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Published version

CURRAN, Thomas, ABACHA, Fatima, HIBBERD, Stephen, ROLFE, Matthew, LACEY, Melissa and GREEN, Jeffrey (2017). Identification of new members of the Escherichia coli K-12 MG1655 SlyA regulon. Microbiology. (In Press)

Microbiology

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--Manuscript Draft--

Manuscript Number:	MIC-D-16-00406R1
Full Title:	Identification of new members of the Escherichia coli K-12 MG1655 SlyA regulon
Article Type:	Standard
Section/Category:	Regulation
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Abstract:	SlyA is a member of the MarR family of bacterial transcriptional regulators. Previously, SlyA has been shown to directly regulate only two operons in Escherichia coli K-12 MG1655, fimB and hlyE (clyA). In both cases SlyA activates gene expression by antagonizing repression by the nucleoid associated protein H-NS. Here the transcript profiles of aerobic glucose-limited steady-state chemostat cultures of E. coli K-12 MG1655, slyA mutant and slyA over-expression strains are reported. The transcript profile of the slyA mutant was not significantly different to that of the parent; however, that of the slyA expression strain was significantly different from that of the vector control. Transcripts representing 27 operons were increased in abundance, whereas 3 were decreased. Of the 30 differentially regulated operons, 24 have been previously associated with sites of H-NS binding, suggesting that antagonism of H-NS repression is a common feature of SlyA-mediated transcription regulation. Direct binding of SlyA to DNA located upstream of a selection of these targets permitted the identification of new operons likely to be directly regulated by SlyA. Transcripts of four operons coding for cryptic adhesins exhibited enhanced expression and this was consistent with enhanced biofilm formation associated with the SlyA over-producing strain.

Identification of new members of the *Escherichia coli* K-12 MG1655 SlyA regulon

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Key words: biofilm, gene expression, MarR family, transcription regulation

Abbreviations used: A_{600}; absorbance at 600 nm; Amp^R, ampicillin resistance; Btn, biotin; Cm^R, chloramphenicol resistance; CRP, cyclic-AMP receptor protein; EMSA, electrophoretic mobility shift assay; Kan^R, kanamycin resistance; OD_{600}; optical density at 600 nm; Tet^R, tetracycline resistance; T^s, temperature sensitive

Running title: The SlyA regulon of *E. coli* K-12 MG1655

Subject category: Regulation

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SlyA is a member of the MarR family of bacterial transcriptional regulators. Previously, SlyA has been shown to directly regulate only two operons in *Escherichia coli* K-12 MG1655, *fimB* and *hlyE* (*clyA*). In both cases SlyA activates gene expression by antagonizing repression by the nucleoid associated protein H-NS. Here the transcript profiles of aerobic glucose-limited steady-state chemostat cultures of *E. coli* K-12 MG1655, *slyA* mutant and *slyA* over-expression strains are reported. The transcript profile of the *slyA* mutant was not significantly different to that of the parent; however, that of the *slyA* expression strain was significantly different from that of the vector control. Transcripts representing 27 operons were increased in abundance, whereas 3 were decreased. Of the 30 differentially regulated operons, 24 have been previously associated with sites of H-NS binding, suggesting that antagonism of H-NS repression is a common feature of SlyA-mediated transcription regulation. Direct binding of SlyA to DNA located upstream of a selection of these targets permitted the identification of new operons likely to be directly regulated by SlyA. Transcripts of four operons coding for cryptic adhesins exhibited enhanced expression and this was consistent with enhanced biofilm formation associated with the SlyA over-producing strain.

INTRODUCTION

The MarR family of transcription regulators are widespread throughout the Bacterial and Archeal kingdoms [1]. MarR family members are homodimeric and bind to palindromic DNA sequences within regulated promoters using a characteristic winged-helix-turn-helix DNA-binding domain [2]. These regulators repress gene expression by promoter occlusion (e.g. MarR; [3]), or activate gene expression by stabilizing RNA polymerase-promoter DNA interactions (e.g. OhrR; [4]), or by antagonizing the action of repressors (e.g. RovA; [5]). These activities of MarR proteins are inhibited upon interaction with cognate signalling molecules, although for many members the natural ligand is unknown [6].

The Salmonella enterica serovar Typhimurium LT2 SlyA protein is one of the best characterized members of the MarR family. The S. enterica serovar Typhimurium slyA mutant is attenuated for virulence, is hypersensitive to oxidative stress and is impaired for survival in macrophages [7, 8]. A consensus DNA binding site has been proposed, TTAGCAAGCTAA [9, 10], and proteomic and transcriptomic comparisons of parent and slyA mutant strains suggest that SlyA can act as both a negative and positive regulator of gene expression, with significant overlap with genes of the PhoPQ regulon involved in cell envelope function, virulence, resistance to anti-microbial peptides and regulation of small RNAs [11-15]. Salmonella enterica serovar Typhimurium 14028s SlyA has also been linked to the stringent response by binding ppGpp resulting in enhanced DNA-binding [16, 17]. The expression of many SlyA-regulated genes is subject to H-NS-mediated silencing

and activation of these genes generally involves an element of antagonism of H-NS repression by SlyA; e.g. [11, 18-22].

The SlyA protein of E. coli MG1655 is 91% identical, 95% similar (over 142 amino acids) to the S. enterica serovar Typhimurium LT2 protein, but is much more poorly characterized. Only two genes, hlyE and fimB (as well as autoregulation of slyA) have been shown to be directly regulated by SlyA [19, 21, 23]. In some other E. coli strains, SlyA regulates capsule synthesis and lipid A palmitoylation in biofilms [18, 19, 22]. Here transcriptional profiling of parent, slyA mutant and slyA over-expression strains reveals the breadth of the E. coli MG1655 SlyA regulon, indicating roles in activating expression of cryptic fimbrial-like adhesins that contribute to enhanced biofilm formation.

METHODS

Bacterial strains, plasmids, oligonucleotides and growth conditions. The bacterial strains plasmids and oligonucleotides that were used are listed in Table 1. Bacterial strains were routinely cultured in Luria Bertani broth or on Luria Bertani agar plates [24]. Aerobic glucose-limited steady-state chemostat cultures of E. coli were established in Evans minimal medium [25] in Labfors 3 fermentation vessels (Infors-HT, Switzerland) with a 1 L working volume, 0.2 h^{-1} dilution rate, 37°C, pH 6.9, 400 rpm stirring rate and sparging with 1 L min^{-1} air. Evans minimal medium consists of: 10 mM NaH_2PO_4, 10 mM KCl, 1.25 mM $MgCl_2$, 20 mM NH_4Cl, 0.02 mM $CaCl_2$, 0.1 mM Na_2SeO_3, 1.5 mM monosodium nitrilotriacetate, 20 mM glucose and 100 ml trace element solution. The trace element solution consisted of (g L^{-1}): ZnO (0.412), $FeCl_3.6H_2O$ (5.4), $MnCl_2.4H_2O$ (2.0), $CuCl_2.2H_2O$ (0.172), $CoCl_2.6H_2O$ (0.476), H_3BO_3 (0.064), $Na_2MoO_4.H_2O$ (0.004) in 0.3% v/v HCl. For generation of cell paste for purification of His-tagged SlyA, E. coli BL21 (λDE3) transformed with pGS2469 was grown in auto-induction medium supplemented with ampicillin (100 mg L^{-1}) [26]. Resistance to chloramphenicol was tested by inoculating Luria Bertani broth (2 ml) containing kanamycin (30 μg ml^{-1}) and either 0, 1, 2, 3 or 4 μg ml^{-1} chloramphenicol with 10 μl of overnight starter cultures (E. coli K-12 MG1655 pET28a or E. coli K-12 MG1655 pGS2468). Triplicate cultures were grown under aerobic conditions for 6 h at 37°C before measuring OD_{600} as a measure of growth. The experiment was carried out twice.

Biofilm assay. Biofilm assays were performed using 96-well plates essentially as described by Tagliabue et al. [27] using M9 minimal medium with 20% (w/v) glucose and 50 μg ml^{-1} kanamycin. Wells containing 200 μl of medium were inoculated (1:10) from an overnight culture of E. coli K-12 MG1655 pET28a or E. coli K-12 MG1655 pGS2468 and then incubated for 16 h under aerobic conditions at 37°C. Growth of cultures was monitored by measuring OD_{600}. The planktonic cells were removed and the remaining biofilm was stained for 5 min with 200 μl 1% (w/v) crystal violet solution. Excess stain was removed by three washes with deionized water before the plate was

air-dried. To quantify the extent of staining, 200 µl ethanol:acetone (4:1) was added to each well, and after incubating for 20 min the amount of biofilm was estimated by measuring A_{600}. Adhesion units were calculated by dividing the A_{600} values for crystal violet-stained adhered cells by the OD_{600} values for the corresponding planktonic cells.

Creation of *E. coli* K-12 MG1655 *slyA* mutant. A PCR-amplified DNA fragment containing the kanamycin cassette from pKD4 flanked by 40 bp DNA homologous to regions surrounding the slyA gene was synthesized using oligonucleotide primers TC7 and TC8 (Table 1). The purified (QiaQuick PCR cleanup, Qiagen) PCR product (5 µg) was used to transform E. coli JRG6072 by electroporation (Hybaid Cell Shock unit; 1800 V, 1 mm path length). The E. coli JRG6072 competent cells were prepared from aerobic Luria Bertani broth batch cultures supplemented with ampicillin (100 mg L^{-1}) at 30°C that had been induced to express the λred recombinase by addition of L-arabinose (1 mM). Kanamycin resistant mutants were selected on Luria Bertani agar plates containing kanamycin (30 mg L^{-1}) at 37°C. Mutation of the slyA gene by insertion of the kanamycin resistance cassette was confirmed by colony PCR using oligonucleotides TC9 and TC10. The slyA mutation was then transduced using bacteriophage P1 to E. coli MG1655 [24].

Transcriptional profiling. Transcriptomic analyses were carried out as described by Rolfe et al. [28] using directly quenched samples from glucose-limited steady-state chemostat cultures (dilution rate 0.2 h^{-1}) for the three E. coli K-12 MG1655 strains; parent, slyA mutant (JRG6457) and slyA over-producer (JRG6636). RNA samples were labelled with Cy5 and the reference E. coli K-12 MG1655 genomic DNA was labelled with Cy3. In total, two independent biological replicates were performed that were hybridised in duplicate (technical replicates) giving four replicates. After hybridization and image capture, data were extracted from the raw image files using Agilent Feature Extraction v11.5 software and analyzed using GeneSpring v7.3.1. Transcriptomic data have been deposited with ArrayExpress (accession E-MTAB-5220).

Purification of SlyA and Western blotting. Cultures (500 ml auto-induction medium supplemented with ampicillin in 2 L conical flasks) of E. coli BL21 (λDE3) pGS2469 were grown at 37°C for 24 h with shaking (250 rpm). Bacteria were collected by centrifugation, the pellet was re-suspended in 15 ml of breakage buffer (20 mM Tris-HCl, 500 mM NaCl, 5% v/v glycerol, pH 7.5), the bacteria were lysed by two passages through a French pressure cell (16,000 psi) and the extract clarified by centrifugation (27,000 g, 15 min, 4°C). The His-tagged SlyA protein was isolated from the cell-free extract by affinity chromatography on a HiTrap chelating column (1 ml) attached to an AKTA prime according to the standard manufacturer's protocol (GE Healthcare). The eluted SlyA was buffer exchanged into 20 mM Tris-HCl, pH 7.4 containing 200 mM NaCl by repeated dilution

and Vivaspin 6 concentration (Sartorius Stedim Biotech). The protein was judged to be >90% pure by Coomassie blue-stained SDS-PAGE and protein concentration was estimated by the BioRad protein reagent protocol [29]. SlyA protein was detected by Western blotting after separation of polypeptides by SDS-PAGE and electrophoretic transfer (100 V for 1 h; transfer buffer: 5.8 g L^{-1} Tris, 2.9 g L^{-1} glycine, 20% v/v methanol, 0.037% w/v SDS) to Hybond-C Extra nitrocellulose membranes (GE Healthcare). The membranes were soaked in a blocking solution, which contained 5% w/v dried skimmed milk in PBS (10 mM phosphate buffer, 137 mM NaCl, 2.7 mM KCl, pH 7.4) and 0.05% v/v Tween 20, for 16 h at 4°C. The blocking solution was then removed and the membranes washed in PBS containing 0.05% v/v Tween 20 before exposure to a 1:1000 dilution of the SlyA antibody (raised in rabbit and provided by Prof. Ian Blomfield, University of Kent) in blocking solution for 1 h at room temperature. After four washes with PBS containing 0.05% v/v Tween 20, the membranes were soaked in blocking solution containing anti-rabbit secondary antibody provided in the Pierce ECL Western Blotting kit and the presence of SlyA was visualized according to the manfacturer's standard protocol (Thermo Scientific).

Electrophoretic mobility shift assays (EMSA). The LightShift Chemiluminescent EMSA kit (Thermo Scientific) was used according the manufacturer's intstructions. Biotin-labelled DNA of target promoter regions was amplified from genomic DNA using the appropriate oligonucleotide primer pairs (Table 1). The core binding assays (20 µl) contained: 2 µl 10x binding buffer (100 mM Tris-HCl, pH 7.5, containing 500 mM KCl and 10 mM dithiothreitol and 1 µg poly (dI•dC). The DNA concentration was ~1 nM and the concentration of SlyA ranged from 0 to 500 nM as indicated. Mixtures were incubated at 25°C for 30 min before separation of SlyA-DNA complexes by native gel electrophoresis, followed by transfer to Hybond-N+ nylon membranes, UV-crosslinking for 60 s at 120 mJ cm^{-2} and detection of labelled DNA using the Nucleic Acid Detection Module (Thermo Scientific).

RESULTS AND DISCUSSION

Enhanced expression of *slyA* in *E. coli* K-12 MG1655 results in altered abundance of transcripts from 30 operons

Previous work has shown that SlyA directly activates the expression of two genes in E. coli K-12 (hlyE and fimB) by antagonising H-NS repression [20, 21, 23]. However, in S. enterica serovar Typhimurium the influence of SlyA is much more extensive, with at least 31 regulated genes resulting in hypersensitivity to reactive oxygen species and attenuation in infection models [7, 8, 12]. The initial aim of this work was to apply transcript profiling to determine the extent of the E. coli K-12 MG1655 SlyA regulon by comparison of steady-state glucose-limited aerobic chemostat cultures of wild-type and slyA mutant strains. Comparison of transcript profiles of wild-type and slyA mutant

cultures grown at a dilution rate of 0.5 h^{-1} (equivalent to a doubling time of 1.4 h) revealed no significant (≥2-fold; $p \leq 0.05$) changes in transcript abundance. Because SlyA translation might be enhanced at low growth rates, due to its unusual UUG start codon [21], steady-state cultures at dilution rates 0.2, 0.1 and 0.05 h^{-1} were established (equivalent to doubling times of 3.5, 6.9 and 13.8 h, respectively). However, once again, when the transcript profiles and growth characteristics of the wild-type and slyA mutant cultures were compared no significant differences were detected. These observations indicated that, under the conditions tested, deletion of the slyA gene had no significant effect on gene expression in E. coli K-12 MG1655, even at low growth rates.

Anti-SlyA serum was used to determine whether SlyA was detectable in E. coli K-12 MG1655 cells grown in glucose-limited chemostats at a dilution rate of 0.2 h^{-1}. In accordance with the transcript profiling, SlyA was not detected (Fig. 1). This suggests that the expression of SlyA is regulated and switched on under conditions other than those imposed here; for example, SlyA protein has been detected by Western blotting extracts from E. coli batch cultures grown in minimal medium with glycerol as the carbon and energy source [21]. To overcome any regulatory barrier to identifying genes potentially controlled by SlyA, a plasmid (pGS2468) to express slyA under the control of its own promoter was constructed. Western blotting showed that SlyA protein was now readily detectable in the transformed E. coli K-12 MG1655 cells grown in glucose-limited chemostats at a dilution rate of 0.2 h^{-1} (Fig. 1). The growth characteristics of the vector control and the slyA expression strains were essentially the same, with similar yields (1.4 ±0.2 g cell dry weight per litre) and no detectable glucose or over-metabolites in the culture supernatants. Therefore, the transcript profiling experiments were carried out with these strains grown in aerobic glucose-limited chemostats at a dilution rate of 0.2 h^{-1}. The transcript profile of the SlyA over-production strain was significantly different from that of the vector control. Transcripts representing 27 operons were increased in abundance and 3 were decreased (Table 2; Fig. 1c). The transcripts exhibiting decreased abundance were: the sgc operon (sgcXBCQAER), which encodes a phosphotransferase system for the uptake of an unknown sugar; fecIR the membrane-bound sensor (FecR) that receives signals from the outer membrane ferric citrate uptake receptor (FecA) for transmission to FecI (σ^{19}), which activates transcription of the fecABCDE operon encoding components of a cytoplasmic membrane bound ferric citrate uptake system; and yecH, which encodes a predicted protein of unknown function (Table 2) [30, 31].

Amongst the up-regulated transcripts were the previously identified SlyA-regulated gene hlyE and slyA itself (Table 2). The latter was not surprising as the slyA gene was present in multi-copy, but despite this the slyA transcript only increased ~3-fold in abundance, yet the SlyA protein level increased from being undetectable in the control to a level equivalent to ~1.5 μM in the cytoplasm (based on the dry weight of E. coli being 3×10^{-13} g with an aqueous volume of 7×10^{-13} ml per cell; [32]). The relatively low level of induction of the slyA transcript when present in multi-copy but

much greater induction of SlyA protein suggests that the slyA promoter is subject to auto-regulation, consistent with the reported SlyA binding at the slyA promoter [19]. It was also notable that the ydhI-K operon, which is divergently transcribed from slyA and not present on the slyA expression plasmid, also exhibited enhanced transcript abundance, suggesting that SlyA is capable of activating expression from divergent promoters; an assertion supported by the enhanced abundances of the divergently transcribed hlyE and C0299 (encodes a small RNA) genes in the presence of SlyA (Table 2).

Twenty-four of the 30 operons (~80%) that showed altered transcript abundance upon over-production of SlyA have also been shown to be associated with H-NS binding sites (Table 2). Thus, it appears that H-NS repressed genes are over-represented in the set of transcripts that increase in abundance when SlyA is expressed, suggesting that SlyA acts by antagonizing H-NS repression at the corresponding promoters; a mechanism that is established for hlyE [20]. H-NS binds DNA by recognizing the structure of A-T-rich minor grooves and silences the expression of horizontally acquired A-T-rich genes (reviewed by [33]). H-NS is thus considered crucial in permitting the acquisition of new genes whilst counteracting the potentially detrimental effects of inappropriate expression of these genes. Counter-silencing by H-NS antagonists, such as SlyA, provides a route to integrate expression of the genes into the regulatory circuits of E. coli under appropriate conditions. Horizontally acquired genes are located within genomic islands, which are regions of bacterial chromosomes containing that are often associated with drug resistance, metabolic adaptability, stress tolerance and pathogenesis. Genomic islands can be recognized by their sequence composition and increased transcript start point densities [32]. The analysis tools GIST (Genomic-island Identification by Signals of Transcription) and IslandViewer have been used to map the genomic islands of E. coli K-12 MG1655 [34]. Notably, 13 of the 30 differentially regulated operons overlapped predicted genomic islands, suggesting a general role for SlyA in the counter-silencing of H-NS repressed horizontally acquired genes under conditions when slyA is up-regulated (Table 2).

The H-NS-repressed casABC operon was up-regulated by SlyA (Table 2). This operon encodes proteins involved in maintaining and utilising the library of foreign genetic elements interspersed between CRISPR sequences which act as the immune system memory of Bacteria and Archaea [35]. CRISPR loci, in general, consist of closely spaced direct repeats separated by short spacer regions of variable sequence. Spacer regions mostly correspond to sections of foreign plasmid or viral sequences which have been integrated. The CRISPR loci are found adjacent to the casABC operon. The fact that the casABC operon was significantly up-regulated by SlyA suggests that this regulator may contribute to viral resistance and immunity in E. coli K-12 MG1655.

Other transcripts that exhibited increased abundance in the presence of SlyA were associated with uptake and metabolism of phenylacetic acid (paaA-K), utilization of alkanesulfonates as alternative sulfur sources (ssuEADCB; divergently transcribed from the elf operon; see below), a cryptic galactosamine transport and catabolism system (agaS-I) and a 2-O-α-mannosyl-D-glycerate phosphotransferase and α-mannosidase (Table 2) [36-39]. Hence, it appears that SlyA plays a role in

regulating systems that expand the repertoire of substrates utilized by E. coli. Increased abundance of the mdtM transcript suggests a role for SlyA in enhancing expression of this multidrug transporter that confers resistance to ethidium bromide and chloramphenicol with mutants exhibiting attenuated growth at alkaline pH [40]. However, simple growth inhibition studies suggested that slyA expression led to increased sensitivity to chloramphenicol (growth yield after 6 h at 37°C in Luria Bertani broth was lowered to ~50% by 2 μg ml^{-1} for the wild-type carrying the empty vector compared to 1 μg ml^{-1} for the wild-type carrying the slyA expression plasmid), rather than increased resistance, perhaps reflecting the complexity of the phenotype of the slyA expression strain.

Several of the SlyA-regulated operons code for proteins involved in membrane function. In S. enterica serovar Typhimurium the majority of genes affected by SlyA encode proteins associated with the bacterial cell envelope and are important for virulence and survival within murine macrophages. Although it has been previously shown that the majority of genes regulated by SlyA in S. enterica serovar Typhimurium are not present in E. coli K-12 [12, 15], a similar propensity for cell envelope proteins being regulated by the E. coli SlyA was evident here. Thirteen (43%) of the 30 operons that exhibited altered expression in SlyA-expressing bacteria were associated with cell-surface/membrane functions (Table 2).

The gspC-O operon is cryptic membrane-associated, H-NS-repressed, transcription unit that was up-regulated by SlyA (Table 2). The gspC-O operon encodes a Type-II Secretion System (T2SS) for the export of endogenous proteins and formation of structural elements of the Gsp secreton, which is thought to facilitate the export of the endogenous endochitinase ChiA, a product of another H-NS silenced gene [41, 42].

Amongst the transcripts with increased abundance in the SlyA over-producing strain were four cryptic operons (elfADCG-ycbUVF, sfmHF, yehDCBA and yadN) encoding fimbrial-like adhesins (Table 2). These four operons were amongst seven putative chaperone-usher fimbrial systems shown to be poorly expressed under laboratory conditions by Korea et al. [43]. Nevertheless, when these operons were individually expressed by placing them under the control of a constitutive promoter six were shown to be functional and expression of the elf (ycb), yad and yeh operons resulted in enhanced biofilm formation on abiotic surfaces, whereas sfm promoted binding to eukaryotic cells [43]. Moreover, all four operons were repressed by H-NS. The increased abundances of the elf, sfm, yad and yeh transcripts upon expression of SlyA is consistent with the cryptic status of these genes under normal laboratory conditions and suggests that these chaperone-usher fimbriae are functional under environmental conditions that enhance slyA expression such that SlyA can operate as an H-NS antagonist (Table 2).

SlyA over-production is associated with enhanced biofilm formation

The observation that SlyA increased transcription of four cryptic fimbrial-like adhesins suggested that the SlyA over-producing strain should exhibit enhanced biofilm production. This was tested using

static cultures of E. coli K-12 MG1655 transformed with pET28a (control) or the slyA expression plasmid pGS2468 in conditions that mirrored the transcript profiling experiment. The data showed a 4-fold increase in biofilm formation when slyA was over-expressed, consistent with the transcript profiling data (Fig. 2).

Identification of new *E. coli* K-12 MG1655 operons that are directly regulated by SlyA

The changes in transcript profiles that were observed upon over-production of SlyA could result from direct interaction of SlyA with the promoter regions of the corresponding genes or indirectly via SlyA-regulated factors. For example, one of the genes up-regulated upon SlyA over-production, leuO, encodes a transcriptional regulator that, like SlyA, operates by antagonising H-NS regulation [44, 45]. Fourteen (52%) of the 27 transcripts that were increased in abundance when SlyA was expressed in E. coli K-12 MG1655 were associated with LeuO binding sites identified in the SELEX-chip study of Shimada et al. [44]. This strong correlation could arise from; (1) the positive effect SlyA has on the expression of leuO resulting in an increase in expression of the entire LeuO regulon, i.e. indirect regulation by SlyA; or (2) SlyA and LeuO have overlapping regulons as a consequence of the fact they both operate by antagonising H-NS-mediated repression. To further investigate the extent of direct SlyA-mediated regulation in E. coli K-12 MG1655 binding of SlyA to ten promoter regions was examined by electrophoretic mobility shift assays (EMSA).

Amongst the transcripts differentially regulated by over-production of SlyA there were three arranged as divergent operons (Fig. 3). Binding of SlyA at the hlyE-C0299 intergenic region was shown previously (Fig. 3) [20]. Two other examples of SlyA-activated divergent operons (slyA-ydhIJK and ssuE-B-elfADCG-ycbUVF) were shown to bind SlyA in EMSA (Fig. 3). Furthermore, SlyA bound at the casA, fecIR, gspCDEF, leuO, mdtM and paaA-K promoters (Fig. 3). The $K_{d(app)}$ values for SlyA binding at these promoters were similar at ~50-100 nM. These experiments indicate that these operons are likely to be directly regulated by SlyA. The sgcXBCQ-sgcAER genes are separated by a sRNA ryjB on the opposite DNA strand (Fig. 3). It is suggested that the sgcXBCQAER is a single transcription unit, but there is no high quality evidence to support this suggestion [46]. Therefore, both the region upstream of sgcX and the intergenic region between sgcQ and sgcA were used in EMSAs with the SlyA protein. No specific interaction was observed with the region upstream of sgcA but interaction, albeit weaker than that observed for the promoter regions analyzed above, was observed when the DNA upstream of sgcX was tested (Fig. 3). These observations suggest that sgcXBCQAER is a single SlyA-repressed transcription unit.

The EMSA experiments indicate that SlyA binds Pssu, Pcas, Ppaa, Pelf, PleuO and Pgsp, all of which are promoter regions of genes or operons proposed to be part of the LeuO regulon (Table 2). This suggests that, perhaps because of the similarity in their mode of action, i.e. antagonizing H-NS repression, the SlyA and LeuO regulons substantially overlap such that upon activation by their respective signals a similar transcriptional response is elicited.

A consensus binding site (TTAGCAAGCTAA) for the Salmonella enterica serovar Typhimurium LT2 SlyA protein was proposed based on footprinting and a limited SELEX analysis [10]. This consensus was further analyzed by site-directed mutagenesis, which suggested the consensus sequence $TTAN_6TAA$ [9]. All the DNA fragments that bound E. coli SlyA in EMSAs (Fig. 3) possessed DNA sequences similar to the previously proposed consensus sequences (Table 3). Site-directed replacement amino acid residues of Salmonella enterica serovar Typhimurium LT2 SlyA identified 16 locations that impaired DNA-binding [9], all these amino acids are conserved in the E. coli SlyA protein, suggesting that these closely related proteins recognize similar DNA motifs.

Concluding remarks

SlyA proteins have been shown to play important roles in regulating gene expression in a wide range of bacterial species. The most common mechanism for SlyA-mediated activation of gene expression is through antagonism of H-NS repression. Here transcript profiling has revealed the breadth of the SlyA regulon (directly and indirectly regulated genes) in E. coli K-12 MG1655 cultures grown under precisely controlled conditions such that any potential effects associated with changes in growth rate/growth phase could not confound the interpretation of the data obtained. Enhanced transcript abundance for several cryptic fimbrial operons in a SlyA over-producing strain and an over-representation of H-NS repressed genes were consistent with the current model of SlyA-mediated gene activation. The SlyA protein was shown to bind at 9 intergenic regions controlling the expression of 11 operons, thus expanding the number of known directly SlyA-regulated genes in E. coli MG1655 from 2 to 13.

FUNDING INFORMATION

TDC was supported by a Biotechnology and Biological Sciences Research Council (BBSRC) UK PhD scholarship; SPH was supported by a Sheffield Undergraduate Research Experience (SURE) project.

ACKNOWLEDGEMENTS

The authors thank Ian Blomfield (University of Kent, UK) for providing the SlyA antiserum and Ruth Roberts (University of Sheffield) for technical support.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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Table 1 Bacterial strains, plasmids and oligonucleotides

Strain or plasmid	Relevant characteristics[a]	Reference or source
Bacterial strain		
E. coli BL21 (λDE3)	E. coli BL21 lysogen for inducible (IPTG) expression of the T7 RNA polymerase	Novagen
E. coli JRG6457	E. coli MG1655 slyA	This work
E. coli JRG6636	E. coli MG1655 pGS2468	This work
E. coli JRG6072	E. coli MG1655 pKD46	This work
E. coli MG1655	Genome sequenced parental strain	[47]
Plasmid		
pET28a	Multi-copy plasmid; Kan^R	Novagen
pGS2468	pET28a derivative for expression of slyA from the slyA promoter; Kan^R	This work
pGS2469	pLATE-51 derivative for over-production of SlyA; Amp^R	This work
pKD4	Source of kanamycin resistance cassette; Amp^R, Kan^R	[48]
pKD46	Plasmid for inducible (L-arabinose) expression of the λred recombinase; Amp^R, T^s	[48]
pLATE-51	Expression vector for production of His-tagged proteins; Amp^R	Thermo Scientific
Oligonucleotide		
TC7	TAAAGCCGCATAATATCTTAGCAAGCTAATTATAAGGAGATTA CACGTCTTGAGCGATT; creation of slyA mutant	This work
TC8	TTGCGTGTGGTCAGGTTACTGACCACACGCCCCCTTCATTCAT ATGAATATCCTCCTTAG; creation of slyA mutant	This work
TC9	CTGACGGTAACCAAATGCAG; PCR of slyA locus	This work
TC10	TTTGCGTGTGGTCAGGTTAC; PCR of slyA locus	This work
TC49	[Btn]ACTCTCTCCTTATAACCAATTG; forward primer for PCR of biotin (Btn)–labelled 355 bp intergenic region between ssuE and elfA	This work
TC50	CGTTATCATCCTGATCTCTT; reverse primer for use with TC49	This work
TC51	[Btn]TGGTGAATATTATTGATCAATTAAT; forward primer for PCR of biotin (Btn)–labelled 344 bp intergenic region between leuO and leuL	This work
TC52	ACTTAACTCCACTGTCACACTTAA; reverse primer for use with TC51	This work
TC53	[Btn]TTGTTCTCCTTCATATGCTC; forward primer for PCR of biotin (Btn)–labelled 414 bp intergenic region between casA and cas3	This work
TC54	CTTCGGGAATGATTGTTATC; reverse primer for use with TC53	This work
TC55	[Btn]TGTTGCTAATAGTTAAATCGC; forward primer for PCR of biotin (Btn)–labelled 257 bp intergenic region between paaA and paaZ	This work
TC56	GTCATCACCTTTACGATTCC; reverse primer for use with TC55	This work
TC57	[Btn]AACAAACAACTCCTTGTCCG; forward primer for PCR of biotin (Btn)–labelled 400 bp region upstream of mdtM	This work
TC58	CCCCGAGGCGCTTTCCAGGC; reverse primer for use with TC57	This work
TC59	[Btn]AGAACTTCCTGTTTTAATTATTG; forward primer for PCR of biotin (Btn)–labelled 179 bp intergenic region between gspA and gspC	This work
TC60	GATGTATGTTCTAATAAAATAGATTG; reverse primer for use with TC59	This work
TC61	[Btn]CCGTCGTTGACTCCATGC; forward primer for PCR of biotin (Btn)–labelled 130 bp intergenic region between sgcA and sgcQ	This work
TC62	GATGGGGATAAGCAGAGC; reverse primer for use with TC61	This work
TC63	[Btn]GCGGAGTGCATCAAAAGT; forward primer for PCR of biotin (Btn)–labelled 291 bp intergenic region between fecI and insA-7	This work
TC64	GCAAGCACCTTAAAATCAC; reverse primer for use with TC63	This work
TC65	[Btn]TTTCATCTCCTTATAATTAGCTT; forward primer for PCR of biotin (Btn)–labelled 200 bp intergenic region between slyA and ydhI	This work
TC66	AAAGTAGATTCCTTTACGACC; reverse primer for use with TC65	This work
TC70	[Btn]AGCTATCTCCGTAGACCGT; forward primer for PCR of biotin (Btn)–labelled 400 bp region upstream of sgcX	This work
TC71	GATTATCTATACTCCCTCTGAATC; reverse primer for use with TC70	This work

[a]Amp^R, ampicillin resistant; Kan^R, kanamycin resistant; T^s, temperature sensitive replication

489 **Table 2** Transcripts exhibiting altered abundance upon over-expression of *slyA* in *E. coli* MG1655

Operon[a]	Fold-change[b]	Gene function[c]	H-NS regulon[d]	LeuO regulon[e]	Overlap with genomic island[f]
ybeT	4.1	conserved outer membrane protein	K	✓	
trkG	3.8	Rac prophage potassium transporter subunit	K,O		IV
ssuE**A**DCB	3.6	aliphatic sulfonate transport and metabolism	G,K,O	✓	
yehDCBA	3.6	chaperone-usher fimbrial operon (cryptic)	K,O	✓	GIST
mngAB	3.4	2-O-α-mannosyl-D-glycerate PTS and α-mannosidase		✓	
casABC	3.3	CRISPR associated genes	K	✓	GIST, IV
yghS	3.1	predicted protein with nucleoside triphosphate hydrolase domain	K,O	✓	
slyA	3.0	DNA-binding transcriptional activator	O		
yfbN	2.8	predicted protein	K,O	✓	IV
paaA-K	2.8	phenylacetic acid degradation			
ybeU-hscD	2.8	predicted tRNA ligase and chaperone	K,O	✓	
elfADCG-ycbUVF	2.7	predicted fimbrial-like adhesin protein (cryptic)	G,K,O	✓	
ygeG	2.7	predicted chaperone	G,K,O		GIST
crfC-yjcZ	2.6	clamp-binding sister replication fork co-localization protein and predicted protein	K,O		
sfmHF	2.6	predicted fimbrial-like adhesin protein (cryptic)	O		IV
agaS-kbaY-aga**B**CDI	2.5	predicted galactosamine-transport and metabolism (cryptic)		✓	
ydhY**V**-T	2.5	predicted oxidoreductase	G,K,O		GIST
yiiE	2.5	predicted transcriptional regulator	K,O		
mdtM	2.5	multidrug efflux system protein			
leuO	2.5	DNA-binding transcriptional activator	G,K,O	✓	GIST
C0299	2.4	sRNA C0299	O		
ycjM**N**-V	2.4	predicted sugar transporter and metabolism	K,O	✓	
yadN	2.4	predicted fimbrial-like adhesin protein (cryptic)	G,K,O	✓	GIST
gspCDEF	2.4	type II secretion system (cryptic)	K,O	✓	
ydhIJK	2.2	predicted proteins	O		
yfdM	2.1	CPS-53 (KpLE1) prophage predicted methyltransferase	O		GIST, IV

hlyE	2.0	hemolysin E (cryptic)	K,O	
yecH	0.5	predicted protein	O	GIST
sgcX**B**CQAER	0.5	predicted sugar transport and metabolism		IV
fecI**R**	0.4	transcription regulation of ferric citrate transport		IV

[a]The fold-change data shown are for the first gene in the operon except where indicated by bold typeface; note that all genes in the operons followed the same pattern of regulation.

[b]Fold-change (≥2-fold, $p \leq 0.05$) is the product of dividing the transcript abundance for the *slyA* over-expression cultures by that for the control cultures.

[c]Gene functions as assigned in Ecocyc.org [46].

[d]Genes associated with H-NS binding were identified from Grainger *et al*. [49] (G), Kahramanoglou *et al*. [50] (K) and Oshima *et al*. [51] (O).

[e]Genes located up- or down-stream of a LeuO binding site identified by Shimada *et al*. [44].

fGenes the overlap with genomic islands in *E. coli* K-12 MG1655 identified by GIST and/or IslandViewer (IV) [34].

499 **Table 3** Candidate SlyA binding sites within the DNA fragments used for EMSA analyses

Promoter region	Possible SlyA binding sites	Location of site relative to start codon
PcasA	**TTA**TTGAAT**TAA**	100 bp upstream of casA
PssuE/elfA	**T**CA**G**GA**T**GA**TAA**	8 bp upstream of elfA
PgspC	**TTA**TATTAG**TAA**	79 bp upstream of gspA
PpaaA	**TTA**AATC**G**C**G**A**A**	239 bp upstream of paaA
	TTATA**A**A**A**AA**TA**G	136 bp upstream of paaA
	TTACTTA**A**C**TA**T	81 bp upstream of paaA
PsgcX	**TTA**TGCT**G**GG**AA**	336 bp upstream of sgcX
	TTTCA**A**CCA**TAA**	188 bp upstream of sgcX
PfecI	**TTA**G**A**A**A**AAC**AA**	109 bp upstream of fecI
PslyA	**TTA**GCAAGC**TAA**	22 bp upstream of slyA
	TTAG**A**TTAA**TAA**	161 bp upstream of slyA
PleuO	**TTA**ATGCAT**TAA**	305 bp upstream of leuO
	TTAAAT**A**TA**TAA**	297 bp upstream of leuO
PmdtM	**T**A**T**A**C**A**C**CT**TAA**	249 bp upstream of mdtM

500

501 Sequences shown are those with the greatest similarity to the previously proposed consensus for the
502 *Salmonella enterica* serovar Typhimurium LT2 SlyA protein (Haider *et al*. [9]; **TTA**N_6**TAA**). Where
503 more than one possible site was present those with the greatest similarity to the consensus sequence
504 TTAGCAAGCTAA proposed by Stapleton *et al*. [10] are shown. Locations of sites are given as the
505 number of base pairs from the start codon of the specified gene to the centre of the proposed binding
506 site.

507

Figure legends

FIG. 1. Changes in the transcript profile of *E. coli* K-12 MG1655 over-producing SlyA. (a) Transformation of *E. coli* K-12 MG1655 with a multi-copy plasmid expressing *slyA* under the control of its own promoter results in detectable SlyA protein in lysed cell suspensions from aerobic steady-state glucose-limited chemostat cultures. The upper panel shows the Coomassie blue-stained SDS-polyacrylamide gel and the lower panel shows the relevant region of a Western blot prepared with the same samples and loadings developed with SlyA antiserum. The gels were loaded as follows: Lane M, SDS-PAGE markers (sizes, kDa, are indicated); lanes 1 and 2, extracts from independent cultures of *E. coli* K-12 MG1655 transformed with the vector pET28a ($SlyA^{WT}$); lanes 3 and 4, extracts from independent cultures of *E. coli* K-12 MG1655 transformed with the expression plasmid pGS2468 ($SlyA^{+}$); lane 5, purified $(His)_6$-SlyA (~10 ng protein loaded). (b) Western blot corresponding to the gel shown in (a). The locations of SlyA and purified $(His)_6$-SlyA are indicated. (c) Graphical representation of the changes in transcript abundance occurring upon over-production of SlyA in *E. coli* K-12 MG1655. Comparison of the fold-changes in transcript abundance of aerobic steady-state glucose-limited chemostat cultures of *E. coli* K-12 MG1655 transformed with either the pET28a ($SlyA^{WT}$) or pGS2468 ($SlyA^{+}$). Each line represents a gene that exhibits a ≥2-fold change in transcript abundance ($p \leq 0.05$) from two biological and two technical replicates i.e. four measurements.

FIG. 2. Biofilm formation by *E. coli* K-12 MG1655 is enhanced by elevated *slyA* expression. Wells containing M9 minimal medium with 20% w/v glucose as a carbon source were seeded with 1:10 inocula of overnight cultures and incubated at 37°C for 16 h. The OD_{600} of the planktonic bacteria was measured before a biofilm assay was carried out. Values shown are the mean and standard deviation (n = 12) *** denotes $p \leq 0.00001$ in a Student's *t*-test.

FIG. 3. Electrophoretic mobility shift assays show specific binding of SlyA to intergenic regions of selected operons. The dashed lines in the diagrams on the left indicate the DNA regions used in the EMSA shown on the right. The arrows indicate the polarity of the genes (names above the arrows). The numbers below the arrows representing genes are the fold-changes in transcript abundance observed upon over-production of SlyA (Table 2). SlyA-binding to the *hlyE-C0299* intergenic region has been reported previously [20]. For the EMSAs, biotin labelled intergenic DNA was prepared as described in the *Methods*. Labelled DNA was incubated with increasing concentrations of purified SlyA protein and protein-DNA complexes were separated by electrophoresis on native polyacrylamide gels. Lanes 1-8: 0, 1, 5, 10, 50, 100, 200, 500 nM SlyA. The locations of the free DNA (D) and the SlyA-DNA

545 complexes (C) are indicated. Note that binding at the *sgcX* upstream region was only
546 evident at the highest SlyA concentratrion tested and the complex (C) was located close to a
547 contaminating DNA species.
548

Figure 1

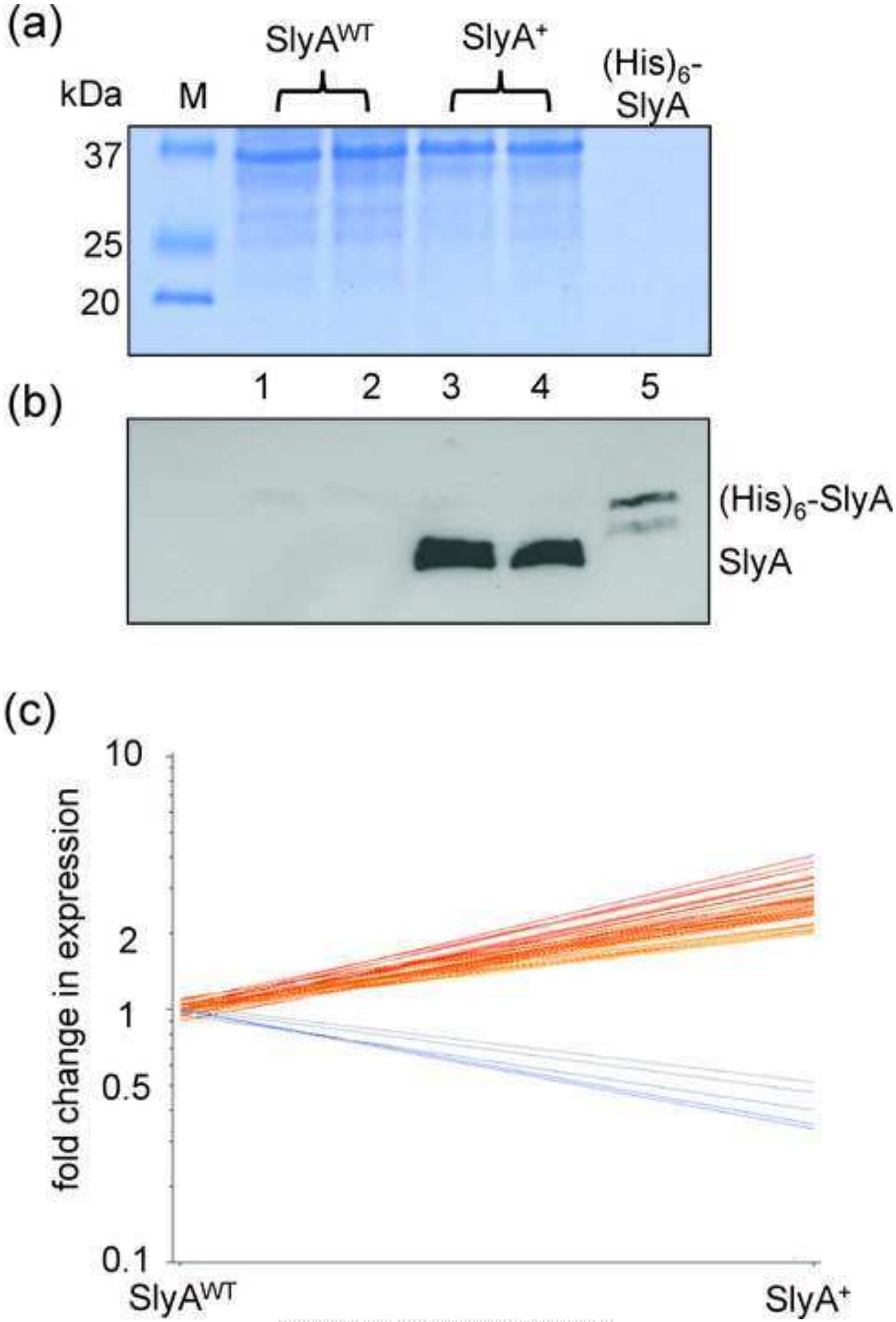

Figure 2

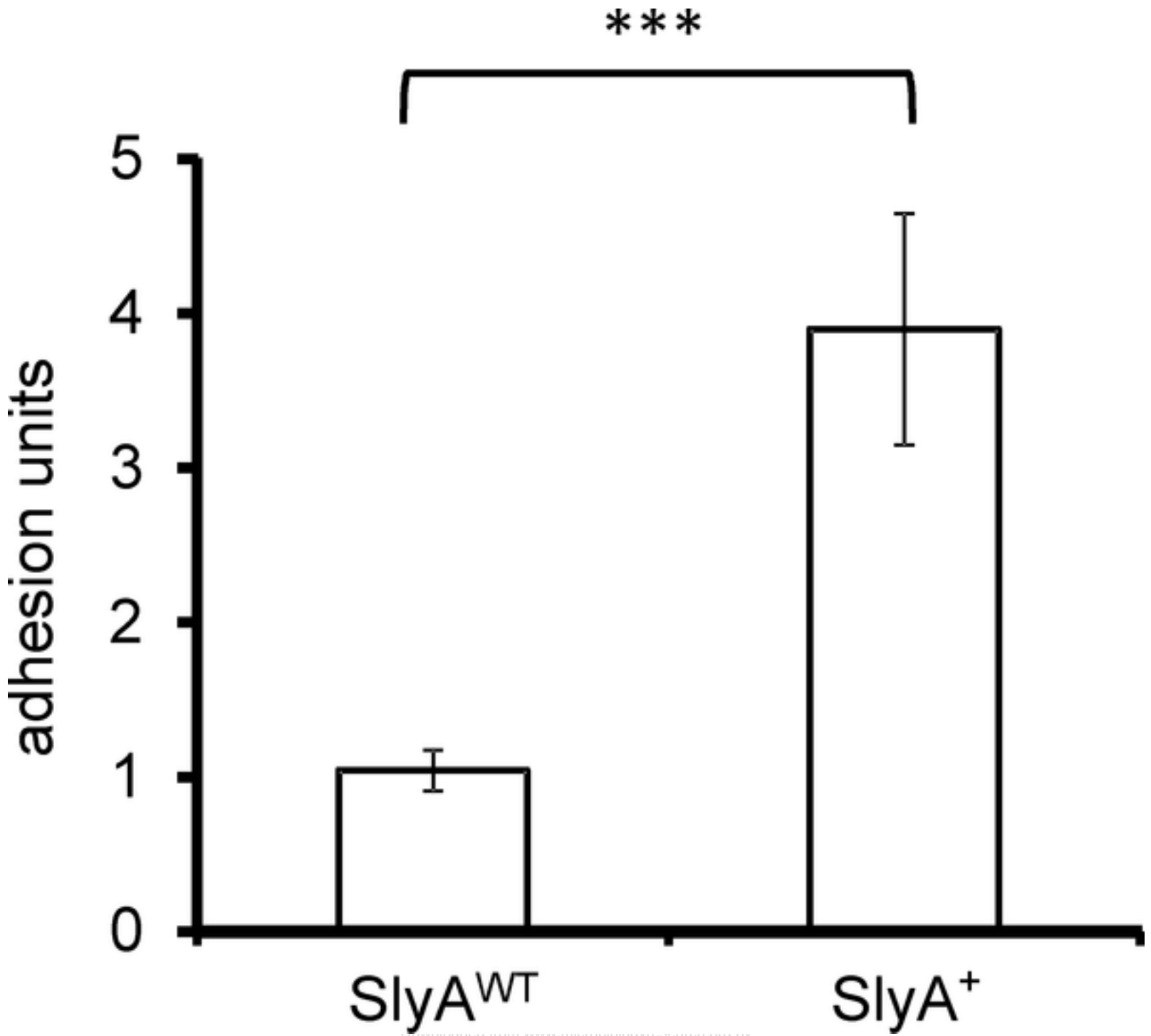

Figure 3