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

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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.

1 # Identification of new members of the *Escherichia coli* K-12

2 # MG1655 SlyA regulon

3

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

12

13 Abbreviations used: A_{600}; absorbance at 600 nm; Amp^R, ampicillin resistance; Btn, biotin;

14 Cm^R, chloramphenicol resistance; CRP, cyclic-AMP receptor protein; EMSA, electrophoretic

15 mobility shift assay; Kan^R, kanamycin resistance; OD_{600}; optical density at 600 nm; Tet^R,

16 tetracycline resistance; T^s, temperature sensitive

17

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

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20 Subject category: Regulation

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30   SlyA is a member of the MarR family of bacterial transcriptional regulators. Previously, SlyA

31   has been shown to directly regulate only two operons in *Escherichia coli* K-12 MG1655, *fimB*

32   and *hlyE* (*clyA*). In both cases SlyA activates gene expression by antagonizing repression

33   by the nucleoid associated protein H-NS. Here the transcript profiles of aerobic glucose-

34   limited steady-state chemostat cultures of *E. coli* K-12 MG1655, *slyA* mutant and *slyA* over-

35   expression strains are reported. The transcript profile of the *slyA* mutant was not significantly

36   different to that of the parent; however, that of the *slyA* expression strain was significantly

37   different from that of the vector control. Transcripts representing 27 operons were increased

38   in abundance, whereas 3 were decreased. Of the 30 differentially regulated operons, 24

39   have been previously associated with sites of H-NS binding, suggesting that antagonism of

40   H-NS repression is a common feature of SlyA-mediated transcription regulation. Direct

41   binding of SlyA to DNA located upstream of a selection of these targets permitted the

42   identification of new operons likely to be directly regulated by SlyA. Transcripts of four

43   operons coding for cryptic adhesins exhibited enhanced expression and this was consistent

44   with enhanced biofilm formation associated with the SlyA over-producing strain.

45

46   **INTRODUCTION**

47   The MarR family of transcription regulators are widespread throughout the Bacterial and Archeal

48   kingdoms [1]. MarR family members are homodimeric and bind to palindromic DNA sequences

49   within regulated promoters using a characteristic winged-helix-turn-helix DNA-binding domain [2].

50   These regulators repress gene expression by promoter occlusion (e.g. MarR; [3]), or activate gene

51   expression by stabilizing RNA polymerase-promoter DNA interactions (e.g. OhrR; [4]), or by

52   antagonizing the action of repressors (e.g. RovA; [5]). These activities of MarR proteins are inhibited

53   upon interaction with cognate signalling molecules, although for many members the natural ligand is

54   unknown [6].

55   The *Salmonella enterica* serovar Typhimurium LT2 SlyA protein is one of the best

56   characterized members of the MarR family. The *S. enterica* serovar Typhimurium *slyA* mutant is

57   attenuated for virulence, is hypersensitive to oxidative stress and is impaired for survival in

58   macrophages [7, 8]. A consensus DNA binding site has been proposed, TTAGCAAGCTAA [9, 10],

59   and proteomic and transcriptomic comparisons of parent and *slyA* mutant strains suggest that SlyA

60   can act as both a negative and positive regulator of gene expression, with significant overlap with

61   genes of the PhoPQ regulon involved in cell envelope function, virulence, resistance to anti-microbial

62   peptides and regulation of small RNAs [11-15]. *Salmonella enterica* serovar Typhimurium 14028s

63   SlyA has also been linked to the stringent response by binding ppGpp resulting in enhanced DNA-

64   binding [16, 17]. The expression of many SlyA-regulated genes is subject to H-NS-mediated silencing

2

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

101   air-dried. To quantify the extent of staining, 200 µl ethanol : acetone (4 : 1) was added to each well, and

102   after incubating for 20 min the amount of biofilm was estimated by measuring A_{600}. Adhesion units

103   were calculated by dividing the A_{600} values for crystal violet-stained adhered cells by the OD_{600} values

104   for the corresponding planktonic cells.

105

106   **Creation of _E. coli_ K-12 MG1655 _slyA_ mutant.**   A PCR-amplified DNA fragment containing

107   the kanamycin cassette from pKD4 flanked by 40 bp DNA homologous to regions surrounding the

108   _slyA_ gene was synthesized using oligonucleotide primers TC7 and TC8 (Table 1).   The purified

109   (QiaQuick PCR cleanup, Qiagen) PCR product (5 µg) was used to transform _E. coli_ JRG6072 by

110   electroporation (Hybaid Cell Shock unit; 1800 V, 1 mm path length).   The _E. coli_ JRG6072 competent

111   cells were prepared from aerobic Luria Bertani broth batch cultures supplemented with ampicillin

112   (100 mg L^{-1}) at 30°C that had been induced to express the λred recombinase by addition of L-

113   arabinose (1 mM).   Kanamycin resistant mutants were selected on Luria Bertani agar plates

114   containing kanamycin (30 mg L^{-1}) at 37°C.   Mutation of the _slyA_ gene by insertion of the kanamycin

115   resistance cassette was confirmed by colony PCR using oligonucleotides TC9 and TC10.   The _slyA_

116   mutation was then transduced using bacteriophage P1 to _E. coli_ MG1655 [24].

117

118   **Transcriptional profiling.** Transcriptomic analyses were carried out as described by Rolfe _et al._

119   [28] using directly quenched samples from glucose-limited steady-state chemostat cultures (dilution

120   rate 0.2 h^{-1}) for the three _E. coli_ K-12 MG1655 strains; parent, _slyA_ mutant (JRG6457) and _slyA_ over-

121   producer (JRG6636).   RNA samples were labelled with Cy5 and the reference _E. coli_ K-12 MG1655

122   genomic DNA was labelled with Cy3.   In total, two independent biological replicates were performed

123   that were hybridised in duplicate (technical replicates) giving four replicates. After hybridization and

124   image capture, data were extracted from the raw image files using Agilent Feature Extraction v11.5

125   software and analyzed using GeneSpring v7.3.1.   Transcriptomic data have been deposited with

126   ArrayExpress (accession E-MTAB-5220).

127

128   **Purification of SlyA and Western blotting.**   Cultures (500 ml auto-induction medium

129   supplemented with ampicillin in 2 L conical flasks) of _E. coli_ BL21 (λDE3) pGS2469 were grown at

130   37°C for 24 h with shaking (250 rpm).   Bacteria were collected by centrifugation, the pellet was re-

131   suspended in 15 ml of breakage buffer (20 mM Tris-HCl, 500 mM NaCl, 5% v/v glycerol, pH 7.5),

132   the bacteria were lysed by two passages through a French pressure cell (16,000 psi) and the extract

133   clarified by centrifugation (27,000 _g_, 15 min, 4°C).   The His-tagged SlyA protein was isolated from

134   the cell-free extract by affinity chromatography on a HiTrap chelating column (1 ml) attached to an

135   AKTA prime according to the standard manufacturer's protocol (GE Healthcare).   The eluted SlyA

136   was buffer exchanged into 20 mM Tris-HCl, pH 7.4 containing 200 mM NaCl by repeated dilution

4

137 and Vivaspin 6 concentration (Sartorius Stedim Biotech). The protein was judged to be >90% pure by

138 Coomassie blue-stained SDS-PAGE and protein concentration was estimated by the BioRad protein

139 reagent protocol [29]. SlyA protein was detected by Western blotting after separation of polypeptides

140 by SDS-PAGE and electrophoretic transfer (100 V for 1 h; transfer buffer: 5.8 g L^{-1} Tris, 2.9 g L^{-1}

141 glycine, 20% v/v methanol, 0.037% w/v SDS) to Hybond-C Extra nitrocellulose membranes (GE

142 Healthcare). The membranes were soaked in a blocking solution, which contained 5% w/v dried

143 skimmed milk in PBS (10 mM phosphate buffer, 137 mM NaCl, 2.7 mM KCl, pH 7.4) and 0.05% v/v

144 Tween 20, for 16 h at 4°C. The blocking solution was then removed and the membranes washed in

145 PBS containing 0.05% v/v Tween 20 before exposure to a 1:1000 dilution of the SlyA antibody

146 (raised in rabbit and provided by Prof. Ian Blomfield, University of Kent) in blocking solution for 1 h

147 at room temperature. After four washes with PBS containing 0.05% v/v Tween 20, the membranes

148 were soaked in blocking solution containing anti-rabbit secondary antibody provided in the Pierce

149 ECL Western Blotting kit and the presence of SlyA was visualized according to the manfacturer's

150 standard protocol (Thermo Scientific).

151

152 **Electrophoretic mobility shift assays (EMSA).** The LightShift Chemiluminescent EMSA kit

153 (Thermo Scientific) was used according the manufacturer's intstructions. Biotin-labelled DNA of

154 target promoter regions was amplified from genomic DNA using the appropriate oligonucleotide

155 primer pairs (Table 1). The core binding assays (20 µl) contained: 2 µl 10x binding buffer (100 mM

156 Tris-HCl, pH 7.5, containing 500 mM KCl and 10 mM dithiothreitol and 1 µg poly (dI•dC). The

157 DNA concentration was ~1 nM and the concentration of SlyA ranged from 0 to 500 nM as indicated.

158 Mixtures were incubated at 25°C for 30 min before separation of SlyA-DNA complexes by native gel

159 electrophoresis, followed by transfer to Hybond-N+ nylon membranes, UV-crosslinking for 60 s at

160 120 mJ cm^{-2} and detection of labelled DNA using the Nucleic Acid Detection Module (Thermo

161 Scientific).

162

163 **RESULTS AND DISCUSSION**

164 **Enhanced expression of *slyA* in *E. coli* K-12 MG1655 results in altered abundance of**

165 **transcripts from 30 operons**

166 Previous work has shown that SlyA directly activates the expression of two genes in *E. coli* K-12

167 (*hlyE* and *fimB*) by antagonising H-NS repression [20, 21, 23]. However, in *S. enterica* serovar

168 Typhimurium the influence of SlyA is much more extensive, with at least 31 regulated genes resulting

169 in hypersensitivity to reactive oxygen species and attenuation in infection models [7, 8, 12]. The

170 initial aim of this work was to apply transcript profiling to determine the extent of the *E. coli* K-12

171 MG1655 SlyA regulon by comparison of steady-state glucose-limited aerobic chemostat cultures of

172 wild-type and *slyA* mutant strains. Comparison of transcript profiles of wild-type and *slyA* mutant

5

173 cultures grown at a dilution rate of 0.5 h^{-1} (equivalent to a doubling time of 1.4 h) revealed no
174 significant ($\geq$2-fold; $p \leq 0.05$) changes in transcript abundance. Because SlyA translation might be
175 enhanced at low growth rates, due to its unusual UUG start codon [21], steady-state cultures at
176 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
177 h, respectively). However, once again, when the transcript profiles and growth characteristics of the
178 wild-type and *slyA* mutant cultures were compared no significant differences were detected. These
179 observations indicated that, under the conditions tested, deletion of the *slyA* gene had no significant
180 effect on gene expression in *E. coli* K-12 MG1655, even at low growth rates.

181   Anti-SlyA serum was used to determine whether SlyA was detectable in *E. coli* K-12
182 MG1655 cells grown in glucose-limited chemostats at a dilution rate of 0.2 h^{-1}. In accordance with
183 the transcript profiling, SlyA was not detected (Fig. 1). This suggests that the expression of SlyA is
184 regulated and switched on under conditions other than those imposed here; for example, SlyA protein
185 has been detected by Western blotting extracts from *E. coli* batch cultures grown in minimal medium
186 with glycerol as the carbon and energy source [21]. To overcome any regulatory barrier to identifying
187 genes potentially controlled by SlyA, a plasmid (pGS2468) to express *slyA* under the control of its
188 own promoter was constructed. Western blotting showed that SlyA protein was now readily
189 detectable in the transformed *E. coli* K-12 MG1655 cells grown in glucose-limited chemostats at a
190 dilution rate of 0.2 h^{-1} (Fig. 1). The growth characteristics of the vector control and the *slyA*
191 expression strains were essentially the same, with similar yields (1.4 ±0.2 g cell dry weight per litre)
192 and no detectable glucose or over-metabolites in the culture supernatants. Therefore, the transcript
193 profiling experiments were carried out with these strains grown in aerobic glucose-limited chemostats
194 at a dilution rate of 0.2 h^{-1}. The transcript profile of the SlyA over-production strain was significantly
195 different from that of the vector control. Transcripts representing 27 operons were increased in
196 abundance and 3 were decreased (Table 2; Fig. 1c). The transcripts exhibiting decreased abundance
197 were: the *sgc* operon (*sgcXBCQAER*), which encodes a phosphotransferase system for the uptake of
198 an unknown sugar; *fecIR* the membrane-bound sensor (FecR) that receives signals from the outer
199 membrane ferric citrate uptake receptor (FecA) for transmission to FecI (σ^{19}), which activates
200 transcription of the *fecABCDE* operon encoding components of a cytoplasmic membrane bound ferric
201 citrate uptake system; and *yecH*, which encodes a predicted protein of unknown function (Table 2)
202 [30, 31].

203   Amongst the up-regulated transcripts were the previously identified SlyA-regulated gene *hlyE*
204 and *slyA* itself (Table 2). The latter was not surprising as the *slyA* gene was present in multi-copy, but
205 despite this the *slyA* transcript only increased ~3-fold in abundance, yet the SlyA protein level
206 increased from being undetectable in the control to a level equivalent to ~1.5 μM in the cytoplasm
207 (based on the dry weight of *E. coli* being 3 x 10^{-13} g with an aqueous volume of 7 x 10^{-13} ml per cell;
208 [32]). The relatively low level of induction of the *slyA* transcript when present in multi-copy but

6

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

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

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

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

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

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

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

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

279

280 **SlyA over-production is associated with enhanced biofilm formation**
281 The observation that SlyA increased transcription of four cryptic fimbrial-like adhesins suggested that
282 the SlyA over-producing strain should exhibit enhanced biofilm production. This was tested using

8

283   static cultures of *E. coli* K-12 MG1655 transformed with pET28a (control) or the *slyA* expression

284   plasmid pGS2468 in conditions that mirrored the transcript profiling experiment. The data showed a

285   4-fold increase in biofilm formation when *slyA* was over-expressed, consistent with the transcript

286   profiling data (Fig. 2).

287

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

289   The changes in transcript profiles that were observed upon over-production of SlyA could result from

290   direct interaction of SlyA with the promoter regions of the corresponding genes or indirectly via

291   SlyA-regulated factors. For example, one of the genes up-regulated upon SlyA over-production, *leuO*,

292   encodes a transcriptional regulator that, like SlyA, operates by antagonising H-NS regulation [44, 45].

293   Fourteen (52%) of the 27 transcripts that were increased in abundance when SlyA was expressed in *E.*

294   *coli* K-12 MG1655 were associated with LeuO binding sites identified in the SELEX-chip study of

295   Shimada *et al.* [44]. This strong correlation could arise from; (1) the positive effect SlyA has on the

296   expression of *leuO* resulting in an increase in expression of the entire LeuO regulon, i.e. indirect

297   regulation by SlyA; or (2) SlyA and LeuO have overlapping regulons as a consequence of the fact

298   they both operate by antagonising H-NS-mediated repression. To further investigate the extent of

299   direct SlyA-mediated regulation in *E. coli* K-12 MG1655 binding of SlyA to ten promoter regions

300   was examined by electrophoretic mobility shift assays (EMSA).

301   Amongst the transcripts differentially regulated by over-production of SlyA there were three

302   arranged as divergent operons (Fig. 3). Binding of SlyA at the *hlyE-C0299* intergenic region was

303   shown previously (Fig. 3) [20]. Two other examples of SlyA-activated divergent operons (*slyA-*

304   *ydhIJK* and *ssuE-B-elfADCG-ycbUVF*) were shown to bind SlyA in EMSA (Fig. 3). Furthermore,

305   SlyA bound at the *casA*, *fecIR*, *gspCDEF*, *leuO*, *mdtM* and *paaA-K* promoters (Fig. 3). The $K_{d(app)}$

306   values for SlyA binding at these promoters were similar at ~50-100 nM. These experiments indicate

307   that these operons are likely to be directly regulated by SlyA. The *sgcXBCQ-sgcAER* genes are

308   separated by a sRNA *ryjB* on the opposite DNA strand (Fig. 3). It is suggested that the *sgcXBCQAER*

309   is a single transcription unit, but there is no high quality evidence to support this suggestion [46].

310   Therefore, both the region upstream of *sgcX* and the intergenic region between *sgcQ* and *sgcA* were

311   used in EMSAs with the SlyA protein. No specific interaction was observed with the region upstream

312   of *sgcA* but interaction, albeit weaker than that observed for the promoter regions analyzed above, was

313   observed when the DNA upstream of *sgcX* was tested (Fig. 3). These observations suggest that

314   *sgcXBCQAER* is a single SlyA-repressed transcription unit.

315   The EMSA experiments indicate that SlyA binds P*ssu*, P*cas*, P*paa*, P*elf,* P*leuO* and P*gsp*, all

316   of which are promoter regions of genes or operons proposed to be part of the LeuO regulon (Table 2).

317   This suggests that, perhaps because of the similarity in their mode of action, i.e. antagonizing H-NS

318   repression, the SlyA and LeuO regulons substantially overlap such that upon activation by their

319   respective signals a similar transcriptional response is elicited.

9

320   A consensus binding site (TTAGCAAGCTAA) for the *Salmonella enterica* serovar
321   Typhimurium LT2 SlyA protein was proposed based on footprinting and a limited SELEX analysis
322   [10]. This consensus was further analyzed by site-directed mutagenesis, which suggested the
323   consensus sequence $TTAN_6TAA$ [9]. All the DNA fragments that bound *E. coli* SlyA in EMSAs (Fig.
324   3) possessed DNA sequences similar to the previously proposed consensus sequences (Table 3). Site-
325   directed replacement amino acid residues of *Salmonella enterica* serovar Typhimurium LT2 SlyA
326   identified 16 locations that impaired DNA-binding [9], all these amino acids are conserved in the *E.*
327   *coli* SlyA protein, suggesting that these closely related proteins recognize similar DNA motifs.
328

Concluding remarks

330   SlyA proteins have been shown to play important roles in regulating gene expression in a wide range
331   of bacterial species. The most common mechanism for SlyA-mediated activation of gene expression
332   is through antagonism of H-NS repression. Here transcript profiling has revealed the breadth of the
333   SlyA regulon (directly and indirectly regulated genes) in *E. coli* K-12 MG1655 cultures grown under
334   precisely controlled conditions such that any potential effects associated with changes in growth
335   rate/growth phase could not confound the interpretation of the data obtained. Enhanced transcript
336   abundance for several cryptic fimbrial operons in a SlyA over-producing strain and an over-
337   representation of H-NS repressed genes were consistent with the current model of SlyA-mediated
338   gene activation. The SlyA protein was shown to bind at 9 intergenic regions controlling the
339   expression of 11 operons, thus expanding the number of known directly SlyA-regulated genes in *E.*
340   *coli* MG1655 from 2 to 13.
341

CONFLICTS OF INTEREST

352   The authors declare no conflicts of interest.
353

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485

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	TAAAGCCGCATAATATCTTAGCAAGCTAATTATAAGGAGATTACACGTCTTGAGCGATT; creation of *slyA* mutant	This work
TC8	TTGCGTGTGGTCAGGTTACTGACCACACGCCCCCTTCATTCATATGAATATCCTCCTTAG; 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

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
ssuEADCB	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-agaBCDI	2.5	predicted galactosamine-transport and metabolism (cryptic)		✓	
ydhYV-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		
ycjMN-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
sgcXBCQAER	0.5	predicted sugar transport and metabolism		IV
*fec**IR***	0.4	transcription regulation of ferric citrate transport		IV

490   [a]The fold-change data shown are for the first gene in the operon except where indicated by bold typeface; note
491   that all genes in the operons followed the same pattern of regulation.
492   [b]Fold-change (≥2-fold, $p \leq 0.05$) is the product of dividing the transcript abundance for the *slyA* over-expression
493   cultures by that for the control cultures.
494   [c]Gene functions as assigned in Ecocyc.org [46].
495   [d]Genes associated with H-NS binding were identified from Grainger *et al.* [49] (G), Kahramanoglou *et al.* [50] (K)
496   and Oshima *et al.* [51] (O).
497   [e]Genes located up- or down-stream of a LeuO binding site identified by Shimada *et al.* [44].
498   [f]Genes the overlap with genomic islands in *E. coli* K-12 MG1655 identified by GIST and/or IslandViewer (IV) [34].

17

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
P*casA*	**TTA**TTG**A**AT**TAA**	100 bp upstream of *casA*
P*ssuE/elfA*	**TCA**GG**A**TG**ATAA**	8 bp upstream of *elfA*
P*gspC*	**TTA**TATTAG**TAA**	79 bp upstream of *gspA*
P*paaA*	**TTA**AATC**G**C**GAA**	239 bp upstream of *paaA*
	TTATA**AAA**A**TAG**	136 bp upstream of *paaA*
	TTACTT**A**AC**TA**T	81 bp upstream of *paaA*
P*sgcX*	**TTA**TGCT**G**GG**AA**	336 bp upstream of *sgcX*
	TTTCA**A**CCA**TAA**	188 bp upstream of *sgcX*
P*fecI*	**TTA**G**AA**A**A**C**AA**	109 bp upstream of *fecI*
P*slyA*	**TTA**GCAAGC**TAA**	22 bp upstream of *slyA*
	TTAGATTAA**TAA**	161 bp upstream of *slyA*
P*leuO*	**TTA**ATGCAT**TAA**	305 bp upstream of *leuO*
	TTAAAT**A**TA**TAA**	297 bp upstream of *leuO*
P*mdtM*	**TATA**CACCT**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

18

Figure legends

509

510 **FIG. 1.** Changes in the transcript profile of *E. coli* K-12 MG1655 over-producing SlyA. (a)

511 Transformation of *E. coli* K-12 MG1655 with a multi-copy plasmid expressing *slyA* under the

512 control of its own promoter results in detectable SlyA protein in lysed cell suspensions from

513 aerobic steady-state glucose-limited chemostat cultures. The upper panel shows the

514 Coomassie blue-stained SDS-polyacrylamide gel and the lower panel shows the relevant

515 region of a Western blot prepared with the same samples and loadings developed with SlyA

516 antiserum. The gels were loaded as follows: Lane M, SDS-PAGE markers (sizes, kDa, are

517 indicated); lanes 1 and 2, extracts from independent cultures of *E. coli* K-12 MG1655

518 transformed with the vector pET28a (SlyA^{WT}); lanes 3 and 4, extracts from independent

519 cultures of *E. coli* K-12 MG1655 transformed with the expression plasmid pGS2468 (SlyA$^+$);

520 lane 5, purified (His)$_6$-SlyA (~10 ng protein loaded). (b) Western blot corresponding to the

521 gel shown in (a). The locations of SlyA and purified (His)$_6$-SlyA are indicated. (c) Graphical

522 representation of the changes in transcript abundance occurring upon over-production of

523 SlyA in *E. coli* K-12 MG1655. Comparison of the fold-changes in transcript abundance of

524 aerobic steady-state glucose-limited chemostat cultures of *E. coli* K-12 MG1655 transformed

525 with either the pET28a (SlyA^{WT}) or pGS2468 (SlyA$^+$). Each line represents a gene that

526 exhibits a $\geq$2-fold change in transcript abundance ($p \leq 0.05$) from two biological and two

527 technical replicates i.e. four measurements.

528

529 **FIG. 2.** Biofilm formation by *E. coli* K-12 MG1655 is enhanced by elevated *slyA* expression.

530 Wells containing M9 minimal medium with 20% w/v glucose as a carbon source were

531 seeded with 1:10 inocula of overnight cultures and incubated at 37°C for 16 h. The OD$_{600}$ of

532 the planktonic bacteria was measured before a biofilm assay was carried out. Values shown

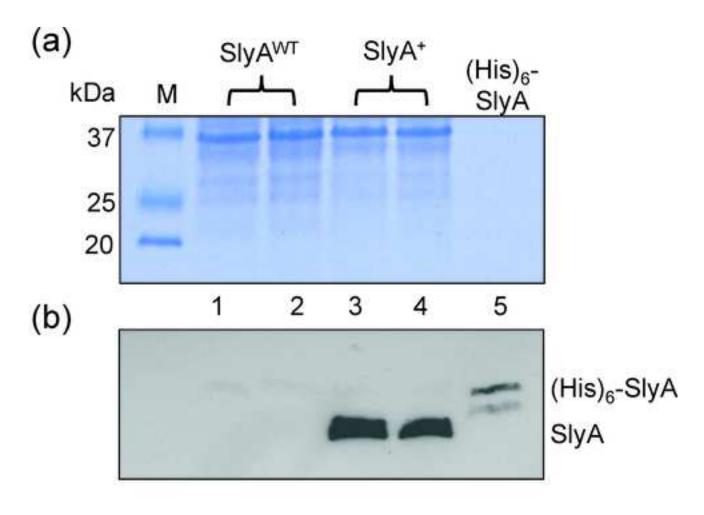533 are the mean and standard deviation (n = 12) *** denotes $p \leq 0.00001$ in a Student's *t*-test.
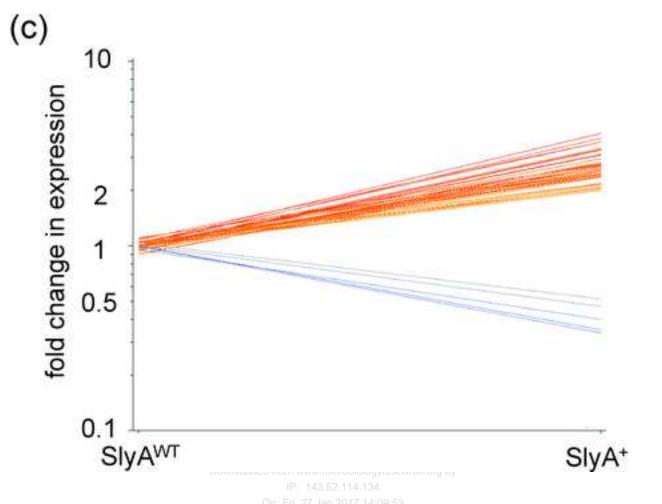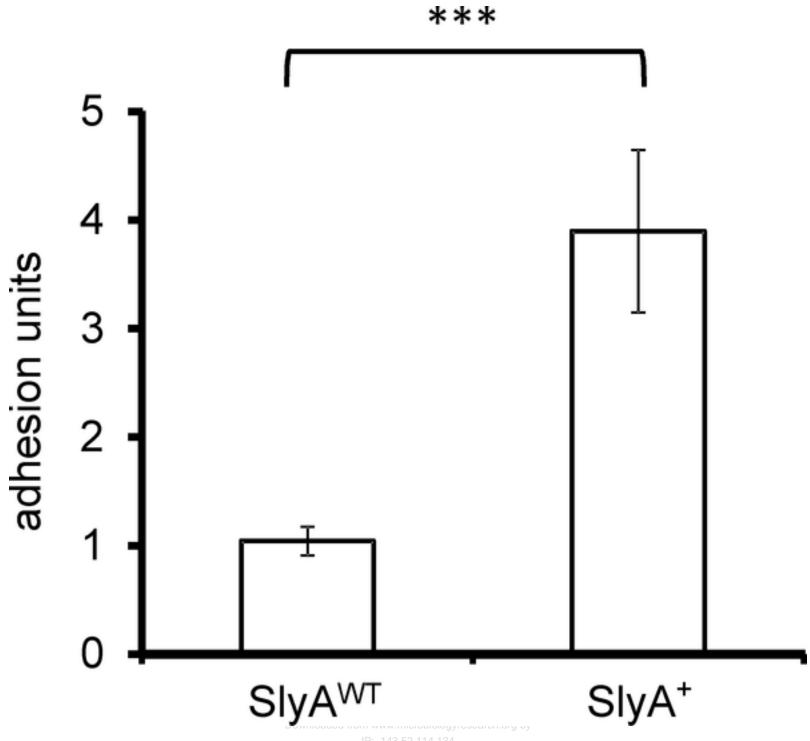
534

535 **FIG. 3.** Electrophoretic mobility shift assays show specific binding of SlyA to intergenic

536 regions of selected operons. The dashed lines in the diagrams on the left indicate the DNA

537 regions used in the EMSA shown on the right. The arrows indicate the polarity of the genes

538 (names above the arrows). The numbers below the arrows representing genes are the fold-

539 changes in transcript abundance observed upon over-production of SlyA (Table 2). SlyA-

540 binding to the *hlyE-C0299* intergenic region has been reported previously [20]. For the

541 EMSAs, biotin labelled intergenic DNA was prepared as described in the *Methods*. Labelled

542 DNA was incubated with increasing concentrations of purified SlyA protein and protein-DNA

543 complexes were separated by electrophoresis on native polyacrylamide gels. Lanes 1-8: 0, 1,

544 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

20

Figure 1

Figure 2

Figure 3