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ORIGINAL ARTICLE

Generating a *SlyA* mutation in *E. coli* and Validating its Effect on Microbial Response to Environmental Stress Factors

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ABSTRACT

Background: *In the human host, environments encountered by bacteria can vary widely. Bacteria experience stress from their initial moment of contact with the host. Therefore, adaptation and survival of the bacteria hinge on their ability to probe the environment and respond appropriately. Capsule expression is one of the responses to stress. In E. coli K5 capsule expression is temperature regulated and SlyA was proven to be involved in E. coli capsule gene expression.* ***Methodology:*** *Using HA1 cells as a model, cells were grown under different environmental conditions using a set of chemical substances and the b-galactosidase activity was measured and used as an indicator of capsule expression. pGEM-T easy vector and a series of PCR were used in cloning and generating a tet-insertional mutation of the slyA gene.* ***Results:*** *Maximum activity of capsule expression was under anaerobic conditions, then microaerophilic with least activity been obtained aerobically. Maximum capsule expression was obtained in presence of 0.4M sucrose or NaCl and 2mM $MgSO_4$ at pH 7.5. In all conditions capsule expression was increased ~2.4 fold in the presence of multicopy slyA plasmid; an effect that disappeared with tet:: slyA.* ***Conclusion:*** *Intestinal environmental cues in terms of anaerobiosis, relatively high osmolarity in addition to the slightly alkaline pH, all can contribute to up-regulating capsule gene expression in E. coli intestinal flora. Many aspects of clinical host-pathogen interactions involve stress responses and adaptive responses allow different pathogenic bacteria to resist host defences. Capsule expression was significantly increased in the presence of a multicopy slyA*

INTRODUCTION

The 16.7-kDa SlyA protein is a member of the MarR/SlyA family of transcription regulators. It was originally described in *Salmonella enterica* serovar Typhimurium as a gene product that can induce -in *Escherichia coli* K-12- a hemolytic phenotype (**S**almo**ly**sin) [1]. Transcriptome and proteome analyses identified a large number of genes as SlyA-regulated genes in many bacteria where it is required for virulence. In *S. enterica* [2, 3]. SlyA controls the function of *Salmonella* pathogenicity islands (SPIs) by directly binding to the *ssrA* promoter, a sensor kinase of the two-component regulatory system (TCS) [4, 5].

One of the best-characterized TCSs in bacteria is the PhoP-PhoQ system in which PhoQ acts as the sensor kinase and PhoP acts as the response regulator. When bacteria are induced to grow under certain conditions, the sensor kinase PhoQ autophosphorylates, then it transphosphorylates the response regulator PhoP [6, 7] that, in turn binds to its target promoters [8] stimulating transcription of PhoP-activated genes [9]. Analysis of the transcriptome of the *S. typhimurium slyA* mutant revealed that many *slyA*-dependent genes are also controlled by the magnesium-sensing PhoP/PhoQ regulatory system [10].

SlyA regulates expression of tens of genes required for virulence and resistance to antimicrobial peptides in *S. enterica* serovar Typhimurium [11]. A SlyA homologue of Yersinia spp., named RovA (Regulator of virulence A), regulates expression of *inv* encoding a surface protein important for microbial invasion in response to a range of environmental signals [12].

In *E. coli* K-12 the haemolytic activity involves the release of the bacterial protein HlyE (ClyA or SheA) via outer membrane vesicles. The expression of *hly*E responds to specific environmental conditions, such as anaerobiosis or glucose starvation [13]. *hly*E regulation is driven by native transcriptional factors including SlyA and H–NS, or by foreign transcriptional factors as SlyA and HlyX, from *Salmonella enterica* serovar Typhimurium and *Actinobacillus pleuropneumoniae*, respectively [2, 14].

E. coli inhabits human large intestine as one of the most predominant colonic microflora. However, some *E. coli* strains are likely to cause extra intestinal infections e.g. *E. coli* K1 and K5. During systemic bacterial spread osmolarity has been reported as one of the environmental cues regulating virulence gene expression in many bacteria. Within the intestinal lumen, enteric pathogens encounter membrane-active antimicrobial peptides [15], bile salts, free fatty acids, enhanced osmolarity, and changing oxygen tensions [16].

Myself and co-workers presented the first evidence of a role for the transcriptional regulator SlyA in the regulation of transcription of the *Escherichia coli* K5 capsule gene cluster [17,18]. Capsule production is an important contributor to microbial pathogenicity through evading the host's immune response. We demonstrated the dependence of transcription on the functional interplay between H-NS and SlyA using a combination of reporter gene fusions, DNase I footprinting, and electrophoretic mobility shift assays, the dependence of transcription on the functional interplay between H-NS and SlyA. We also showed that the *E. coli slyA* gene expression is temperature-regulated, positively autoregulated, and independent of H-NS. In *E. coli* K12 SlyA also regulates genes involved in responses to acid and heat stress and a variety of metabolic functions [19].

In this paper, I aimed at studying the effect of some environmental cues, namely, anaerobiosis, pH and osmolarity changes on the *E. coli* K5 capsule gene expression in HA1 cells [17, 18] and validating the effects- if any- using a generated insertional mutation in the *E. coli SlyA* gene.

METHODOLOGY

Bacterial Strains, plasmids, growth conditions and chemicals:

Unless otherwise stated, all growth media were purchased from (Oxoid), chemicals from (AnalaR) and antibiotics from (Sigma). Strains were routinely grown in sterilised Luria-Bertani medium (LB). Bacteria were routinely grown in LB medium at 37°C, as indicated, and supplemented with antibiotics obtained from (Sigma) as appropriate at the following concentrations: 100 ug/ml ampicillin and 50 ug/ml tetracycline. pGEM^{R} -T easy vector system (Promega-UK) was used for generating the insertional *slyA* mutation [20].

Plasmid pACYC-*SlyA* pACYC184 containing slyA coding region and promoter[19] was used as a positive control. For screening of recombinants using insertional inactivation of the *lacZ*` cassette, solid media were supplemented with 2µl ml^{-1} X-gal (2% (v/v) solution in dimethylformamide) and 1µl ml^{-1} IPTG (100mM solution in water-filter sterilised) (Bioline). Strain HA1 (with single copy F1/*lacZ*/λRS45-lacZ) was transformed with the plasmids used and grown at 37°C.

Growth under different oxygen conditions:

Brain heart infusion (BHI) broth was used, aerobic conditions were established by inoculating 10ml BHI with HA1 with shaking in at least 50ml universal. Growth under micro-aerophilic and anaerobic conditions was produced by inoculating warm universals filled with BHI to minimise the oxygen content within the culture vessel and using Anaerocult^{R} (Merck 1.16275) and AnaeroGen (Oxoid) sachets respectively in gas jars.

Growth under different pH conditions:

The effect of pH on *E. coli* capsule expression was tested in HA1 using the method reported in White-Ziegler *et al.*,[21]; 20ml of double strength BHI (Merk 1.10493) was added to 13.33ml of the appropriate 300mM buffer. 1M NaCl or HCl were used to obtain the correct pH value and the total volume was then made up to 40ml using distilled water. The resultant BHI medium was filter-sterilised before use. The pH values used were 5.5, 6.5, 7.5, and 8.5. The pH values were obtained using 300mM MES, MOPS and TAPS respectively. Cells were grown in the pH-adjusted BHI for 14 hours at 37^{o}C with shaking and then re-grown in the same conditions till mid-exponential phase.

Growth under different Mg^{2+} concentrations:

The effect of Mg^{2+} on capsule expression was tested in HA1 using the method reported above [21] with the modification of using M9 minimal salt medium (pH 7.4) and supplemented with 0.1mM $CaCl_2$, 2mM MgSO4, Thiamine (10mg ml^{-}), glucose (4mg ml^{-1}), and appropriate amino acids (50mg ml^{-1}). 20ml M9 minimal medium with $MgSO_4$ to obtain final concentrations of (0.05, 0.1, 0.5, 1, 2, 5 and 10mM) in a total volume made up to 40ml using distilled water). The resultant minimal medium was filter-sterilised before use.

Growth under different osmotic conditions:

HA1 cells were grown on M9 medium supplemented with sucrose (0.1, 0.2, 0.3 and 0.4M) as well as on BHI (0.2, 0.4, 0.6 and 0.8M).

β-galactosidase assays: Overnight cultures grown at 37°C were diluted 1:100 into fresh, prewarmed LB plus antibiotics, and grown to an OD600 of ~0.4–0.6. Assays were performed as described [22]. Assays were performed in triplicate and repeated at least four times.

DNA manipulations. All DNA cloning procedures were performed as described by Sambrook et al. [23]

Disruption of the E. coli SlyA gene

Two pairs of primers were used in 3 separate PCR reactions aiming at inserting a *HindIII* restriction site about the middle of *SlyA* gene of the *E. coli* strain (HA1) [Fig. 1a]. P1 and P2 primers carried a 5` *XbaI* and *HindII* restriction sites respectively (underlined); SlyA F1 (P1): 5`- **TCT AGA** ATG AAA TTG GAA TCG CC-3` and SlyA R1 (P2): 5`- AAG CT T AAC CCT TTT TCT T CC-3`. Using these primers should produce a 258bp fragment. The other primer pair, P3 and P4 had the following sequence; SlyA F2: (P3) 5`- AAG CTT AAG AAA AAG GGT TAA CC-3` and SlyA R2 (P4) 5`- TCT AGA CCC AAA GGC CTG TAA CTC-3` and should produce when used a 246bp fragment. The DNA template for PCR reactions 1&2 was the *E. coli* K12 *SlyA* gene. Then a third PCR reaction using the external primers P1 and P4 was carried out to amplify a 504bp *SlyA* fragment with a unique central *Hind*III site. The DNA template for the

third PCR reaction was a mixture of products of PCR reactions 1 and 2. That 504bp *SlyA XbaI* fragment was then cloned into the pGEM^{R} -T easy vector system (Promega-UK) following the manufacturer's recommendations [20]. The cloned *SlyA* gene was then disrupted by the insertion of a 2.7kb *Hind*III - *Hind*III tetracycline resistance cassette (kindly supplied by Haly-pBL2-H).

Transformation:

Plasmid DNA was extracted using the alkaline lysis method described by Birnhoim and Doly [24]. *E. coli* cells were made competent using calcium chloride. Plasmid DNA (0.1-1mg in water) was mixed with (100ml of fresh or 200ml of competent cells stored at -80^{o} C) and placed on ice for 30 minutes. The cells were then heat shocked by incubation at 42°C for 90-120 seconds and then back to ice for 2 more minutes [17].

Polymerase chain reaction:

PCR was performed in a 25µl reaction volume containing 1µl (50 ng) of extracted DNA, 1µl of each pair of primers 12.5µl of 2x Taq premix Mastermix (Sigma) and 9.5µl sterile double-distilled water. The PCR program involved an initial denaturation step at 95°C for 5 min followed by 35 cycles of a denaturation step at 95°C for 30 seconds, a primer annealing step at 54- 60°C for 30 seconds, an extension step at 72°C for 30 seconds, and a final step at 72°C for 10 min. In each reaction, a positive and a negative control were included as appropriate. The negative control contained all the reagents without template DNA. All PCR products were visualyzed by agarose gel electrophoresis stained with 0.5µg/ml ethidium bromide, and photographed under a UV transilluminator by using a Digital Kodak Science 120 system [17].

RESULTS

Generation of an insertional *slyA* mutant *E. coli* HA1:

As shown in Figure 1 (a and b); 3 sequential PCR reactions produced 2 *Xba*III-*Hind*III *slyA* gene fragments from *E. coli*. These 2 fragments were then mixed and used as a template for the third PCR that finally produced a *slyA* gene with a centrally inserted *Hind*III site. This final product was then successfully cloned into the pGEM-T easy vector. The inserted *Hind*III site was then used to generate an insertional *slyA* mutation using a 2.7kb *Hind*III- *Hind*III *tet* cassette as shown in figure 2. The resulting 6.2kb cloned molecule was then transformed into the competent HA1 cells that was then used to test effect of multiple copy *slyA* mutation on capsule gene expression and growth under some environmental stresses. In all cases a pACYC-*SlyA* transformed HA1 served as a positive control and β-galactosidase assays were carried out in triplicates and repeated at least 4 times to evaluate the activity of the *E. coli* capsule gene expression

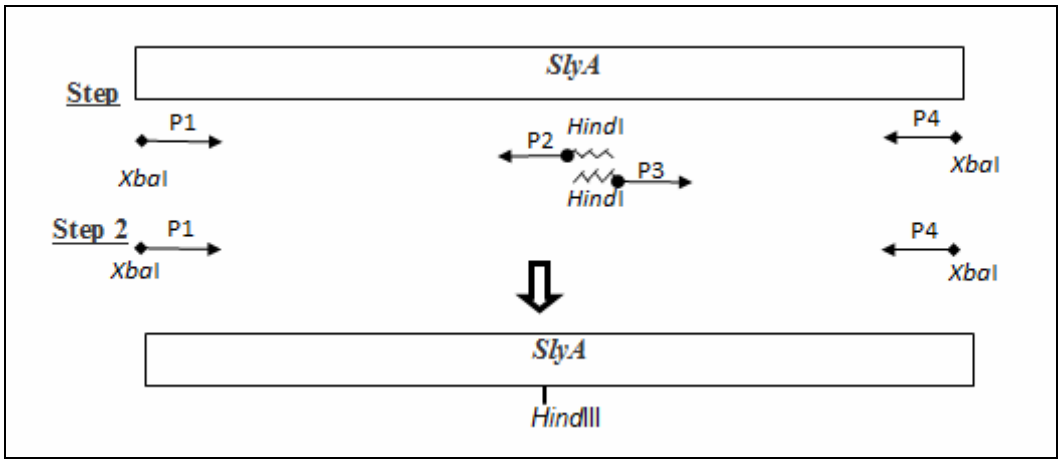

Fig. 1a: Insertion of a *Hind*III site in the middle of *SlyA*

The experiment was carried out in two steps; Step (1) involved 2 PCR reactions the first using primers P1&P2 while primers P3&P4 were used for the second PCR reaction [P2&P3 had *Hind*III sites at their overlapping ends]. In Step 2 a mixture of both PCR products from step (1) was used as a template of a third PCR using primers P1&P4; this step generated a *SlyA* with a *Hind*III unique central site.

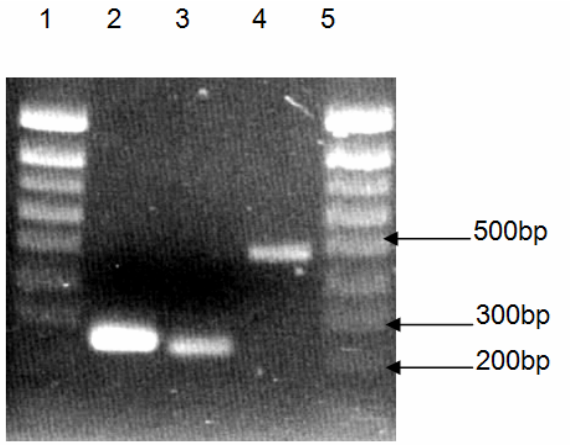

Fig. 1b: PCR amplification of *SlyA* fragments
An Ethidium bromide stained gel electrophoresis showing PCR products of steps 1&2; The gel was loaded as follows: lanes 1&5; 1kb DNA size marker with arrows pointing to fragment sizes of 500, 300, and 200bp. Lane 2; 258bp PCR product using primers P1&P2. Lane 3 shows a 246bp PCR product using primers P3&P4. Lane 4 shows a 504bp PCR product using primers P1&P4 and using a mixture of PCR products in lanes 2&3 as a template.

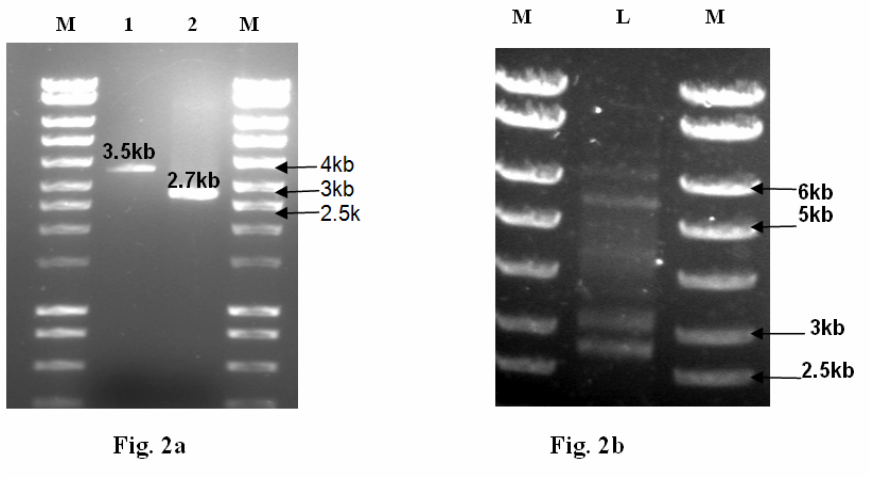

Fig. 2: Generating an insertion mutation of the *SlyA* gene
Fig 2a: An Ethidium bromide stained gel electrophoresis showing the 3.5 kb (pGEM-T easy vector into which the 504bp *slyA* PCR generated fragment) in lane 1; lane 2 has the *Hind*III PCR generated 2.7kb *tet* cassette. Lanes marked M have the 1kb DNA size marker.
Fig 2b: The gel shows (in lane L) the products of ligation reaction of the 2 bands in lanes 1 and 2 of Fig 1a; The expected desired ligation produced a 6.2kb band. Lanes marked M have the 1kb DNA size marker.

The effect of some environmental factors on *the E. coli* capsule gene expression:

The effect of Oxygen concentration:

The main habitat for *E. coli* in human body is the anaerobic colonic environment. A question was addressed about a possible role of intestinal anaerobiosis in over-expression of *E. coli* capsules that may help in intestinal colonisation. Trying to investigate the effect of oxygen availability on region 1 promoter activity HA1 cells were grown on brain-heart infusion broth (BHI) under aerobic, microaerophilic, and anaerobic conditions at 37°C and β-galactosidase activity of these cells were measured. It was found that there were significant differences in the β-galactosidase activities in each of the three tested conditions (P value <0.05%) with maximum activity of region 1 was under anaerobic conditions, then microaerophilic with least activity been obtained aerobically (Table 1) suggesting a role for anaerobiosis as an environmental cue stimulating capsule gene expression.

The effect of osmolarity:

HA1 cells were grown on minimal medium supplemented with varying sucrose concentrations (range 0.1, 0.2, 0.3 and 0.4M) and also on BHI supplemented with NaCl in a range of concentrations (0.2, 0.4, 0.6, and 0.8M) at 37°C till mid-exponential

phase. When the β-galactosidase activity of these cells was measured, a direct proportional relationship between the increase in osmolarity and region 1 promoter activity in HA1 with maximum expression at 0.4M sucrose. Using NaCl maximum expression was obtained also with 0.4M NaCl that was significantly higher than that with 0.6 and 0.8M (Table 1).

The effect of Magnesium availability

When the β-galactosidase activity of HA1 cells was measured. It was found that the presence of Mg^{2+} significantly increased region 1 promoter activity up to a certain limit after which the activity dropped again. The optimal level was found with 2mM $MgSO_4$ and was not significantly different from that with 5mM $MgSO_4$ (Table 1).

Table 1: b- galactosidase activity of test strains under different environmental conditions

Tested environmental stress factor	**b- Galactosidase activity in Miller units**			
	Test strain	**HA1**	**HA1p*slyA***	**HA1p*tet::slyA***
Oxygen concentration				
Aerobic		78.4	184.1	78.1
Microaerophilic		130.3*	307.5	129.98
Anaerobic		139.8	335.52	140.2
I. Osmotic changes				
i). Using Sucrose				
M9		40.1	94.24	40
0.1M		42.4	100	41.9
0.2M		43.9	104.16	44
0.3M		46.4	109.9	46.6
0.4M		53.4*	126.5	52.9
ii). Using NaCl				
BHI		106.8	252	106.3
0.2M		115.8	272.1	116.1
0.4M		116.5*	274.94	116.4
0.6M		96	230.4	95.7
0.8M		70.4	166.14	70
II. Magnesium ion concentration				
0.5mM		120.1	283.2	121
1mM		124.5	295	125.2
2mM		206.6	487.5	207
5mM		212.7*	499.8	213.3
10mM		100	240.3	99.6
III. The effect of pH				
pH5.5		75.3	177.7	75
pH6.5		80.7	189.64	80.4
pH7.5		127*	299.72	126.8
pH8.5		116.6	393.17	116.1

* Represents the maximum activity obtained with each growth condition with HA1 wild type, HA1 transformed with the high copy plasmids carrying *slyA* (HA1pslyA) and its *tet* insertional mutant form (HA1*tet::slyA)*.

The effect of pH changes

When HA1 was grown on brain heart infusion broth supplemented with 2mM $MgSO_4$ buffered to have final pH ranges of 5.5, 6.5, 7.5 and 8.5. The β-galactosidase activity assays of the test strains revealed a direct proportional increase in region 1 promoter activity with increase in pH value with a peak of region 1 promoter activity at pH 7.5 (P value <0.05) (Table 1).

The effect of slyA: Capsule expression was significantly increased in the presence of a multicopy *slyA* compared to the wild type HA1 under all studied growth conditions (~2.4-fold), an effect that was nullified by the *tet* induced mutation of the gene.

DISCUSSION

In the human host, environments encountered by bacteria can vary widely, for example, from the external skin to mucosal surfaces of the intestine or the airways, or deeper tissues and the blood stream. Bacteria experience stress from their initial moment of contact with the host. Therefore, adaptation and survival of the bacteria hinge on their ability to probe the environment and respond appropriately [25].

HA1 cells were used to investigate the role of some environmental factors on region 1 group 2 capsule gene expression. Data obtained with growth in different oxygen availability conditions revealed an increase in region 1 promoter activity under microaerophilic and anaerobic conditions suggesting that anaerobiosis may be one of the environmental cues sensed by *E. coli* (the normal habitat is the microaerophilic environment of the intestine) stimulating capsule expression. This finding suggests that upregulation of capsule genes may be required at some stage during intestinal mucosal colonisation. These results were similar to those reported by Favre-Bonte *et al.,* [26] who reported that capsulated strains of *Klebsiella pneumoniae* persist in the intestinal tract of mice at 10^8 CFU/g of feces compared to 10^4 CFU/g in the isogenic capsule deficient mutant. Additionally, mixed infection experiments revealed that capsule mutant strain was rapidly outcompeted by the wild type. The ArcB/ArcA system in *E. coli*, appear to sense cellular respiration [27].

The suggested role of anaerobiosis would address the possibility of involvement of FNR (**F**umarate **N**itrate **R**eductase), a transcriptional activator that binds at the target promoters and found to be involved in regulating virulence gene expression in other pathogenic bacteria for example, *L.monocytogenes S. gordonii* and *S. pyogenes* (GAS). The identification of a putative FNR consensus sequence 5' region 1 promoter transcription start point may augment this hypothesis [28].

The idea that magnesium limitation might induces the expression of some virulence factors in *E. coli* as well as other gram-negative bacteria was based on the assumption that low intracellular Mg^{2+} especially when linked to the low pH, conditions that simulate the intravacular conditions inside macrophages. These conditions were reported in *Salmonellae* to be under the control of the PhoPQ system that regulates many *in vivo* induced virulence genes [29]. To investigate the role of such a system (if any) in region 1 K5 capsule expression, HA1 cells were grown on minimal medium (on which cells were grown with variable magnesium concentration). β-galactosidase activity assays showed a peak of capsule gene expression at Mg^{2+} of 0.2M in HA1. Considering that the osmolarity of human blood is in the order of 150mM based on magnesium ions [30], these data would suggest a possible role for Mg^{2+} during systemic *E. coli* infection when bacteria need to over-express capsule as a virulence factor to protect itself against specific as well as non-specific host immune mechanisms.

Looking further at the effect of osmolarity on region 1 capsule gene expression, Sucrose dependant data revealed a proportionate increase in β-Galactosidase activity with maximum expression obtained at 0.4M sucrose (the highest tested concentration). Data obtained with NaCl maximum expression was also obtained with 0.4M NaCl. Osmolarity with the intestinal environment is 300mM based on NaCl, this may suggest over expression of capsule genes for intestinal colonisation. Adaptation to hyperosmolarity is particularly required for respiratory pathogens [29].

Additionally, the effect of pH has been looked at and data obtained with HA1 showed that maximum expression of region 1 genes was obtained at slightly alkaline conditions (pH 7.5). The biological relevance of acid stress to pathogenesis has been clearly shown in many organisms [31]

It can be concluded that intestinal environmental cues in terms of anaerobiosis, relatively high osmolarity in addition to the slightly alkaline pH can all contribute to up-regulating capsule gene expression in *E. coli* intestinal flora. Many aspects of clinical host-pathogen interactions involve stress responses and adaptive responses allow different pathogenic bacteria to resist host defences [32] and capsule expression was significantly increased in the presence of a multicopy *slyA*

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