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TITLE: Characterization of Putative RpoS-regulated Outermembrane Proteins Slp and OmpX in *Escherichia coli* O157:H7

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ABSTRACT

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E. coli O157:H7 is a food pathogen with exceptional tolerance to certain stressful growth conditions. RpoS is a master regulator for bacterial stress adaptation and in particular, for acid resistance genes. The role of outermembrane proteins (Omps) in bacterial acid tolerance has not been investigated extensively. Omp profiles of strain EDL933 (FRIK 47) and its isogenetic derivative *rpoS* mutant were compared. Omps down-regulated in the *rpoS* mutant include Slp and OmpX. Putative RpoS-dependent promoter consensus sequences were identified in both *slp* and *ompX*. The expression levels of *slp* and *ompX* in the *rpoS* mutant were reduced compared to FRIK 47. Inactivation of *slp* reduced bacterial survival in pH3 at a similar level to the *rpoS* mutant. Our findings suggest that RpoS regulates production of two Omps, Slp and OmpX, and Slp is a key component of RpoS-dependent acid resistance system in *E. coli* O157:H7.

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Honors Thesis

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ABSTRACT

E. coli O157:H7 is a food pathogen with exceptional tolerance to certain stressful growth conditions. RpoS, or σ^s, is known to be a master regulator for bacterial stress adaptation. In particular, RpoS–regulated genes involved in acid resistance are strongly induced during growth in low pH. The role of outermembrane proteins (Omps) in bacterial acid tolerance has not been investigated extensively. In this study, we initially compared the Omp profiles of strain EDL933 and its isogenic derivative *rpoS* mutant. The data showed that Omps down-regulated in the *rpoS* mutant during stationary phase include Slp and OmpX. Putative RpoS-dependent promoter consensus sequences were identified in both *slp* and *ompX*. Consistently, the expression levels of *slp* and *ompX* in the *rpoS* mutant were shown to be reduced as compared to those of the wild-type strain during early stationary growth. Inactivation of *slp* reduced bacterial survival in pH3 medium at a similar level to the *rpoS* mutant while the *ompX* mutant survived comparably to wild-type strain. Findings in this study suggest that RpoS regulates production of two Omps, Slp and OmpX, and Slp is a key component of RpoS-dependent acid resistance system in *E. coli* O157:H7.

INTRODUCTION

Enterohemorrhagic *Escherichia coli* (EHEC) is an important class of microorganisms in the food industry. Sporadic outbreaks of EHEC-contaminated food or water sources have led to numerous hospitalizations annually. Serotype O157:H7, the most prevalent in North America, causes serious illnesses including hemorrhagic colitis and hemolytic uremic syndrome. It has been hypothesized that the pathogenicity of *E. coli* O157:H7 may be attributed to its ability to withstand acidic conditions.

Optimal cellular adaptation including outermembrane protein (OMP) expression is one of the mechanisms through which stress from an acidic environment may be alleviated. OMPs consist of porins, adhesion proteins, lipoproteins, and secretory proteins amongst many others. The composition of OMPs is dependent on many regulatory pathways. One global regulator that has been reported to regulate the expression of many OMPs is the sigma factor σ^s (RpoS) subunit of RNA polymerase (1).

RpoS is highly conserved between species and plays a critical role in survival of the organism in various non-optimal conditions such as nutrient-limitation and extreme acidity. Dong and Schellhorn (2) reported that RpoS has a global effect on gene expression in *E. coli* O157:H7 strain EDL933. When they compared the gene expression levels of the wild-type versus an *rpoS* mutant, a significant number of genes were up- or down-regulated in the strains that lacked RpoS. Several OMP genes were among the 1,000 genes observed to be affected by the absence of *rpoS* (2).

Initially, we analyzed the OMP profile of *E. coli* O157:H7 strain EDL933 (FRIK 47) versus an isogenic *rpoS* mutant at stationary phase using SDS-PAGE. One band that was greatly decreased in the *rpoS* mutant was sequenced using MALDI-TOF spectrometry. The results revealed the presence of three proteins: Dps, Slp, and OmpX.

Extensive studies have shown Dps to be an RpoS-regulated acid tolerance gene. Choi et al. (3) reported reduced survivability of an O157:H7 *dps* null mutant as compared to the parent strain. This finding was

further confirmed by the work of Jeong et al. (4) which also identified an extended -10 region of *dps* where RpoS could regulate its expression.

Starvation lipoprotein (Slp) is a lipoprotein induced during carbon starvation and in stationary phase (5). It has been suggested that RpoS may be a regulator of *slp* expression. Furthermore, *slp* exists within the vicinity of a number of genes that are responsible for acid tolerance including the glutamate decarboxylase system, GAD (Figure 1), which has been shown to be regulated by RpoS (6). This region has been deemed an acid tolerance island. *Slp*, because of its location, is thus potentially acid tolerance related. *dctR*, a transcription factor gene which is located adjacent to *slp*, has been reported to be co-transcribed with *slp* in *E. coli* strain K12 (6).

OmpX is an adhesion protein. Vogt and Schulz (7) reported that the crystal structure of OmpX revealed that it may be involved in mechanisms of virulence. The location of *ompX* on the *E. coli* genome is near *dps* (Figure 2).

In this study, our objective was to determine the role of RpoS-regulated genes in acid tolerance of *E. coli* O157:H7. We analyzed the upstream promoter regions of *slp* and *ompX*. The presence of the RpoS-binding consensus sequence in both genes' upstream promoter region indicates that *slp* and *ompX* may be regulated by RpoS. RT-PCR and qPCR analysis revealed slightly decreased expression of both genes in an *rpoS* deletion mutant as compared to the wild-type (FRIK 47). Mutant strains containing interrupted genes of *slp* and *ompX* were generated. The *slp* mutant showed lower tolerance in acid than FRIK 47 while the *ompX* mutant did not seem to be affected by the acidic conditions. The results of this study suggest that a lipoprotein, Slp, is a key component of RpoS-dependent acid resistance system in *E. coli* O157:H7.

MATERIALS AND METHODS

Bacterial strains and culture conditions. We used *E. coli* O157:H7 strain EDL933 (FRIK 47) along with an *rpoS* deletion mutant and a *dps* deletion mutant (Table 1). In addition, two mutant strains were generated that contained null mutations in *slp* and *ompX*. Cells were routinely grown aerobically at 37°C in Luria-Bertani (LB) broth (1.0% Bacto tryptone, 0.5% yeast extract, 0.5% NaCl), by shaking at 150 rpm and on LB agar (15g L^{-1} agar). When appropriate, chloramphenicol (final concentration of 25 $\mu g \cdot ml^{-1}$, Cm) was added to the agar and broth solutions. Unless otherwise noted, strains were initially grown overnight (20-24 hours) in test tubes containing 2 ml LB broth, subcultured in 5-10 ml LB in a 250 ml flask, and incubated to the desired cell density. Cell density was monitored at OD_{600} using a spectrometer (Beckman Coulter, Fullerton, CA).

Outermembrane protein extraction and analysis. Initially, we analyzed the OMP profile of *E. coli* O157:H7 strain EDL933 (FRIK 47) versus an isogenic *rpoS* mutant at stationary phase using SDS-PAGE. Overnight cultures were inoculated in a dilution of 1:100 into fresh LB media and incubated at 37°C with shaking. After 6 hours, cells were collected, pelleted, and immediately stored at -20°C. Cells were washed with and resuspended in 20 mM Na_3PO_4. Pulsating sonication was used to lyse cells. Cellular debris (excluding inner- and outermembrane proteins) was pelleted out of solution. The supernatant was filtered with 0.22 µm filter and 2% Sarkosyl (Invitrogen) was added. The solution was incubated for at least 1 hour

to solubilize inner-membrane proteins. Ultracentrifugation at 90,000 rpm was used to pellet out the remaining insoluble fraction of outermembrane proteins. The protein pellet was resuspended in 2X SDS and concentrations were measured using the BSA assay. 40 ug of protein was analyzed by SDS-PAGE. Coomassie blue was used to stain the gel overnight and acetic acid was used to destain the gel. One band that was greatly decreased in the *rpoS* mutant was identified using MALDI-TOF spectrometry.

RpoS-binding consensus sequence analysis of upstream promoter region. To identify a putative RpoS-dependent promoter of *slp* and *ompX* genes, 200 bp upstream sequences of those genes were obtained from the JCVI-CMR database. These regions were analyzed by aligning with RpoS-binding consensus sequences determined previously (3,8); the consensus sequence from position -13 reads 5'-KCTATACTTWWWWR-3' where K stands for G or T, W stands for A or T, and R stands for A or G. The -35 region is known to be variable in RpoS-regulated genes.

Construction of *slp* and *ompX* mutants. Inactivation of *slp* and *ompX* in FRIK 47 was performed by inserting the suicide vector, pKnock containing a chloramphenicol resistance gene and the internal fragment of each gene, by homologous recombination. Initially, a 291 bp internal fragment of *slp* and a 268 bp region of *ompX* were amplified by polymerase chain reaction (PCR) (Table 2). The products were analyzed for the correct size on 1% agarose gel then purified using GENECLEAN TURBO kit (BP bio). An end conversion enzyme was used to cut the AAA overhang and phosphorylate the ends of the PCR products. The PCR fragments subsequently were blunt-ligated to the SmaI site of the pKnock vector. Ligations were performed at a ratio of 1:10. Transformation into competent cells *E. coli* S17-λpir was done with 45 seconds of heat shock at 40°C, and cells were plated onto LB+Cm agar plates overnight. A transformant with pKnock harboring the PCR fragment was selected by colony PCR using a primer pair from flanking regions of SmaI site of the vector (see below). S17-λpir containing the correct recombinant pKnock-Cm plasmid was conjugated with FRIK 47. Selection on tellurite and chloramphenicol identified mutant cells in which the recombination pKnock-Cm had integrated into the chromosome by single crossover homologous recombination. Gene disruption was confirmed by PCR (see below).

Colony PCR analysis. *E. coli* S17 and FRIK 47 colonies grown on LB agar plates overnight at 37°C were used as PCR template for confirmation of transformation and conjugation, respectively. Confirmation reactions contained screen-pKnock-F and screen-pKnock-R primers (Table 2). Confirmation of the final mutant was done using *slp*-confirm-F and screen-pKnock-F for *slp* and *ompX*-confirm-F and screen-pKnock-R for *ompX*. PCR conditions included a 5-min soak at 94°C and 30 cycles of 94°C for 30 sec, 54°C for 30 sec, and 72°C for 3 min, followed by a 5-min extension at 72°C. Presence of a PCR product at expected size when checked by 1% agarose gel electrophoresis confirmed the mutant clone while the false positive mutants gave negative PCR reults.

RNA preparations. To analyze the level of *dps*, *slp*, and *ompX* transcription, an overnight culture in LB medium was diluted 100 fold and grown at 37°C with shaking (150 rpm) for 6 hours. Cells were collected and immediately pelleted and stored at –20°C. Total RNA was extracted with Trizol reagent (Sigma), following the manufacturer's protocol. Final RNA pellet was resuspended with 100 µl nuclease-free distilled H_2O. RNA concentration was determined by absorbance at 260 nm. Duplicate measurements were performed for each RNA sample and an average value was obtained as a final concentration. Total RNA was digested with the RQ1 RNase-free DNase reagent (Promega) based on the manufacturer's

protocol. For all RNA extracts, DNA contamination was assessed by performing a control PCR reaction alongside RT-PCR analysis.

RT-PCR analysis. Reverse-transcriptase PCR (RT-PCR) was carried out using an AccessQuick one-step RT-PCR system (Promega) according to the manufacturer's protocols. Primer pairs used for RT-PCR are in Table 2. cDNA synthesis was conducted at 52°C for 45 min. Subsequent PCR conditions included a 5-min soak at 94 °C and 20-30 cycles of 94 °C for 30 sec, 54 °C for 30 sec, and 72°C for 1 min, followed by a 5-min extension at 72 °C. The number of cycles varied according to the level of expression of the various mRNAs to ensure that the comparison was performed in the linear range of the amplification: 24 cycles for *dps*, *slp*, and *ompX*, and 18 cycles for 16S rRNA. 16S rRNA was used as the internal control to confirm that equal amounts of total RNA were used in each reaction. The RT-PCR reaction (25 µl) contained the following components: 300 µg total DNase-treated RNA, 10 pmol each of forward and reverse primer and 0.5 unit of reverse transcriptase. 10 µl of each RT-PCR product were analyzed by 1.5% agarose gel electrophoresis, and the intensity of the bands visualized by ethidium bromide staining was compared to assess the mRNA level of each gene.

cDNA synthesis. Total RNA derived from FRIK 47 and *rpoS* mutant were used to synthesize cDNA as follows. Strand cDNA using 800 ng of total RNA was synthesized with SuperScript™ II RT (Invitrogen) using 2.5 pmol of random hexamer primers (Fermentas) according to the manufacturer's protocol. The residual RNA was eliminated by an RNase treatment (Invitrogen). cDNA was column-purified using GENECLEAN TURBO (BP bio) and was diluted eight times with Nuclease-free water. 2.5 µl of the diluted cDNA was subsequently used in 25 µl qPCR reaction, which was carried out in triplicates with the SYBR Green master mix (Clontech) and iClycler® thermal cycler (BioRad).

qPCR analysis. qPCR was used to carry out quantitative analysis of RNA content. Total RNA was extracted as described above and the same primer pairs as RT-PCR analysis were used. Clontech system was used according to the manufacturer's protocols to prepare qPCR reactions. The following cycle condition was used for PCR reaction: 30 s at 94°C, 30 s at 54°C, and 30 s at 72°C for extension. *ompR* was used as the internal control. The qRT-PCR reaction (25 µl) contained the following components: 2.5 µl cDNA (see above), 10 pmol of each primer, and 12.5 µl of SYBR green master mix. Fold differences in the amount of *slp*, *ompX*, and *dps* mRNA (target) relative to the *ompR* mRNA (control) in *rpoS* mutant (mutant) as compared to wild-type strain (WT) were calculated using the following sets of equations.

$$\Delta Ct = Ct_{target} - Ct_{control}$$

$$\Delta(\Delta Ct) = \Delta Ct_{mutant} - \Delta Ct_{WT}$$

$$fold\ difference = 2^{-\Delta(\Delta Ct)}$$

Microscopic Examination. 10 µl of an overnight culture were heat-fixed and stained with crystal-violet dye. Microscopic examinations were made with an Olympus Light Microscope and pictures were captured with an attached camera.

Bioscreen Analysis of Growth and Survival. Overnight cultures grown in LB at 37°C with shaking were diluted to an OD_{600} of 1. Cells were pelleted (16.1 rcf, 1 min) and resuspended in fresh LB to obtain

an OD_{600}=2. The suspension was inoculated into 100-well plate with LB (for growth analysis) and LB acidified with 6N HCl to pH=3 (for acid survival analysis). Final OD_{600} was 0.5. OD_{600} of the cultures was monitored for 12 hours. Triplicates of each culture were averaged to obtain average values.

Acid Challenge Assay. Overnight cultures were diluted to OD_{600}=1. Cells were pelleted (4000 rpm, 1 min) and resuspended in fresh LB to obtain an OD_{600}=1. The suspension was inoculated into culture tubes in a 1:100 dilution with LB acidified with 6N HCl to pH=3. Tubes were incubated at 37°C with shaking and sampled periodically for 6 hours. Plate count method was used to detect viable cells.

RESULTS and DISCUSSION

SDS-PAGE analysis of OMPs of FRIK 47 and rpoS mutant

OMPs during early stationary phase from FRIK 47 and *rpoS* were compared using SDS-PAGE (Figure 3). A band between 15 and 20 kDa showed decreased expression in the *rpoS* mutant when compared to FRIK 47. OmpX is around 18 kDa and thus hypothesized to correspond to the reduced band. MALDI-TOF analysis (data not shown) identified 3 proteins: OmpX, Dps, and Slp.

Identifying Consensus Sequence for RpoS-binding in slp and ompX

Regulatory regions including ~200 bp upstream of the *slp* and *ompX* loci were analyzed for identification of the putative RpoS-dependent promoter in reference to the RpoS consensus sequence (8) and the previously determined RpoS promoter in the *dps* locus (3). The sequence from the -35 position to +1 position of the gene is as follows: TTGACA - (15 bp nonspecific sequence) - KCTATACTTWWWR, where K stands for G or T, W stands for A or T, and R stands for A or G. The presence of the characteristic extended -10 region of the RpoS consensus sequence (Figure 4) as well as overall consensus similarity were inspected by multiple sequence alignments of upstream sequences of *slp* and *ompX* and several known RpoS promoters of several other genes including *dps* (not shown). The predicted RpoS consensus sequences for *slp* and *ompX* are shown in Figure 4. Identification of the putative RpoS promoters in *slp* and *ompX* loci implies that RpoS directly regulates these genes as it does in *dps*. However, further genetic evidence is required to confirm this.

Expression of slp, ompX, and dps in FRIK 47 and rpoS mutant

To determine whether RpoS controls expression of *slp* and *ompX* during stationary phase growth, mRNA levels of the genes between wild-type strain and rpoS mutant were compared using semi-quantitative RT-PCR with RNA derived from stationary cells (OD_{600} of 2). Slight reduction in *slp* mRNA level was detected in the *rpoS* mutant as compared to FRIK 47 while both *ompX* and *dps* mRNA levels were indistinguishable between the two strains (Figure 5a). To determine the RpoS effect during early stationary phase, expression of the three genes were assessed by quantitative real-time RT-PCR. The expression level of *rpoS*/FRIK 47 was 0.49 for *dps*, 0.49 for *slp*, and 0.64 for *ompX* (Figure 5b). This result indicates that expression of *slp*, *ompX*, and *dps* is not entirely dependent on RpoS during stationary phase growth under high nutrient (LB) condition. It was also suggested that the RpoS effect on expression of RpoS-dependent genes such as *dps* varies and can be temporal in different growth phases and conditions (3). Different growth and stress conditions such as low pH and low nutrition may increase

cellular levels of RpoS and thus expression of RpoS-regulated genes. Characterization of these conditions eliciting RpoS-mediated *slp* and *ompX* up-regulation is subjected to further investigation.

Construction and phenotypic characterization of slp and ompX mutants

Amongst RpoS-regulated acid tolerance systems that have been extensively studied is the glutamate-dependent (GAD) system. This system confers bacteria intracellular pH homeostasis via proton-consumption during glutamate decarboxylation. Genes required for this system including decarboxylase genes and the anti-porter gene are encoded in the region named acid fitness island (AFI) (6). *slp* is the first gene encoded in the AFI and its role in either the GAD system or acid tolerance is not clearly defined. The involvement of *ompX* in acid tolerance is not known. To address whether *slp* and *ompX* is involved in acid tolerance in *E. coli* O157, *slp* and *ompX* were inactivated in this study.

Whether *slp* and the immediate downstream gene, *dctR*, are co-transcribed was checked by cross-over RT-PCR as shown in Figure 5a. The presence of a *slp-dctR* co-transcript in the total RNA derived from FRIK 47 was tested. The negative result using *slp*-F and *dctR*-R primers indicates that *slp* and *dctR* are transcribed independently. *dctR* encodes a putative transcriptional regulator of which function is not known. This result also supports that the *slp* mutation does not confer a polar effect on *dctR* and downstream *gad* genes which allows us to address *slp*-specific function. The *ompX* gene was also disrupted by the same approach used in *slp* inactivation and both mutants were initially characterized in cell morphology and growth rate.

As shown in Figure 7, microscopic slides of heat-fixed and stained *slp* mutant cells revealed indistinguishable cell morphology from that of FRIK 47. Interestingly, *ompX* mutant cells exhibited elongated morphology.

Comparison of growth rates by monitoring cell density for 12 hours revealed that *slp* mutation did not affect growth (Figure 8). The growth of the *slp* mutant, as well as the *rpoS* and *dps* mutants, was comparable to that of FRIK 47. In contrast, *ompX* mutant showed altered growth rate which was characterized by the lack of exponential growth for the first 2-4 hours. It is plausible that the altered growth rate is associated with its elongated cell shape but it is not clear what causes the phenotypic feature observed in the *ompX* mutant.

Characterization of slp and ompX inactivation on acid tolerance

Similar growth rates in standard LB growth condition (pH7) between FRIK 47, *slp* mutant, *rpoS* mutant enable direct comparison of acid tolerance phenotypes among those strains. Initially, growth rates of the four strains, FRIK 47, *slp*, *rpoS*, and *ompX*, in LB-acidified to pH3 was compared based on optical density over 12 hours (Figure 9). All strains were shown to be unable to grow exponentially in this condition which is consistent with a previous study (Mand et al., unpublished).

It has been previously shown that *E. coli* O157:H7 strain EDL933 can survive in pH3 for several hours, and this acid tolerance property requires RpoS function (Mand et al., unpublished data). To test if either Slp and OmpX plays a role in the RpoS-dependent acid tolerance in pH3, the survival of FRIK 47, *slp*, *rpoS*, and *ompX* after exposure to LB-acidified to pH3 after various times was examined (Figure 10). As expected, while FRIK 47 survived well up to 2-4 hours post exposure, the *rpoS* mutant was defective in survival. The *slp* mutant was also impaired in acid tolerance similar to the *rpoS* mutant. Both mutants

yielded low proportions of viable cells after 2 hours, 0.29 and 0.12, respectively, and undetectable cell counts occurred after 4 hours of acid exposure (Figure 10). In contrast, the *ompX* mutant showed an increase in viable count after 2 hours which suggests that the elongated cells septated into multiple cells. The survival proportion of FRIK 47 reached 0.25 at 4 hours while that of *ompX* mutant reached 0.23 at 6 hours. Altogether, acid tolerance phenotypic analysis strongly suggests that Slp is a key component of RpoS-dependent acid tolerance system in *E. coli* O157:H7. Whether Slp functions independently to the downstream GAD system remains to be elucidated.

CONCLUSION

Bacterial outer membrane proteins (OMPs) are involved in diverse functions including nutrition uptake, secretion, and virulence. It has been shown that subsets of OMPs are under the control of RpoS (1, 2). Despite multiple studies on diverse OMP functions, the role of OMPs in acid tolerance is not well understood. RpoS is a master regulator promoting bacterial adaptation to such stress conditions by coordinately regulating multitudes of functions. In particular, RpoS plays a substantial role in acid tolerance of *E. coli* O157:H7. The aim of this study was to identify OMPs that are involved in acid tolerance of this bacterium and to delineate the regulatory role of RpoS in OMP production. Slp and OmpX were initially identified as Omps down-regulated in the absence of RpoS. Promoter sequence analysis and expression data suggest that RpoS controls expression of both *slp* and *ompX* genes. Acid tolerance analysis revealed that both *rpoS* and *slp* mutants were equally defective in survival at pH 3 suggesting that the RpoS-Slp regulatory cascade is crucial to acid tolerance in *E. coli* O157:H7.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or Plasmid	Relevant genotype, phenotype, or characteristic(s) [a]	Reference/Source
E. coli		
FRIK 47	EDL933	Perna et al. 2001
rpoS	FRIK 2785/*rpoS*	Laboratory stock
dps	FRIK 2827/*dps::nptI*	Choi et al. 2000
ompX	FRIK 47/*ompX*::Cm	this study
slp	FRIK 47/*slp*::Cm	this study
S17-λpir	*recA, thi, pro, hsdR*-M+. RP4-2Tc::Mu Km::*Tn*7	Laboratory stock
S17-*ompX*	S17-λpir containing pKnock-*ompX*	this study
S17-*slp*	S17-λpir containing pKnock-*slp*	this study
Plasmids		
pKnock-Cm	Broad-host-range suicide vector; Cm^r RP4, *ori*T; *ori*R6K	Laboratory stock
pKnock-*slp*	Internal fragment of *slp* cloned into pKnock-Cm	this study
pKnock-*ompX*	Internal fragment of *ompX* cloned into pKnock-Cm	this study

[a]Abbreviations; Cm^r, Chloramphenicol resistance

Table 2. Primer pairs for PCR reactions

Primers	Primer Sequences (5′ → 3′)
dps-F	GGGACCACTCAAGTTATCAACAG
dps-R	GGTGTCGTCATCTTTCGCTTC
slp-F	GTGCACTCATACTCAGCCTTTC
slp-R	CAAGGAAGCCGCTTTGTCTG
ompX-F	CTCAGGGCCAAATGAACAAAATG
ompX-R	CTGGTGTCGTGTTTGTAGGTC
16S-F	ATACCTTTGCTCATTGACGTTACC
16S-R	CCAGTCATGAATCACAAAGTGGTAAG
ompR-F	GTGAGTTTGCGGTACTGAAGG
ompR-R	CTGGATCTTCTTCCACCATGC
Screen-pKnock-F	ACACAGGAACACTTAACGGCTG
Screen-pKnock-R	GAAGTGATCTTCCGTCACAGG
slp-confirm-F	TGATAAGGATAGTAACATGAACATGAC
ompX-confirm-F	GAAGTGATCTTCCGTCACAGG
dctR-R	CAACATTACGTCGATCTCTTCTTC

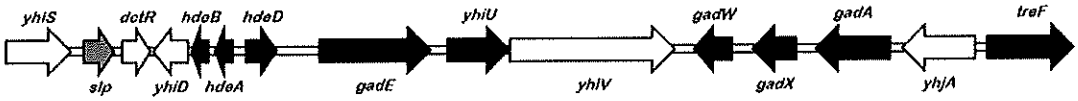


Figure 1. Acid tolerance island of E. coli O157:H7 genome. The 78 min region of the *E. coli* chromosome contains a number of acid stress-related tolerance genes. *Slp* is located within this acid tolerance island and is hypothesized to be involved in acid response. Pink arrow represents the *slp* gene, yellow arrow represents *dctR* gene, black arrows represent acid tolerance genes, and white errors represent genes not responsive during acid stress. Direction of the arrows represents direction of transcription.

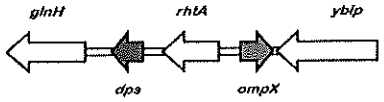


Figure 2. *dps* and *ompX* locus. *dps* and *ompX* are located around the 18 min region of the *E. coli* genome. Green arrow represents *dps*, orange arrow represents *ompX*, and white arrows represent other genes in the vicinity. Direction of the arrows represents direction of transcription.

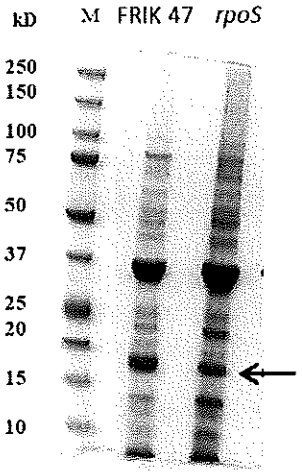
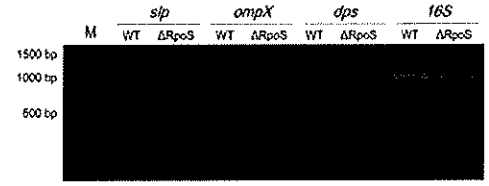


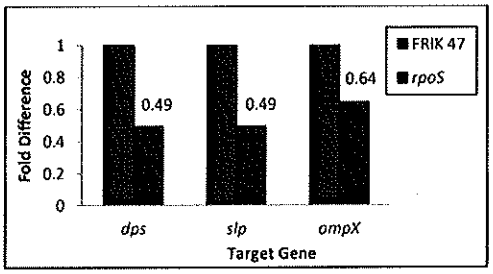
Figure 3. SDS-PAGE gel of FRIK 47 versus *rpoS* mutant. 40 µg of protein of each culture was analyzed using SDS-PAGE gel. A band at ~18 kDa (arrow) showed reduction in the *rpoS* mutant compared to FRIK 47. Three proteins were identified from this band: Slp, OmpX, and Dps.

	-35			-13	-10		+1
Consensus:	TTGACA	-----15 bp ---		KC	TATACT	TWHWH	R
dps	GA	ATAGCG	GAACACATAGCCGGT	GC	TATACT	TAATCTC	G
ompX	GT	TAGACA	TCAGAAATCGCGAAGAGTTTC	CC	ATTAAT	TATTAAAAC	T
slp	AG	ATGAAA	ACTATAAAGAAATATC	TC	TATTATG	GTTTT	A

Figure 4. Consensus sequence analysis of upstream promoter region of *dps*, *ompX*, and *slp*. An upstream consensus sequence for the binding site of RpoS was used to analyze the upstream regions of *ompX* and *slp*. *Dps*, a known RpoS-regulated gene, was analyzed for comparison. Highlighted letters represent those which are a direct match to the consensus sequence. In the sequence, K = G or T, W = A or T, R = A or G.

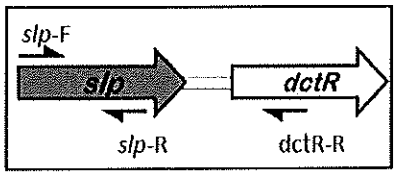


5a.

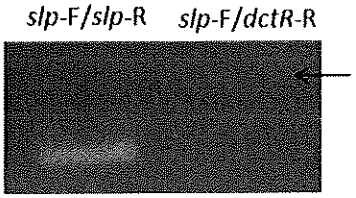


5b.

Figure 5a-b. PCR analysis of *dps*, *slp*, and *ompX* expression in FRIK 47 versus *rpoS* mutant. ON cultures of FRIK 47 and *rpoS* mutant cultures were sub-cultured in LB broth at 37°C. RNA was collected after cultures reached early stationary phase of OD=1 and OD=2. RT-PCR (a) of the OD=2 culture revealed slight differences between WT and mutant. The *16S* gene was a loading control. Lane M represents the marker lane. The decreases were quantified using qPCR analysis (b) of the OD=1 culture; fold differences of *dps*, *slp*, and *ompX* are displayed above each corresponding *rpoS* mutant bar. Samples were standardized using *ompR* as an internal control. RpoS regulation appears to be transient during early stationary phase possibly due to involvement of other regulators.



6a.



6b.

Figure 6a-b. Independent transcription of *slp* and *dctR*. Primers were designed for *slp* and *dctR* at the approximated locations as represented by the black half arrows (a). PCR reactions were analyzed on 1.5% agarose gel (b). There was no detectable evidence of co-transcription of *slp* and *dctR*. The black arrow indicates the approximate location of a positive band for *slp-dctR* PCR product.

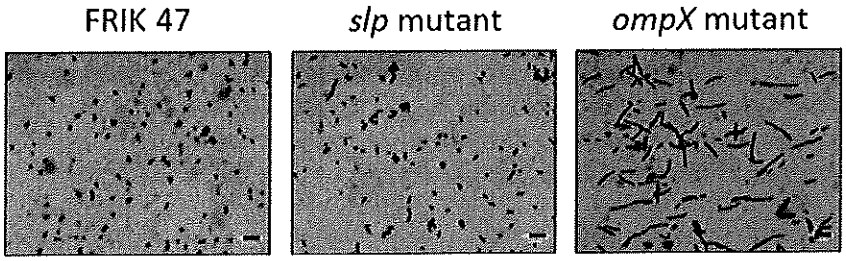


Figure 7. Cell morphology of strains. Overnight cultures were heat fixed and stained with crystal-violet dye. *slp* mutant and FRIK 47 (WT) exhibited similar phenotypes while ompX mutant cells appeared to be elongated. Size bar = 10 μm.

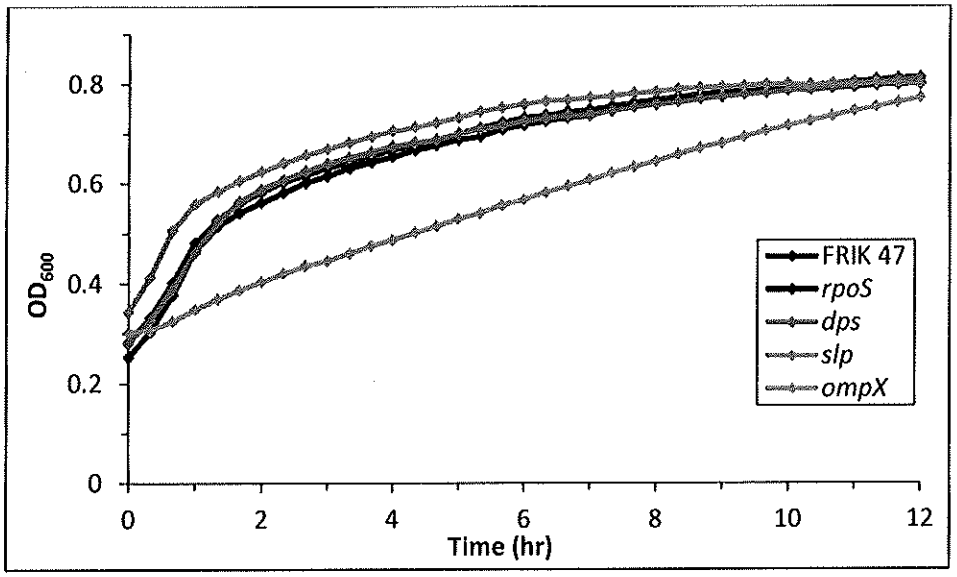


Figure 8. Growth rates of strains in LB-pH7. Overnight cultures were inoculated into fresh LB (pH7) in 100-well plate with a starting inoculum of OD_{600}=0.5. Cultures were monitored for 12 hours. *slp* mutant had similar growth pattern to FRIK 47, *rpoS* mutant, and *dps* mutant. *ompX* showed slower growth rate.

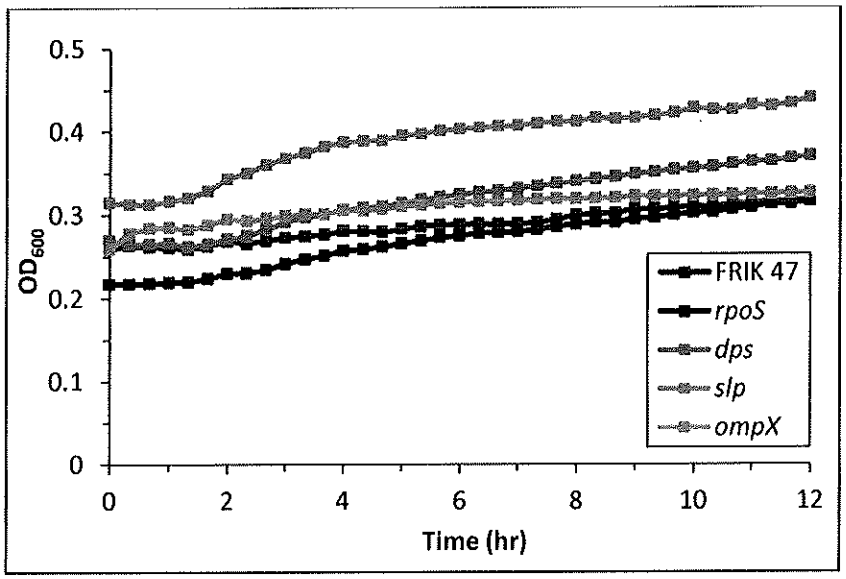


Figure 9. Growth rate in LB-pH3. Overnight cultures were inoculated into 100-well plate with LB acidified with 6N HCl to pH of 3 and a final OD=0.5. Cultures were monitored for 12 hours. All strains experienced suppressed growth at pH 3.

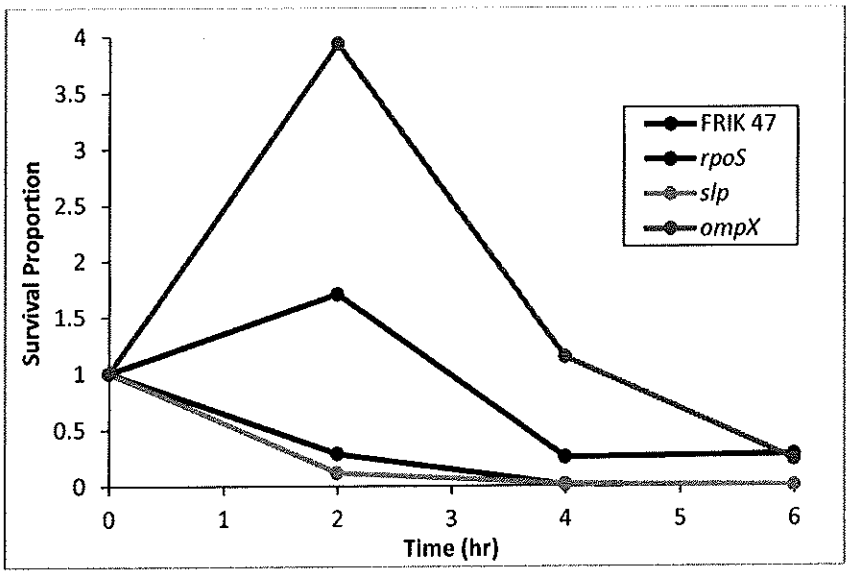


Figure 10. Acid tolerance of strains in LB-pH3. Overnight cultures were diluted 1:100 into culture tubes of LB acidified with 6N HCl to pH of 3. Cultures were incubated at 37°C with shaking and sampled periodically for 6 hours. Plate count analysis revealed *rpoS* and *slp* mutants to have least survivability to acid.

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