codons of the slyA gene fused to the N-terminally truncated *lacZ* gene in the low copy number plasmid pRS415 (Table 1). SlyA expression increased rapidly during growth, with maximal β-galactosidase activity at the late exponential phase, and declined through stationary phase (Fig. 6). In the absence of SlyA, expression of the slyA-*lacZ* fusion at 37 °C during midexponential phase was reduced ~2-fold relative to the parent strain (Table 5). Confirmation of this positive SlyA autoregulation was obtained using quantitative reverse transcription-PCR on RNA extracted from strains MS101 and MS101pRec5 grown at 37 °C, where the presence of excess SlyA resulted in a greater than 7-fold increase in the yield of slyA transcript (data not shown). When strains P90CpDC6 and P90CslyA::kanpDC6 were grown at 20 °C, there was a 2-fold reduction in β-galactosidase expression, and this temperature regulation was independent of SlyA (Table 5). One candidate for regulating SlyA expression in response to temperature was H-NS, which has been shown to directly repress transcription from the rovA promoter in *Yersinia pseudotuberculo-
sis (35). To investigate if slyA expression was H-NS dependent, β-galactosidase expression from plasmid pDC6 was also measured in strain P90C::hns::kan at both the K5 capsule permissive and nonpermissive temperatures. Inactivation of hns did not substantially alter expression of the slyA-lacZ fusion (Table 5). The ratio of expression between the wild-type and hns mutant cells was similar at both temperatures (Table 5). Taken together, these data indicate that expression of the E. coli slyA gene is maximal during exponential growth, is temperature-regulated, is positively autoregulated, and is independent of H-NS.

Two transcription start points were identified in PslyA using primer extension analysis, located at G and T residues 57 bp (P1) and 100 bp (P2), respectively, upstream of the initiating TTG of the slyA gene (data not shown). Direct binding of SlyA to three sites in PslyA was demonstrated using DNase I footprinting. Two sites were located between positions −404 and −361 (Fig. 7A) and between −141 and −108 (Fig. 7B). The DNA sequence of PslyA 3′ to P1 is almost identical to that found in Salmonella, including the presence of a perfect SlyA consensus sequence. Footprinting analysis also demonstrated that SlyA bound downstream of the transcription start points between positions +20 and +54, a region that contains the putative SlyA binding consensus sequence (data not shown).

**DISCUSSION**

The data presented here demonstrate for the first time the involvement of SlyA in the regulation of Group 2 capsule gene transcription. Inactivation of the slyA gene resulted in a dramatic reduction in transcription from the PR1-lacZ fusion in strain HA1; moreover, multicopy slyA caused significant activation of transcription at both the capsule permissive and nonpermissive temperatures. SlyA was also shown to bind preferentially to sequences upstream of the transcription start point.
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in PR1 using EMSA and DNase I footprinting analysis. These data are consistent with a role for SlyA in the activation of capsule gene transcription. The discovery of its role in capsule gene regulation in *E. coli* would seem to fit the proposed role of SlyA in *S. typhimurium* (*SlyA*<sub>ST</sub>) as a regulator of cell membrane and surface-exposed virulence factors (24) and, combined with data from proteomic studies (36), suggests that SlyA may play a similar overall role in *E. coli*.

The capacity of *SlyA*<sub>ST</sub> to induce a hemolytic phenotype upon *E. coli* K-12 (19) is a consequence of the ability of overexpressed *SlyA*<sub>ST</sub> to alleviate H-NS-mediated silencing of expression of a normally cryptic hemolysin, HlyE (ClyA) (37, 38). SlyA was also found to be dispensable for *clyA* transcription in the absence of H-NS (34, 38). Similarly, RovA of *Y. pseudotuberculosis* is only required for transcription *in vivo* at the *rovA* and *inv* promoters of this bacterium in the presence of H-NS (28).

At all three promoters, this phenomenon has been attributed to SlyA/ RovA behaving as an H-NS antirepressor. In the case of PR1, our data indicate that SlyA is not merely acting as an H-NS antirepressor. Specifically, the observations that the increase in transcription induced by multicopy *slyA* was H-NS-dependent and that in an *hns* mutant multicopy *slyA* failed to restore the level of transcription to that seen in the wild type at 37 °C (Table 3) indicate that H-NS is required for maximal SlyA-mediated activation of transcription from PR1. However, SlyA appears capable of activating transcription to some extent in the absence of H-NS, something that was also recently demonstrated for RovA (35). The requirement for H-NS for maximal SlyA-mediated activation of transcription from PR1 would explain the observation that *hns* mutants have reduced transcription from PR1 at 37 °C (9).

By EMSA and DNase I footprinting, we could demonstrate the direct binding of H-NS upstream of the transcription start point of PR1 and identify three H-NS binding sites with characteristically large, poorly defined footprints similar to those observed with H-NS binding to other promoters (39–41). These sites correspond to regions of the promoter with predicted curvature, a prerequisite for specific H-NS binding (14). Examination of the binding characteristics of H-NS and SlyA simultaneously revealed changes in binding site occupation and DNA conformation relative to the individual protein-DNA complexes. Competitive EMSA analyses indicate that despite their overlapping binding sites, SlyA and H-NS were able to bind to this promoter simultaneously, forming higher order SlyA-DNA•H-NS complexes. Indeed, at low SlyA concentrations, the binding of both H-NS and SlyA to the promoter was enhanced. Similar observations have been made of the binding behaviors of Fis and H-NS at the *virF* and *rrnB* P1 promoters under certain conditions (42, 43) and synergistic binding of Lrp and H-NS to the *rrnB* P1 promoter (44). More detailed analysis of simultaneous H-NS and SlyA binding was achieved with competitive DNase I footprinting. This showed that as the relative concentrations of SlyA and H-NS changed, unique footprints were generated at both the SlyA-I and SlyA-II sites. SlyA inhibited the binding of H-NS to the SlyA-I site. The presence of both proteins induced a novel digest pattern at the SlyA-II site, although the two DNase I-hypersensitive sites characteristic of H-NS binding were retained, confirming the continued binding of H-NS at this site. Our interpretation of these data is that in the presence of both H-NS and SlyA, H-NS rather than SlyA is bound at the SlyA-II site and that this binding is affected by the binding of SlyA at the SlyA-I site. The observation that the binding of SlyA to the SlyA-II site was heparin-sensitive would be in keeping with the preferential binding of H-NS at this site. This is in contrast to the *rovA* and *inv* promoters, where a competitive footprinting study suggested that in these cases, H-NS was displaced by RovA, hence the interpretation that RovA behaves as an H-NS antirepressor (28). Clearly, this is quite different from the interaction between SlyA and H-NS at PR1. The reason why the remodeled nucleoprotein complex is conducive to transcription is unclear, but the simplest interpretations are that the promoter conformation induced by simultaneous SlyA and H-NS binding favors either open complex formation or the binding of a further Class I or Class II
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transcriptional activator. An alternative, but perhaps less compelling, explanation could involve the direct interaction of SlyA with H-NS, resulting in the formation of a transient heteromeric complex. The ability of H-NS to form heteromeric complexes has been shown for other regulators, such as StpA (45), and it is able to interact directly with flagellar motor protein, FlG (46). Experiments are currently under way to determine whether SlyA interacts with H-NS.

Based on the data presented here, the regulation of transcription from PR1 will be dependent on the ratio of H-NS and SlyA in the cell. It is known that H-NS is very abundant, with an estimated 10^5 molecules of H-NS per genome (47). The levels of SlyA have not been quantified but are likely to be much lower. However, both proteomic and genomic studies in E. coli and S. typhimurium indicate that the SlyA regulon consists of about 50 genes (23, 48). As such, SlyA may be localized to fewer sites in the genome. Therefore, although our data indicate that the relative levels of SlyA and H-NS will be important in regulating transcription from PR1 at 37 °C, at this stage, we cannot exclude the possibility that other proteins also play a role in regulating transcription. With respect to temperature regulation, it seems likely that the reduced expression of SlyA at 20 °C will alter this ratio and reduce transcription. The temperature-dependent sensor responsible for orchestrating the switch from activation to repression (and vice versa) is unknown. However, the observation that H-NS bound PR1 equally at both temperatures would suggest that it is not simply a consequence of altered H-NS binding at 37 °C. Of course, superimposed on the regulation described here will be the input via BipA that has yet to be characterized in detail.

Finally, we investigated the regulation of the E. coli slyA promoter. First, we demonstrated that in E. coli, slyA is positively autoregulated, with SlyA binding to three sites within its own promoter. The identification of a SlyA binding site downstream of the slyA transcription start point is unusual for a transcriptional activator. However, this has also been observed with SlyA binding at the ugtL promoter in Salmonella (49). In addition, RovA binding downstream of ProvA has been proposed to be the basis of negative feedback in which RovA is able to inhibit its own expression in the presence of high levels of the protein (28). Second, we showed that expression of the E. coli slyA gene was temperature-regulated, being expressed at higher levels at 37 °C than at 20 °C and that this regulation was independent of H-NS. As such, we can rule out the possibility that H-NS-dependent activation of capsule gene transcription is due to H-NS activity at the slyA promoter. Our data showing the lack of role for H-NS in the expression of slyA is in agreement with the absence of SlyA from proteomic and transcriptomic based studies of changes in E. coli gene expression in an hns mutant (13). Finally we show that the E. coli slyA gene was maximally expressed during logarithmic growth with reduced expression in stationary phase. This is in contrast to expression of the slyA gene from S. typhimurium that is negatively autoregulated and maximally expressed in the stationary phase of growth (21, 22). Therefore, despite being closely related, the SlyA homologues of E. coli and S. typhimurium appear to be under different regulatory control. This idea is supported by a recent report suggesting that the SlyA_{ST} regulon is distinct from E. coli, overlapping substan-

tially with the PhoP/PhoQ regulon (23). Interestingly, the rovA gene has also been shown to be temperature-regulated, being preferentially expressed at 25 °C, the temperature at which the RovA-regulated invasin gene is maximally expressed (27). However, unlike the temperature regulation of the E. coli slyA gene, a clear role for H-NS in repression of rovA transcription at 37 °C has been demonstrated (28).

In summary, we have demonstrated that SlyA and H-NS play an integral and novel role in regulating transcription of the E. coli K5 polysaccharide gene cluster. Additionally, we show that expression of slyA is temperature-dependent, autoregulated, and independent of H-NS. Further studies are now under way to decipher the complex regulation of the PR1 promoter.

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