SlyA Protein Activates fimB Gene Expression and Type 1 Fimbriation in Escherichia coli K-12

Received for publication, June 3, 2011, and in revised form, July 13, 2011 Published, JBC Papers in Press, July 15, 2011, DOI 10.1074/jbc.M111.266619

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We have demonstrated that SlyA activates fimB expression and hence type 1 fimbriation, a virulence factor in Escherichia coli. SlyA is shown to bind to two operator sites (O_{SA1} and O_{SA2}) situated between 194 and 167 base pairs upstream of the fimB transcriptional start site. fimB expression is derepressed in an hns mutant and diminished by a slyA mutation in the presence of H-NS only. H-NS binds to multiple sites in the promoter region, including two sites (H-NS2 and H-NS3) that overlap O_{SA1} and O_{SA2}, respectively. Mutations that disrupt either O_{SA1} or O_{SA2} eliminate or reduce the activating effect of SlyA but have different effects on the level of expression. We interpret these results as reflecting the relative competition between SlyA and H-NS binding. Moreover we show that SlyA is capable of displacing H-NS from its binding sites in vitro. We suggest SlyA binding prevents H-NS binding to H-NS2 and H-NS3 and the subsequent oligomerization of H-NS necessary for full inhibition of fimB expression. In addition, we show that SlyA activates fimB expression independently of two other known regulators of fimB expression, NanR and NagC. It is demonstrated that the rarely used UUG initiation codon limits slyA expression and that low SlyA levels limit fimB expression. Furthermore, Western blot analysis shows that cells grown in rich-defined medium contain ~1000 SlyA dimers per cell whereas those grown in minimal medium contain >20% more SlyA. This study extends our understanding of the role that SlyA plays in the host-bacterial relationship.

Bacterial-host attachment is a key step in colonization and pathogenesis. Although the type 1 fimbrial adhesin of Escherichia coli is produced by the majority of non-pathogenic as well as pathogenic strains of the bacterium, it has been implicated as a virulence factor in urinary tract and other infections (1–4). Type 1 fimbriate cells attach to uroplakin receptors in the bladder to facilitate invasion and the subsequent formation of intracellular communities thought to be required for chronic-recurrent UTI (5, 6). The adhesin is able to deliver LPS to TLR4 and even directly activate the TLR4-MyD88 pathway (5–7). This interaction produces an innate immune response in the host, including the release of pro-inflammatory cytokines IL-6, IL-8, and TNF-α (5, 6).

Like many cell surface virulence factors, type 1 fimbriation is controlled by phase variation that produces a mixture of expressing (fimbriate) and non-expressing (afimbriate) bacteria. Phase variation of type 1 fimbriation in E. coli requires the site-specific inversion of a short (~300 bp) segment of DNA (fimS) that contains a promoter for the fimbrial structural operon (8–10). Inversion is catalyzed by tyrosine family recombinases FimB and FimE, which are encoded by genes situated adjacent to the fimbrial structural genes (11, 12). Whereas FimB promotes low frequency (<10^{-7} per cell per generation) phase switching in either direction, FimE can generate rates of fimbriate to afimbriate switching as high as 0.8 per cell per generation (13–15).

The expression of both fimB and fimE, as well as the inversion itself, are controlled by multiple signals including the availability of the branched chain amino acids and alanine, temperature, sialic acid (Neu5Ac), and N-acetylglucomosamine (GlcNAc) (15–17) among others. In addition to the signals noted above, fimB expression, and hence type 1 fimbriation, is also enhanced by the alarmones guanosine tetra- and pentaphosphate (ppGpp) (18). The response to many of the signals described above should decrease the fimbriate cell population during host inflammation, and we have proposed the raison d’être for the regulation observed in E. coli K-12 is to help balance the host-parasite interaction (16, 17, 19). Inversion of fimS can also be catalyzed by homologous recombinases encoded at a distant location in some clinical isolates (20, 21), raising the possibility that OFF-to-ON phase switching is less sensitive to such signals in strains showing greater pathogenicity.

The MarR-family member SlyA was originally identified as a regulator of virulence in Salmonella, where it is required for intracellular survival and systemic pathogenesis (22). SlyA is also found in E. coli where it was first shown to activate expression of the cryptic hemolysin gene hlyE (also known as clyA or shea) (23). Proteomic analysis of the SlyA regulons of enteroinvasive E. coli (EIEC) and Salmonella revealed that SlyA positively or negatively controls the expression of over 30 proteins in each bacterium (24). This study, together with more recent...
work (25), shows that there is little overlap in the SlyA regulons of the two organisms. Perhaps surprisingly, all of the SlyA regulon members identified in EIEC, including those involved in heat and acid stress responses and a variety of metabolic functions, are also found in *E. coli* K-12. However, SlyA has also been shown to activate expression of the K5 capsule, which is a virulence factor in UPEC (26, 27).

Notwithstanding these differences between the SlyA regulons of *E. coli* and *Salmonella* noted above, in all cases where the mechanism of SlyA control has been characterized in detail, SlyA regulates gene expression by interacting with the abundant nucleoid-associated protein H-NS (27–30). However while SlyA antagonizes H-NS repression of the majority of genes which it activates in both *E. coli* and *Salmonella*, remodeling of the H-NS nucleoprotein complex facilitates activation of K5 capsule in *E. coli* (27). Here we show that SlyA antagonizes the inhibitory effect of H-NS on *fimB* expression. SlyA is thus a novel activator of type 1 fimbriation in *E. coli*.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains, Plasmids, Media, and Growth Conditions**

Descriptions and genotypes of bacterial strains are listed as supplemental material [strains-pdf]. All the bacterial strains used are derivatives of the *E. coli* K-12 strain MG1655 (31). All plasmids used for allelic exchange are derivatives of the chloramphenicol resistant, temperature-sensitive vector pMAK705 (32). All reagents were obtained from Sigma unless otherwise indicated. Media used included L broth (5 g of sodium chloride (Fisher Scientific), 5 g of yeast extract (Oxoid), and 10 g of tryptone (Becton-Dickinson & Co.) per liter) and L agar (L broth with 1.5% agar (Difco)). Sucrose agar used to select recombinant *E. coli* were cloned into derivatives of the temperature-sensitive vector pMAK705 (25–30). Following a further 3 h of incubation, the cultures were cooled on ice for 10 min, and the cells were then pelleted by centrifugation at 8000 × g for 10 min at 4 °C. Cell pellets were frozen at −20 °C and then resuspended in Lysis Buffer (50 mM Tris-HCl, 2 mM EDTA, pH 8.0). Lysozyme (100 μg/ml) and Triton X-100 (0.1%) were added and the mixture was incubated for 20 min at room temperature. For DNase I treatment, MgCl₂ (10 mM) and DNase I (20 μg/ml) were added, and samples were incubated at room temperature for a further 30 min, or until the viscosity was reduced. Supernatant was collected by centrifugation at 12,000 × g for 10 min at 4 °C prior to loading onto a glutathione-Sepharose 4B column (GE Healthcare) equilibrated with Binding Buffer (140 mM NaCl, 2.7 mM KCl, 10 mM HEPES, 1.8 mM KH₂PO₄, pH 7.3). The column was then washed with 5 column volumes of Binding Buffer three times. The GST-His-tagged SlyA was eluted by adding 0.5 ml of Elution Buffer (50 mM Tris-HCl, 0.25 mM NaCl, 10 mM reduced glutathione, pH 8.0) to ml bed volume of glutathione-Sepharose 4B. Fractions enriched for GST-His-tagged SlyA were pooled and dialyzed against 50 mM Tris-HCl, pH 8.0. The GST-His-tag was removed by cleavage with His-tagged recombinant tobacco etch virus protease (Promega). The cleavage reaction was applied to a Nickel affinity column and tag-free SlyA was eluted with a 50 mM imidazole solution (50 mM imidazole, Tris-HCl 50 mM, 0.25 mM NaCl, pH 8.0). Purified SlyA was finally dialyzed against 50 mM Tris-HCl, pH 8.0.

**Western Blot Analysis of SlyA**—1.5 mg of purified SlyA protein was prepared for antibody generation at Eurogentec. After immunization of 2 rabbits with 4 injections each during 28 days (Speedy polyclonal packages), preimmune, medium, and final bleeds were collected. The final bleed serum only was used for Western blot analysis. For Western blot analysis, *E. coli* strains were grown in rich defined or minimal media with rapid aeration to reach an A₀₆₀₀ of 0.2. Cells were cooled on ice for 10 min, pelleted by centrifugation at 8000 × g for 10 min at 4 °C, and then lysed and treated with DNase I as described above. After centrifugation at 12,000 × g for 10 min at 4 °C, the supernatant of cell lysates was collected, and the concentration of total pro-
protein was determined by the Bradford method. 68.5 ng of purified SlyA and 62 μg of total protein from cell extracts were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 5% stacking and 17.5% separating gels. The proteins were transferred from the gel onto polyvinylidene difluoride membrane (PVDF, Millipore) for 1 h by using a current of 0.15–0.20 A. The membrane was blocked by incubation with 5% Dried Skimmed Milk in PBS/T (phosphate-buffered saline + 0.2% Tween 20) for 40 min at room temperature, then incubated with primary antibody (1:1000 rabbit serum) for 1 h at room temperature. After three washes with PBS/T, the membrane was incubated with secondary antibody (1:5000 goat anti-rabbit IgG-peroxidase, Sigma-Aldrich) for 30 min at room temperature, and again washed three times with PBS/T. Proteins were visualized with enhanced chemiluminescence. Western blot films were scanned and processed using ImageJ to quantify SlyA. The plot area of SlyA bands of each sample was generated using primers EMStim03f (5’-CCCGGATCC-TTAGTGACCAAAGC-3’) and EMStim03r (5’-CCCGTCGAC-TAAAATTTTGCAG-3’) primers slyAf3 (5’-CCCGGATCC-TTAGCAAG-3’) and EMSa sly01r (5’-CCCGT-GCATGGTCTCATACAGGACG-3’). DNA ManiPulations—Plasmid DNA was isolated with a kit from Qiagen. Restriction and DNA modifying enzymes were purchased from either Promega or New England Biolabs. PWO DNA polymerase used in PCR reactions was obtained from Roche. PCR and restriction endonuclease digestions were carried out according to the manufacturers’ recommendations. Deletion and replacement mutations were constructed using standard PCR techniques (38) and/or restriction endonuclease digestions, and used to replace wild type sequences cloned into derivatives of the temperature-sensitive vector, pMAK705 (32). Replacement mutations rm21–35, 14–17 bp in length, each contained a SacII restriction endonuclease site (5’-CCCGCCGG). Replacement mutations rm39 – 41, 12 bp in length, were identical and contained a BsiWI restriction endonuclease site (5’-CTGCAG). DNA sequencing was performed by Lark Technologies/Cogenics (now Beckman Coulter Genomics). Kanamycin resistance cassettes of Keio collection origin (39) were cured as described (40). All other molecular genetic procedures were carried out according to standard protocols (38, 41).

EMSA and DNaseI Footprinting—Purified tag-free SlyA was produced by GenScript, and H-NS was obtained as a generous gift from Sylvie Rimskey. The fim DNA fragment used in EMSA was generated using primers EMSAfim03f (5’-CCCGGATCC-TTAGTGACCAAAGC-3’) and EMSAfim03r (5’-CCCGTCGAC-TAAAATTTTGCAG-3’). A positive control for SlyA experiments containing SlyA promoter DNA was generated using primers slyAB (5’-CCCGGATCC-TTAGTGACCAAAGC-3’) and EMSAsly01r (5’-CCCGT-GCATGGTCTCATACAGGACG-3’), spanning the region reported to contain a SlyA binding site (27). A negative control was amplified from pBluescript as previously reported (21). Polyacrylamide gels were cast containing 5% acrylamide (Bio-Rad) and 2% glycerol (Fisher Scientific). Reaction buffer contained 10 mM Tris (Fisher Scientific) pH 9.0, 50 mM KCl (Fisher Scientific), and 0.1% Triton X-100, as used previously (42). Reactions were carried out in a final volume of 10 μl. Each DNA fragment was incubated at a final concentration of 11 nM with varying concentrations of SlyA or H-NS for 10 min, before addition of 2 μl of loading buffer (40% sucrose, 35 mM Tris, pH 8.0). Electrophoresis was immediately carried out in 1 X TBE at 160 V before staining for 15 min in 0.5 μg/ml ethidium bromide.

The fim03 fragment (Fig. 1) was made by PCR with either the fim03f or fim03r oligonucleotide labeled by [γ-32P]ATP and polynucleotide kinase. DNase footprinting was carried out in two different buffers: the H-NS buffer described by Bouffartigues et al. (43) (2007) 40 mM Heps, 60 mM potassium glutamate, pH 8.0, 5 mM MgCl2, 0.05% Nonidet P40, 5 mM DTT, 0.5 mg/ml BSA, and the “SlyA” buffer described by Zhao et al. (44) 10 mM Tris, pH 7.5, 10 mM NaCl, 1 mM MgCl2, 1 mM EDTA, 5 mM DTT, 5% glycerol, 0.5 mg/ml BSA. Complexes were formed at 25°C in a final volume of 40 μl and DNase attack was carried out for generally 1 min with a final concentration of 50 ng/ml DNase. H-NS protects DNA from DNaseL and digestion times were increased in the presence of >100 nM H-NS. Reactions were stopped with 100 μl of aqua phenol pH 8.0 and 200 μl of 0.4 M sodium acetate (pH 5.0) 2.5 min EDTA, 10 μg/ml sonicated herring sperm DNA was added. The digested DNA was phenol extracted, ethanol precipitated, and separated on 6% denaturing acrylamide gels. The radioactive gels were analyzed by phosphoimagery. The size marker is PB322 DNA digested withMspI (New England Biolabs) and labeled with [γ-32P]ATP and polynucleotide kinase.

RESULTS

SlyA Activates fimB Expression—A set of adjacent scanning-replacement mutations, extending from −38 to −184 bp upstream of the fimB transcription start site, were constructed and characterized in a chromosomal FimB-LacZ protein fusion background. This was initially carried out as part of an analysis of how the regulators NanR and NagC activate fimB expression (45). Whereas none of these mutations affected the responses to NanR or NagC (data not shown), the first two adjacent mutations (rm21 and rm22; Fig. 1) did diminish fimB expression (Fig. 24). The nucleotide sequence mutated in rm21 and rm22 contains a close match (5’-TTAGCATGATAA; hereafter called Osa, boxed in Fig. 1B) to a consensus recognition sequence for the transcriptional regulator SlyA (5’-TTAGCAAGCTAA (42)), suggesting that SlyA might be an activator of fimB expression.

To test this hypothesis, the ΔslyA mutation of the Keio collection (39) was transduced into the FimB-LacZ fusion. It was found that expression of the fusion was diminished almost 4-fold in the mutant background following growth in rich-defined MOPS glycerol medium (Fig. 2). Moreover, in contrast to NanR and to NagC (data not shown), rm21 and rm22 had little effect on fimB expression in the absence of SlyA (Fig. 2A). In a control experiment it was also found that the effect of the ΔslyA mutation on fimB expression was complemented by an ectopic copy of slyA placed in the chromosome at lac. This construct (lacUV5-slyA<sub>junc</sub>) included the slyA open reading frame, together with 65 bp of upstream DNA, placed downstream of the lacZYA promoter (Fig. 2B).

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SlyA Binds to the fimB Regulatory Region—To determine whether SlyA binds to the putative operator O_{SA1}, the interaction of SlyA with the fimB promoter region was investigated by EMSA and DNase I footprinting (Fig. 3). For this analysis, a 297-bp PCR product that includes 282 bp of fim sequences, extending from −45 to −327 bp upstream of the fimB transcriptional start site (Fig. 1) was used.

In EMSA, SlyA produced a loss of the free fim DNA, whereas it had no effect on the pBluescript DNA negative control as expected (27) (Fig. 3A). SlyA also bound to its own promoter as expected from previous work (27, 42) (data not shown). However, SlyA produced only a faint discrete mobility-shifted band with either the fim (arrow in Fig. 3A) or slyA substrates. In repeat experiments, between 16 and 27 nm SlyA dimer was required to diminish the amount of the free fim DNA by 50% (not shown).

DNase I footprinting demonstrated that SlyA bound to a region of DNA ～40 bp long, spanning O_{SA1} and extending around 20 bp further upstream relative to the fimB transcriptional start site (Fig. 3B). A DNase I hypersensitive site near position −161 appears to delimit the downstream protected region. Thus SlyA binds to O_{SA1} as predicted. The nucleotide sequence adjacent to O_{SA1} contains a second potential SlyA operator site (5′-CTAGGGACCTAA; hereafter called O_{SA2}), situated 3 bp upstream of O_{SA1}. Both O_{SA1} and O_{SA2} are altered by rm21 (Fig. 1). The whole 40 bp region is simultaneously protected by different concentrations of SlyA suggesting it corresponds to a single site. Despite the fact that the SlyA consensus is only 12 bp long, most SlyA-protected regions described in the literature are at least 40 bp long and are often bounded by hypersensitive DNase I cleavages (27, 28). A weaker level of protection was also observed in a region closer to the fimB promoter. However, mutation of this region did not alter the response of fimB expression to SlyA and the potential importance of this region was not investigated further (data not shown).

Mutagenesis of Potential SlyA Operators—To examine the possible roles of O_{SA1} and O_{SA2} further, two additional mutations which targeted O_{SA1} (rm39) and O_{SA2} (rm40) specifically were constructed and their effects on fimB expression were characterized (Fig. 1 and 4A). Surprisingly, although the O_{SA2} mutation decreased fimB expression substantially in the slyA background, the O_{SA1} mutation affected fimB expression to only a small extent. However, in both of the rm slyA double mutants, the rm mutations each largely suppressed the activating effect of SlyA on fimB expression, supporting the hypothesis that both O_{SA1} and O_{SA2} are SlyA operators as proposed.

It is notable that although all the four mutations rm21, 22, 39, and 40 eliminated the majority of the slyA mediated stimulation of fimB expression they had different effects on the basal fimB expression in the ΔslyA mutant. Expression in the rm22 and
One hypothesis to explain these results is that the mutations rm21, rm22, and rm39 also affect the binding of a repressor of fimB expression. SlyA Activates fimB Expression by Antagonizing H-NS Repression—It has been shown that SlyA antagonizes H-NS repression of hlyE expression, apparently by steric hindrance when the two proteins compete for overlapping operators (28, 48). Moreover it is known that H-NS inhibits fimB expression (49). As shown in Fig. 4B, the activating effect of SlyA on fimB expression is suppressed in an hns mutant. Indeed in the absence of H-NS, SlyA actually seems to inhibit fimB expression. While these results demonstrate that SlyA can exert opposing effects on fimB expression, it is apparent that SlyA has a net activating effect on fimB expression in the wild type background by somehow antagonizing H-NS repression.

H-NS Binding to the fimB Upstream Region with and without SlyA—Prior work showed that H-NS binds in the vicinity of the fimB promoter, but the location of the binding sites was not determined (49). In DNase I footprinting experiments, we have found that H-NS bound to an extensive region of the fimB promoter, producing specific protection at short (10–20 bp) sequences in a region that extended from about 500 bp upstream to about 150 bp downstream of the transcriptional start site (data not shown) similar to that observed at other H-NS sites (for example, see (29, 43)). As predicted from the results described above, H-NS bound to regions overlapping the DNA sequence protected by SlyA. In particular, H-NS protected two regions (called H-NS2 and H-NS3) which overlapped the SlyA binding site, and more weakly a third region (H-NS4) further upstream (Figs. 1 and 5A, lanes 2–4). The H-NS2 and H-NS3 (5'/H11032-ATGATAATAG and GCGATTATTC, appearing on the top and bottom stands in Fig. 1, respectively) each contain 5/10 bp matches to the H-NS site (5'-TCGATAAATT) proposed by Lang et al. (51). H-NS4 does not contain a discernable match.

Adding SlyA (2 μM) had no effect on H-NS binding and only when H-NS binding was lost (at 25 and 50 nM H-NS) was SlyA binding to its site detected (Fig. 5A, lanes 5, 6, 11, and 12). These experiments were performed in a Hepes-glutamate buffer previously described for use with H-NS (43). We noted however...
that many workers have preferred to study SlyA binding using lower salt buffers (42, 44). Using the buffer used by Zhao et al. (44), SlyA bound to the same sites although with higher affinity (Fig. 5 B, lanes 2 and 3). In this buffer H-NS also bound to the H-NS2 and H-NS3 sites, but protection was extended in the direction of fimB (site H-NS1) and reduced at H-NS4 (Fig. 5 B, lanes 4 and 5). H-NS1 actually contains a 7/10 match to the H-NS consensus. In addition, there were several hypersensitive DNaseI cleavages (indicated by arrows), which were not observed in the “H-NS” buffer, suggesting that the structure of the H-NS-DNA complex is dependent upon the ionic conditions. This observation is in agreement with a recent publication which demonstrated that H-NS binds to DNA in two different modes, a “stiffening” mode and a “bridging” mode, depending upon salt and divalent cation composition (50). The low salt, low Mg2+ “SlyA” buffer is compatible with the “stiffening” mode of H-NS binding, which was proposed to be implicated in the gene silencing function of H-NS. Thus, it is interesting to note that in the “SlyA” buffer, SlyA was capable of displacing H-NS from its binding sites, although it did require a 10-fold molar excess of SlyA (lanes 6 and 7). It should also be noted that these experiments were performed on linear DNA and the native supercoiled chromosome could influence the binding characteristics.

Mutation rm21, which might be expected to disrupt both O53 and H-NS2, seems mainly to eliminate SlyA activation. This suggests that H-NS binding to H-NS3 alone might be sufficient for repression of fimB expression. However the more substantial increase in fimB expression seen in the ΔslyA derivatives of the other O53 mutants rm22 and rm39 (Figs. 2A and 4) relative to the single ΔslyA mutant suggests that this is not the case. We have not been able to detect a decrease in H-NS binding to the fimB promoter PCR fragment in these mutants by EMSA (data not shown), though, suggesting that small local changes in H-NS binding can affect the overall repression of fimB expression by H-NS. The consensus match within H-NS3 overlaps with the SlyA operator O53 by 1 bp. We note that the rm40 mutation of the SlyA operator O53 coincidently improves the homology between the H-NS consensus and the match contained within H-NS3 by altering the first base of this sequence from G to T (the C to A transversion on the top strand shown in Fig. 1). Thus the unexpectedly large decrease in fimB expression seen in the rm40 mutant might reflect both diminished SlyA binding to O53 coupled to increased binding of H-NS to H-NS3.
SlyA Limits fimB Expression and Produces Differential Control of fimB Expression in Rich and Minimal Medium—The slyA open reading frame starts with an unusual UUG codon (46), suggesting that a poor level of translation initiation of slyA limits the protein expression. In agreement with this hypothesis, it was found that replacing the native UUG initiation codon with an AUG codon enhanced the level of SlyA that was detected by Western blot analysis over 5-fold (Fig. 6). The lacUV5-slyAUG construct also increased SlyA levels to well above that produced by the wild type and enhanced fimB expression to above wild type levels too (Figs. 6 and 2B, respectively). Thus poor translation initiation of slyA limits slyA expression and hence fimB expression.

The level of expression of poorly-translated proteins generally rise as growth rate declines because of diminished competition with highly translated ribosomal and other proteins for limiting translation factors (47). This suggested that SlyA should have a larger effect on fimB expression in minimal medium than it would in rich medium. In agreement with this prediction, SlyA levels were higher in minimal medium than in rich medium (Fig. 6) and the effect of SlyA on fimB expression was enhanced too (Fig. 7). We estimated that there are ~1036 dimers (the mean of duplicate estimates of 1012 and 1060) of SlyA in exponential-phase cells grown in rich defined medium and ~21% more SlyA in cells grown in minimal medium (the mean of 1280 and 1230). It should be noted that in estimating the levels of SlyA in the different media, we have taken into account the ~3-fold higher total protein content of cells grown in rich medium than in minimal medium (37).

The Effect of SlyA on the Response of fimB Expression to NanR and NagC—The mutations rm21 and rm22 have no effect on the ability of either NanR or NagC to activate fimB expression (data not shown), suggesting that SlyA controls fimB expression independently of either NanR or NagC. As anticipated, combination of the slyA and nanR or nagC mutations decreased fimB expression levels further than did the single mutations alone, as did inclusion of either Neu5Ac (the inducer of NanR) or GlcNAc (the inducer of NagC) in the growth medium of the slyA mutant (Fig. 8). Thus fimB expression is activated by multiple factors, with SlyA having a greater effect than either NanR or NagC have.

The Effect of SlyA on Type 1 Fimbriation—Low levels of fimB expression limit FimB-mediated recombination, and hence OFF-to-ON phase switching, under the conditions used in this study (16). As anticipated, the rate of FimB recombination was over 12-fold lower in the slyA mutant (Table 1). Furthermore, as judged by a decreased level of yeast cell agglutination, deletion of slyA in the wild type (strain MG1655) also diminished type 1 fimbriation as expected (Fig. 9).
In contrast, SlyA had almost no effect on the expression of a FimE-LacZ fusion (data not shown). Moreover, the level of yeast agglutination of a strain containing the fim switch locked in the ON or fimbriate orientation was unaffected by SlyA (data not shown). Thus the effect of SlyA on fimB expression described in detail above seems to be sufficient to explain how SlyA affects both FimB recombination and type 1 fimbriation.

**DISCUSSION**

The phase-variable expression of type 1 fimbriation in E. coli is regulated by a range of environmental signals. Some of these, such as the availability of the branched-chain amino acids and alanine, directly affect the DNA inversion controlling phase variation mediated by both FimB and FimE to alter phase switching from both the afimbriate to fimbriate phase and *vice versa*. Others, however, including exogenous sialic acid and N-acetylglucosamine, exert a specific effect on the direction of phase variation by controlling *fimB* expression selectively. Here we identify SlyA as a novel and specific activator of *fimB* expression and hence type 1 fimbriation.

In initial experiments it was found that mutagenesis of the DNA sequence extending from −184 to −155 bp upstream of the *fimB* transcriptional start site diminished *fimB* expression. Inspection of the wild type sequence in this region showed that it contains a close match (5′-TTAGCATGATAA; extending from −179 to −168 bp) to the consensus binding site for SlyA (5′-TTAGCAAGCTAA) proposed by Stapleton et al. (42). The *fimB* sequence, which we have called *OSA1*, differs from the consensus at only two positions (1A changed to T and 3C changed to T, according to the numbering adopted by Haider et al. (5′-T−5T−4A−3G−2C−1A1A7G3C4T5A6A)) (52). Furthermore, the sequences 5′-T−5T−4A and 5′-T5A6A, shown by Haider et al. (52) to be most important for SlyA binding, are conserved in *OSA1*. In DNase I footprinting experiments it was shown that SlyA binds to *OSA1*, thus protecting the DNA from digestion. Deletion of *slyA* diminished *fimB* expression and this defect was complemented by an ectopic copy of *slyA* placed under control of the lacUV5 promoter at *lac*. Moreover mutations that disrupt *OSA1* (rm21, 22, and 39) greatly diminish the activating effect of SlyA on *fimB* expression. These results show that SlyA is an activator of *fimB* expression and that *OSA1* is required for this effect. However the *fimB* binding site characterized by DNase I footprinting extends further upstream than *OSA1* and includes a second potential SlyA operator (5′-CTAGGGACCTAA; *OSA2*, extending from −194 to −183) three bases pairs upstream of *OSA1*. *OSA2* differs from the SlyA consensus at four positions (−9T changed to C, −2C changed to G, −1A changed to G and 2G changed to C). Mutations that disrupt *OSA2* (rm35 and rm40) also significantly reduce the activating effect of SlyA on *fimB* expression. Thus SlyA requires the region covering both *OSA1* and *OSA2* to be able to activate *fimB* expression fully.

H-NS is an inhibitor of *fimB* expression and was shown previously by EMSA to bind to the *fimB* promoter region (49). Here we have shown that the activating effect of SlyA on *fimB* expression is suppressed in the absence of H-NS. In common with a number of other systems which SlyA controls (29, 48, 53), it thus seems that SlyA activates *fimB* expression by somehow preventing H-NS binding to the *fimB* promoter. At least two models have been proposed for how SlyA antagonizes H-NS binding in other systems: “displacement,” implying direct competition between the two proteins for overlapping binding sites, and “remodeling,” where the binding of SlyA alters, but does not prevent, H-NS binding (28, 29, 30). Two of the H-NS binding sites that we have identified (H-NS2 and H-NS3) overlap the SlyA operator sites *OSA1* and *OSA2*, respectively, and at higher concentrations, SlyA does prevent H-NS binding to both H-NS2 and H-NS3 under one of the conditions tested. On the other hand, we see no evidence in footprinting experiments that the two proteins can bind simultaneously to the overlapping operator sites. However we do note that SlyA might affect H-NS binding outside the immediate SlyA protected regions; for example the hypersensitive cleavages in the downstream part of the H-NS footprint of Fig. 5B (near positions −110 and −80 lanes 4 and 5) are also attenuated in the presence of SlyA (Fig. 5B, lanes 6 and 7).

Mutations of *OSA1* (rm21, 22, and 39) that would be expected to diminish both SlyA and H-NS binding to their overlapping sites have only a modest effect on *fimB* expression in the otherwise wild type background. We note that a similar effect was reported for mutations simultaneously affecting overlapping SlyA and H-NS binding sites in the *phoPQ* promoter of *Salmonella typhimurium* (54). Conversely, a mutation of *OSA2* (rm40) that would, if anything, be expected to enhance H-NS binding to H-NS3 by increasing the match to consensus, decreased *fimB* expression to a very low level. We propose that H-NS binding to H-NS2 and H-NS3 serve as nucleation sites for oligomerization of H-NS along the DNA that is necessary for full inhibition of *fimB* expression (51, 55, 56). We suggest that the simplest inter-
pretation of our data is that SlyA activates fimB expression by binding to O_S,A1, and O_S,A2 to prevent, presumably by steric hindrance, H-NS binding to the overlapping operator sites H-NS2 and H-NS3, respectively.

We have shown previously that fimB expression is activated by the regulators NanR (sialic acid-responsive) and NagC (N-acetylglucosamine 6P-responsive) that bind to operators (O_NR and O_NC1 + O_NC2 respectively) located between around 760 and 470 bp upstream of the fimB promoter (16, 17, 45). One possibility that we have considered is that SlyA affects the ability of these proteins to activate fimB expression. However, mutants lacking SlyA remain fully responsive to NanR and to NagC and their respective inducers Neu$_5$Ac and GlcNAc. Thus fimB expression is under complex and independent regulation by multiple factors.

We have been unable to show an effect of ppGpp on the binding activity of SlyA for fimB, as has been reported for SlyA binding to the divergent pagD-pagC promoters of Salmonella (44). These results emphasize the complexity of the regulation of fimB expression and of FimB recombination and the need for further analysis of both.

We estimate there are ~1000 SlyA dimers present in exponential-phase cells grown in rich defined glycerol medium. SlyA, along with Lrp, somehow enhances the inhibitory effect that type 1 fimbriae exerts over motility (57). While for the basis for this effect requires further investigation, these and our results show that SlyA not only activates type 1 fimbriation, but that it also helps to coordinate adherence with other cellular functions. Overall SlyA controls a regulon of at least 40 proteins in EIEC (24) and our observation that SlyA is moderately abundant is consistent with this observation. Among these, SlyA represses the expression of ivy (ykfE), which encodes an inhibitor of C-lysozyme. The intact outer membrane is an effective barrier against lysozyme, and we suppose that slyA expression might be suppressed, and hence ivy induced, if the bacterial outer membrane is damaged or even if its integrity is threatened by host defenses or other stresses. Both type 1 fimbriae and the K5 capsule are attached to the outer membrane, and we suggest that SlyA may contribute to a novel signaling pathway linking cell structural robustness to the expression of these cell surface virulence factors.

Our results also show that slyA expression is limited by its poorly translated UUG initiation codon. These observations are consistent with the conclusion that SlyA both limits fimB expression and that it has a greater effect on fimB expression in minimal medium than it does in rich-defined medium. However, these results also suggest that oxidative and nitrosative stress, which limit methionine biosynthesis (58, 59), could lead to even lower levels of slyA translation and hence fimB expression. Because SlyA controls fimB expression independently of either NanR or NagC, such an effect could restrict fimB expression very substantially when combined with enhanced levels of sialic acid in the course of host inflammation (16, 19, 45). We thus propose that the regulation of fimB expression by SlyA provides an additional mechanism that helps to limit type 1 fimbriation, and hence host adherence and invasion, when host defensive mechanisms are activated.
