# GLOBAL EVALUATION OF THE *ESCHERICHIA COLI* PROTEOME DURING STATIONARY PHASE

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# ABSTRACT

*Escherichia coli* survives in both nutrient rich nutrient-limited environments. As such, understanding the gene and protein level activity that occurs during stationary phase is considered an important aspect of bacterial survival. *Escherichia coli* has been studied for decades providing substantial insight into gene expression profiles in exponential phase and recently, during adaptation to stationary phase. This led to the discovery of RpoS as a growth phase-dependent sigma factor. Further studies indicated that there are many genes that are expressed in an RpoS-independent but stationary phase-specific manner. However, proteins represent the functional molecules of the cell. Additionally, protein expression does not always correlate with the corresponding gene expression patterns. Therefore, to obtain an in depth understanding of the proteins that play a role in long-term growth in *E. coli*, TMT- (Tandem Mass Tags) based quantitative proteomic analysis was performed to identify proteins that are preferentially expressed during prolonged starvation. We identified proteins that were both positively and negatively regulated by RpoS during stationary phase, such as GadA and TnaA, respectively. RpoS levels peaked during early stationary phase and declined thereafter. However, proteins that were RpoS-dependent continued to increase during prolonged stationary phase. Additionally, we identified proteins that were expressed in an RpoS-independent manner during stationary phase. This suggests that protein expression during early stationary phase is distinct from prolonged stationary phase. Furthermore, RpoS-independent proteins may also play an important role during long-term growth.

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# 1.0 Chapter 1

# Introduction

## 1.1 *Escherichia coli*

*Escherichia coli* (*E. coli*) is a Gram-negative, rod-shaped, facultatively anaerobic bacterium. *E. coli* is a model organism from which many of the current concepts of molecular biology, such as DNA replication and gene expression, have been derived (Cooper, 2000). Most *E. coli* strains are harmless and live commensally within their primary habitat, human and animal gastrointestinal tracts (Kaper *et al.*, 2004). An unavoidable consequence of being a gut microbe is that it is regularly excreted into the external environment. Once *E. coli* is excreted from the host, it encounters rapid changes in conditions, such as nutrient deprivation, changes in temperature and pH, near UV-radiation and oxidative and osmotic stress. Despite being adapted to the anaerobic and nutrient-rich conditions within the gut, *E. coli* quickly adapts to its environmental changes to survive (Cooper, 2000).

Many of the cellular stresses in the external environment are encountered in the normal growth cycle in bacterial batch culture as a result of increasing cell density. The normal growth cycle in *E. coli* consists of 4 phases; lag phase, exponential phase, stationary phase and death phase. When *E. coli* is introduced into nutrient-rich media, like many other bacteria, the cells go through a phase where they must adjust to their new environment (Monod, 1949). This is known as the lag phase. Following this, the cells utilize the nutrients in their surrounding environment to grow and divide exponentially. This is referred to as the exponential phase (or log phase) during which cellular functions such as growth and cellular transport are prioritised (Reeve *et al.*, 1984). However, as those nutrients become depleted and waste products accumulate, *E. coli* cells enter

stationary phase (Monod, 1949). During this phase, cells are starved and the number of dying cells is equal to the number of cells that are growing and dividing. Therefore, there is no observable difference in the number of cells. Stationary phase is a physiological growth phase during which genes and proteins that enable the cell's resistance to stress increase in expression, therefore, increasing survival. These genes and proteins are expressed at a higher level compared to the cells growing in exponential phase. In batch culture, waste products continue to accumulate during this phase, which eventually causes the number of cells to decline exponentially (Tormo *et al.*, 2010). This is known as the death phase. Here, we focus on the stationary phase since these conditions most closely mimic the behavior of the bacterium under starvation and multiple stress conditions.

## 1.1 Stationary Phase

When the cells enter stationary phase, they maintain metabolic activity, but at a lower rate compared to when the cells are in the exponential phase (Schwalbach *et al.*, 2012). Starved cells have an overall reduced metabolic rate, but endogenous metabolism is maintained at a lower rate. As such, starved cells can maintain an energy source (ATP), which can be utilized for the transport of substances in and out of the cell (Siegele and Kolter, 1992). During stationary phase, bulk protein synthesis is turned off and the rate of turnover for proteins is increased, however, many proteins are specifically induced upon entry into stationary phase (Groat *et al.*, 1986).

In the external environment, bacteria rarely encounter conditions that are optimal for growth. Because of this, bacteria have adopted specific regulatory mechanisms that either allow them to remove the stressor, or sustain themselves in a physiological state that reduces the damaging effects of the stressor (Hengge-Aronis, 1999). For example, when exposed to a slightly acidic environment, microorganisms show increased resistance to acid stress (Park *et al.*, 2017). Glutamate-dependent acid stress response, which is mediated by proteins such as GadA and GadB,

consumes an intracellular proton in the decarboxylation reaction to maintain a less acidic intracellular pH (Mates *et al.*, 2007). In contrast to specific stress responses, alternative sigma factor RpoS is triggered upon entry into stationary phase in response to multiple stresses. RpoS controls up to 10% of *E. coli* genome upon entry into stationary phase (Weber *et al.*, 2005). These genes prepare *E. coli* for survival in starvation conditions by altering their expression pattern to produce a more resistant cell. Other stress responses are also triggered including osmotic stress (McCann *et al.*, 1993), temperature stress and oxidative stress (Lacour and Landini, 2004; Lange *et al.*, 1995; McCann *et al.*, 1993). In addition to these physiological changes, *E. coli* also undergoes morphological adaptations upon entry into stationary phase. As a result of a reduction in cell division, the characteristic large rod-shaped cells are reduced to smaller, spherical cells (Nyström, 2004). BolA, a transcriptional regulator, is responsible for the spherical morphology and is mainly controlled by RpoS (Lange and Hengge-Aronis, 1991a; Santos et al., 2002). RpoS is the master regulator during stationary phase and controls the expression of many genes by binding the RNA polymerase (RNAP) sigma subunit and initiating transcription of specific genes.

### 1.1.1 RpoS Regulation during Stationary Phase

Sigma factor binding mediates recognition of promoter regions by RNA polymerase, and therefore initiation of transcription (Maeda *et al.*, 2000). The RNAP enzyme mediates the transcription of DNA to RNA. In *E. coli,* this enzyme consists of four protein subunits; β, β′, α, and σ. The RNAP core enzyme consists of subunit structure $\alpha_2\beta\beta'$ combined with a sigma subunit. There are seven sigma factors in *E. coli,* namely, RpoD, RpoS, RpoN, FliA (RpoF), RpoE, FecI and RpoH. RpoD is the primary sigma factor in *E. coli* and is responsible for the transcription of most genes expressed during the exponential growth phase (Jishage and Ishihama, 1998). RpoD is maintained at a constant level during the transition from exponential phase to stationary phase

although the transcription of genes under the control of this sigma factor decreases (Jishage and Ishihama, 1995).

Under stationary phase conditions, *E. coli* must regulate its transcription so that the expression of genes essential to its survival are activated and those that are not immediately needed are suppressed (Pletnev *et al.*, 2015). Alternative sigma factor ($\sigma^s$), encoded by the *rpoS* gene, is a key regulator during stationary phase and other stress response conditions (Hengge-Aronis, 1996; Martinez-Garcia *et al.*, 2001). RpoS has the lowest affinity for the RNAP core enzyme (Kusano *et al.*, 1996), yet it can out-compete RpoD. Many factors improve the ability of RpoS to bind to RNAP. One such factor is the anti-sigma factor Rsd, which is specific to RpoD. The expression of Rsd increases in stationary phase, and enables RpoS to out-compete RpoD for the enzyme base of RNAP by reducing the affinity of RpoD to RNAP (Jishage *et al.*, 2002).

During the exponential phase, the level of RpoS expression is low, however, once *E. coli* enters stationary phase, RpoS expression levels are significantly induced (Lange and Hengge-Aronis, 1994). RpoS accumulates during stationary phase and is also responsible for the transcription of approximately 100 genes (Ishihama, 2000; Lacour and Landini, 2004; Martinez-Garcia *et al.*, 2001; Schellhorn *et al.*, 1998). RpoS accumulation in stationary phase is highly controlled and conserved in response to different external stimuli, indicating its essential role during starvation as well as other stress conditions (Hengge-Aronis, 2002; Martinez-Garcia *et al.*, 2001).

Many studies have been dedicated to elucidating the regulatory pathways under the control of RpoS. Studies utilizing *rpoS* mutant strains show that oxidative stress genes, including catalase and superoxide dismutase, which are induced upon entry into stationary phase, are regulated by RpoS (Lange *et al.*, 1995). Oxidative stress genes are also controlled by regulatory proteins such

as Fur, SoxRS, and OxyR (Chiang and Schellhorn, 2012). Induction of amino acid and carbohydrate metabolism is RpoS-dependent, which supports the role of RpoS in response to starvation conditions (Lacour and Landini, 2004). Additionally, indole production, which is an essential signal molecule during stationary phase, is controlled by RpoS. RpoS also regulates genes implicated in protein synthesis (Lacour and Landini, 2004) and is required for the expression of acid resistance genes (*gadA, gadB, gadC*) during stationary phase (Mates *et al.*, 2007), osmotic shock genes (*osmC)* and heat shock proteins (Hengge-Aronis, 1996). RpoS also regulates genes that determine cell morphology (*bolA)* in response to environmental changes (Lange and Hengge-Aronis, 1991b). Interestingly, RpoS dependent gene expression differs based on the stress condition and the strain background (Dong and Schellhorn, 2009a). For example, tryptophanase (TnaA) is negatively regulated by RpoS during early stationary phase in *E. coli* K-12 MG1655; however, in *E. coli* O157:H7, TnaA is expressed at a lower level in the Δ*rpoS* strain than in the wildtype. RpoS also plays a major role in pathogenesis as some genes expressed in response to starvation, such as HPII catalase (*katE)* and superoxide dismutase (*sodC),* are also considered as virulence factors (Dong and Schellhorn, 2009a; Fang et al., 1992; Sly et al., 2002). Despite RpoS controlling 10% of the *E. coli* genome upon entry into stationary phase, many proteins that are upregulated by RpoS upon entry into stationary phase are controlled by other regulators during exponential phase and late stationary phase.

### 1.1.2 Other Stationary Phase Regulators in *E. coli*

RpoS-dependent expression differs based on growth condition, which can be due to additional regulators that act in conjunction with RpoS. There is increasing evidence that suggests that RpoS is not the sole regulator during stationary phase as there are genes that are RpoS-independent whose expression is elevated during stationary phase (Schellhorn *et al.*, 1998).

Transitioning from exponential phase to stationary phase, multiple regulators including alternative sigma factors, cyclic adenosine monophosphate (cAMP), H-NS and (p)ppGpp, which are important for adaptation to stationary phase, play a key role (Botsford and Harman, 1992; Magnusson *et al.*, 2005). cAMP and (p)ppGpp each control the expression of specific genes, but they also influence each other in the transition from exponential to stationary phase (Blot *et al.*, 2006).

Histone-like nucleoid-structuring protein (H-NS) plays an important role in the global regulatory network (Barth *et al.*, 1995). H-NS is a DNA binding protein, encoded by *hns,* with an affinity for binding AT-rich curved DNA regions (Yamada *et al.*, 1991). The expression of hundreds of genes may be affected by H-NS, a nucleotide organizing protein, including stress response genes such as genes implicated in acid shock and cold shock (Dorman, 2007; Giangrossi *et al.*, 2005; La Teana *et al.*, 1991). The AT-rich regions can act as nucleation sites for further oligomerization. This oligomerization can repress the transcription of genes by preventing RNAP from binding to a promoter region or by trapping RNAP that is already bound to DNA (Schroder and Wagner, 2000). In the absence of *hns,* RpoS along with certain RpoS-dependent genes are elevated (Yamashino *et al.*, 1995). For example, the members of two well-known RpoS-dependent operons, *csgBA* and *hdeAB,* increase in expression in the absence of *hns*. However, not all RpoS-controlled genes are repressed by H-NS (Barth *et al.*, 1995).

cAMP accumulates when carbon is limited (Notley-McRobb et al., 1997)and activates many genes that are implicated in scavenging for an alternative carbon source when bound to the cAMP receptor protein (CRP) (its effector protein) (Mandelstam and Halvorson, 1960; Notley-McRobb et al., 1997; Pastan and Adhya, 1976). cAMP-CRP along with ArcA are two transcription factors that have been implicated in regulating *rpoS* transcription. ArcA acts as a repressor by

binding to two sites in *rpoS*, one of which overlaps with the binding site for cAMP-CRP, which activates *rpoS* expression (Park *et al.*, 2013). cAMP-CRP can be a positive and negative regulator depending on the condition (Lange and Hengge-Aronis, 1994; Ueguchi *et al.*, 2001). For example, transcription of *csiD* is initiated by RpoS and cAMP-CRP. Its expression occurs from a single promoter, which is slightly upregulated when leucine is present in the growth medium. This indicates that the leucine-responsive regulatory protein, Lrp, positively regulates the *csiD* promoter in the presence of leucine. Lrp positively and negatively influences the expression of many genes. Lrp is responsible for promoting anabolic activity and suppressing catabolic activity during stationary phase. Additionally, Lrp expression varies depending on growth phase, where, there are high levels of Lrp when the growth rate is low (such as during stationary phase), which stimulates anabolic operons and represses catabolic operons. Therefore, growth rate and Lrp expression are inversely related. Furthermore, Lrp is positively regulated by the universal stress alarmone, (p)ppGpp, which also changes inversely with the growth rate (Landgraf *et al.*, 1996). (p)ppGpp mediates the stringent response, which is a response to amino acid starvation, iron limitation, heat stress and other conditions (Traxler *et al.*, 2008).

### 1.1.3 The Stringent Response

The stringent response is triggered in response to a host of stress conditions including amino acid deprivation, fatty acid limitation, iron limitation, as well as carbon and phosphorus starvation. This phenomenon is mediated by the nucleotide guanosine3', 5'-bis(diphosphate), ppGpp, which is a major regulator in bacterial physiology as it responds rapidly to many different stresses (Hauryliuk *et al.*, 2015; Magnusson *et al.*, 2005; Sands and Roberts, 1952). It does so by downregulating rRNA biosynthesis, DNA replication, and ribosomal proteins and upregulating of RpoS, stress proteins and amino acid biosynthesis (Magnusson *et al.*, 2005). During amino acid

starvation, *E. coli* limits cellular growth and other resource consuming processes and prepares the organism's defensive and adaptive processes to promote survival (Magnusson *et al.*, 2005; Srivatsan and Wang, 2008). There are two proteins that control the concentration of ppGpp in the cell, namely, RelA and SpoT (Gentry and Cashel, 1996). The difference between the two is that RelA, also known as ppGpp synthetase I, can only synthesize ppGpp, whereas SpoT, known as ppGpp synthetase II and hydrolase, synthesizes and degrades ppGpp (Gentry and Cashel, 1996). Furthermore, both proteins respond to different cues.

RelA responds to amino acid starvation while SpoT responds to iron (Vinella *et al.*, 2005), carbon (Xiao *et al.*, 1991), phosphorous (Spira *et al.*, 1995) and fatty acid limitation (Battesti and Bouveret, 2006). In amino acid starvation, uncharged tRNAs accumulate within the cell. These excessive uncharged tRNAs enter the ribosomal A site and activate the ribosome-associated enzyme RelA (Wendrich *et al.*, 2002). At this point, RelA catalyzes the transfer of pyrophosphate from ATP (or GTP) to GDP, synthesizing ppGpp. On the other hand, SpoT acts as a ppGpp hydrolase under favourable conditions, which explains the lower concentrations of ppGpp in the cell prior to starvation and other stress conditions (Murray and Bremer, 1996). SpoT is associated with an acyl carrier protein that allows it to sense the level of fatty acids within the cell, and trigger ppGpp synthetase II activity in response to starvation (Battesti and Bouveret, 2006). ppGpp, along with anti-sigma factor Rsd, enables RpoS to out-compete RpoD for the enzyme base of RNAP by reducing the affinity of RpoD to RNAP (Jishage *et al.*, 2002). The stringent response has been shown to play a role in biofilm formation as well as intrinsic antibiotic resistance in *E. coli* (Greenway and England, 1999). Once favourable conditions return, ppGpp levels decrease and the stringent response is reversed. ppGpp serves as a regulator during multiple environmental changes,

allowing the cells to adapt to their changing environment, suggesting it is an important element in the regulatory network that enables cells to survive non-optimal conditions (Traxler *et al.,* 2008).

## 1.2 Unbiased Proteomic Approaches

Omics technologies have provided us with tools required to examine differences in DNA (genomics), RNA (transcriptomics) and protein (proteomics) (Hasin *et al.,* 2017). These experiments are most often carried out in high-throughput assays that eventually produce large amounts of data on characteristics of the cell of interest and have enabled significant advances in the understanding of changes that occur at the molecular level. Recently, there have been many studies that analyze mRNA expression using multiple transcriptomic tools. The results are then used to infer the protein content of the cell organism of interest. However, mRNA can be modified post-transcriptionally and regulated at the level of protein translation. These modifications can result in differences in transcriptomic and proteomic analyses. Therefore, it is important to combine transcriptomics and proteomics to gain a comprehensive understanding of the biological processes and pathways that occur within a given cell or organism (Greenbaum *et al.*, 2003). The function of individual proteins, as well as their implication in pathways that control protein expression in cells, is crucial for a complete understanding of the many processes occurring within living cells.

Proteomics is the large-scale study of proteins. There are many different techniques that are used to assess protein expression within an organism. An example of this is two-dimensional gel electrophoresis (2-DE), which is used to detect differences in expression between different organisms or differing conditions in the same organism (Greenbaum *et al.*, 2003). Here, protein expression in different samples can be compared both qualitatively and quantitatively. 2-DE can be combined with mass spectrometry (MS) to identify protein abundance as well as study protein

function. Though this technique has been the mainstay for protein expression analysis, it is a difficult process to automate for large scale analyses. Furthermore, experiments show little reproducibility and the proteins are not immediately compatible with MS after separation. Because of this, it is difficult to make a comparison between samples on different gels (Thompson *et al.*, 2003). Difference gel electrophoresis (DIGE) is an advancement of 2-DE, which uses fluorescent tagging of protein samples with different dyes. This enables the images of a gel containing fluorescently tagged proteins to be superimposed on one another, and pattern differences can be identified. DIGE overcome the limitations of using 2-DE by running all labeled samples on the same gel. However, the same protein labeled with multiple products does not migrate with itself, producing multiple products for each protein (Thompson *et al.*, 2003). Multidimensional protein identification technology (MudPIT) is a technique that digest proteins to peptides, separates them using 2D-chromatography based on charge and hydrophobicity, and subsequently analyses them using MS (Schirmer *et al.*, 2003).

MS is now being widely used to analyze biological samples. Because of the depth of information that is acquired using MS, it has become an essential tool for proteomics. Quantitative proteomics is a powerful approach that has been used widely in the field of microbiology to understand the dynamics of the global protein within an organism (Otto *et al.*, 2014). Protein abundance in different conditions can be used to highlight the importance and role of individual proteins during these conditions. Proteomic analyses can be carried out using label-free methods, which enable relative and absolute quantitation. Recently, most proteomic analyses entail labeling of peptides within experimental groups or by individual samples, that can then be differentiated by MS. For example, stable isotope labeling by amino acids (SILAC), isotope-coded affinity tag

(ICAT) and isobaric tag labeling. Quantitative proteomics is crucial to our understanding of global protein expression.

As mentioned above, there are two common approaches for protein-based quantitation, namely, label-free or label-based approaches (isotopic, metabolic, and isobaric labeling). Advancements in liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) have simplified the identification and quantification of thousands of proteins in complex proteome samples. Label-free quantitation provides high-throughput data at a low cost, however, it has many limitations (Nahnsen *et al.*, 2013; Théron *et al.*, 2014). Label-free quantification requires accurate mass measurements and reproducible peptide retention times by liquid chromatography, which can introduce bias in the peptide ion intensities obtained (Callister *et al.*, 2006; Karpievitch *et al.*, 2012; Megger *et al.*, 2014). This contributes to a higher number of missing values. The effect of these biases can be lessened by employing metabolic labeling strategies, such as SILAC. However, the metabolic labeling approach requires unique conditions for growth which increase the total cost and time for development. Finally, these challenges can be overcome by employing isobaric chemical tags such as tandem mass tags (TMT) (McAlister *et al.*, 2012; Thompson *et al.*, 2003).

### 1.2.1 Tandem Mass Tags

Tandem mass tags (TMT) are isobaric chemical tags used for MS-based quantification and identification of biological macromolecules such as proteins, peptides, and nucleic acids. Isobaric tags have the same masses and chemical properties but differ in the distribution of heavy isotopes around their structure (Thompson *et al.*, 2003). TMT allows for relative quantitation of up to 10 different samples in a single LC-MS/MS analysis, which improves throughput and percentage coverage by avoiding missing values, which is common in label-free based approaches (Ping *et al.*, 2018). In an experiment, protein samples are digested, typically using trypsin, to produce

peptides. Individual samples are then labeled, each with a different isobaric tag and then combined and subsequently analyzed in single LC-MS/MS analysis. The tags are cleaved from the peptides at a specific linker region upon an energy collision-induced dissociation during MS/MS (Thompson *et al.*, 2003). The fragmented peptide ions are then analyzed for assignment to a peptide sequence and the cleaved isobaric tags are quantitated. MS software, such as Proteome Discoverer and MaxQuant, can quantify TMT.

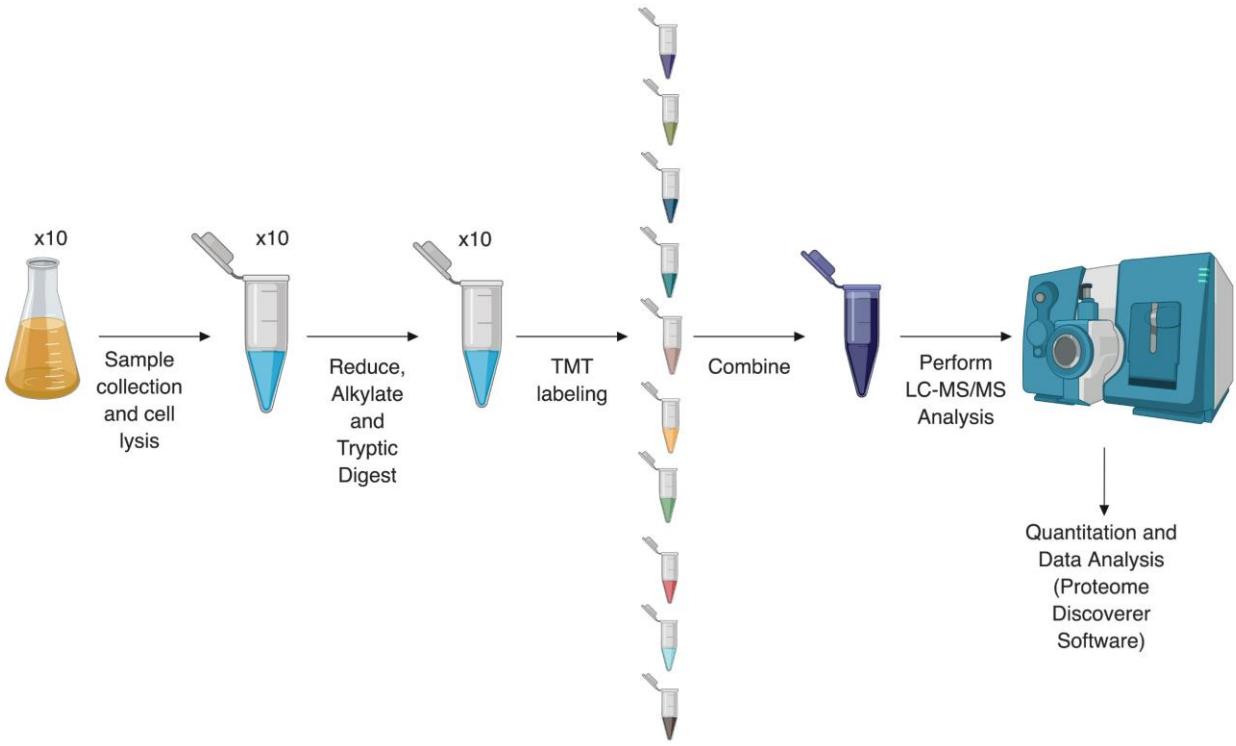


**Figure 1. Overview of TMT labeling for Mass Spectrometry**.
Samples of interest are collected, and the cells are lysed. Crude protein extracts are reduced, alkylated and digested using the protease trypsin. Up to 10 individual samples are labeled with different isobaric tags before being combined for LC-MS/MS analysis. The tags are cleaved upon high energy collision-induced dissociation. The fragmented peptide ions are used for identification while the tags are used to quantitate relative peptide intensities.

## 1.3 Rationale

It is well-known that RpoS induces translation of specific proteins in response to stress, specifically, nutrient limiting stress, which is encountered upon entry into early stationary phase (Hengge-Aronis, 2002). However, a large fraction of the *E. coli* genome is induced during stationary phase compared to exponential phase, and this is not RpoS-dependent, suggesting that RpoS-dependent genes may only represent a small proportion of stationary-phase genes (Schellhorn *et al.*, 1998). Furthermore, a large fraction of the genome continues to increase in expression during prolonged stationary phase that does not require RpoS (Fig. 2). This suggests that besides RpoS, there are other regulatory mechanisms that control expression during prolonged stationary phase and therefore, enable the bacterium's survival. Figure 2 summarizes the changes that occur when cells transition from exponential phase to stationary until net growth ceases in late stationary phase in batch culture. During the exponential phase, expression of RpoS and its regulon members is relatively low but increases during post-exponential phase (PEX). However, during prolonged stationary phase (PSP), RpoS expression decreases, yet there are a set of genes that are continuously induced during prolonged stationary phase (Fig. 2). These genes may be important for adaptation under nutrient-limited conditions. In our lab, we have previously conducted experiments to determine these regulatory mechanisms by employing transcriptomic technologies such as RNA-seq and microarray analysis. However, mRNA can be modified post-transcriptionally and regulated at the level of proteins. Protein expression during prolonged stationary phase is understudied. Post-transcriptional modifications can result in differences in transcriptomic and proteomic analyses (Greenbaum *et al.*, 2003). Furthermore, proteins are ultimately responsible for most cellular phenotypes, therefore, expression at the level of the proteome elucidates the proteins that may be important for the adaptation and survival of *E. coli*

under nutrient limiting conditions. Moreover, it is important to integrate the data obtained from transcriptomic studies with that of proteomic studies for an in-depth understanding of *E. coli*'s survival during starvation conditions.

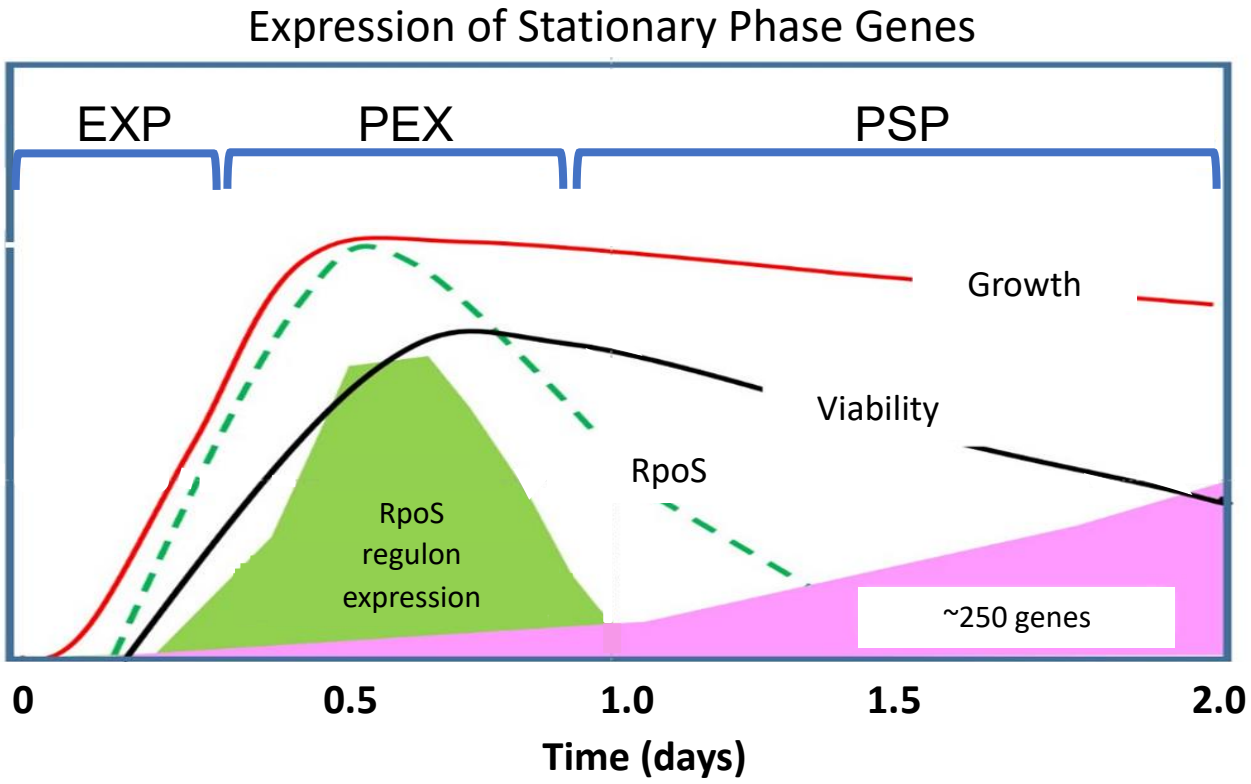


**Figure 2. Major changes in the expression of RpoS and its regulon members.**
The expression of members of the RpoS and its regulon member peaks during PEX. The expression of RpoS declines thereafter. However, approximately 250 genes continue to be expressed during PSP.
**EXP** = Exponential, **PEX** = Post Exponential, **PSP** =Prolonged Stationary

## 1.4 Objectives

1. To identify which proteins are upregulated in *E. coli* during prolonged stationary phase.
2. To determine which proteins that are upregulated during prolonged stationary phase in *E. coli* are RpoS-dependent or independent.
3. To determine the class of proteins to which these upregulated proteins belong and determine their role in long-term growth.
4. To determine the proteome profile of sigma factors as well as DNA binding factors during extended growth.

# 2.0 Materials and Methods

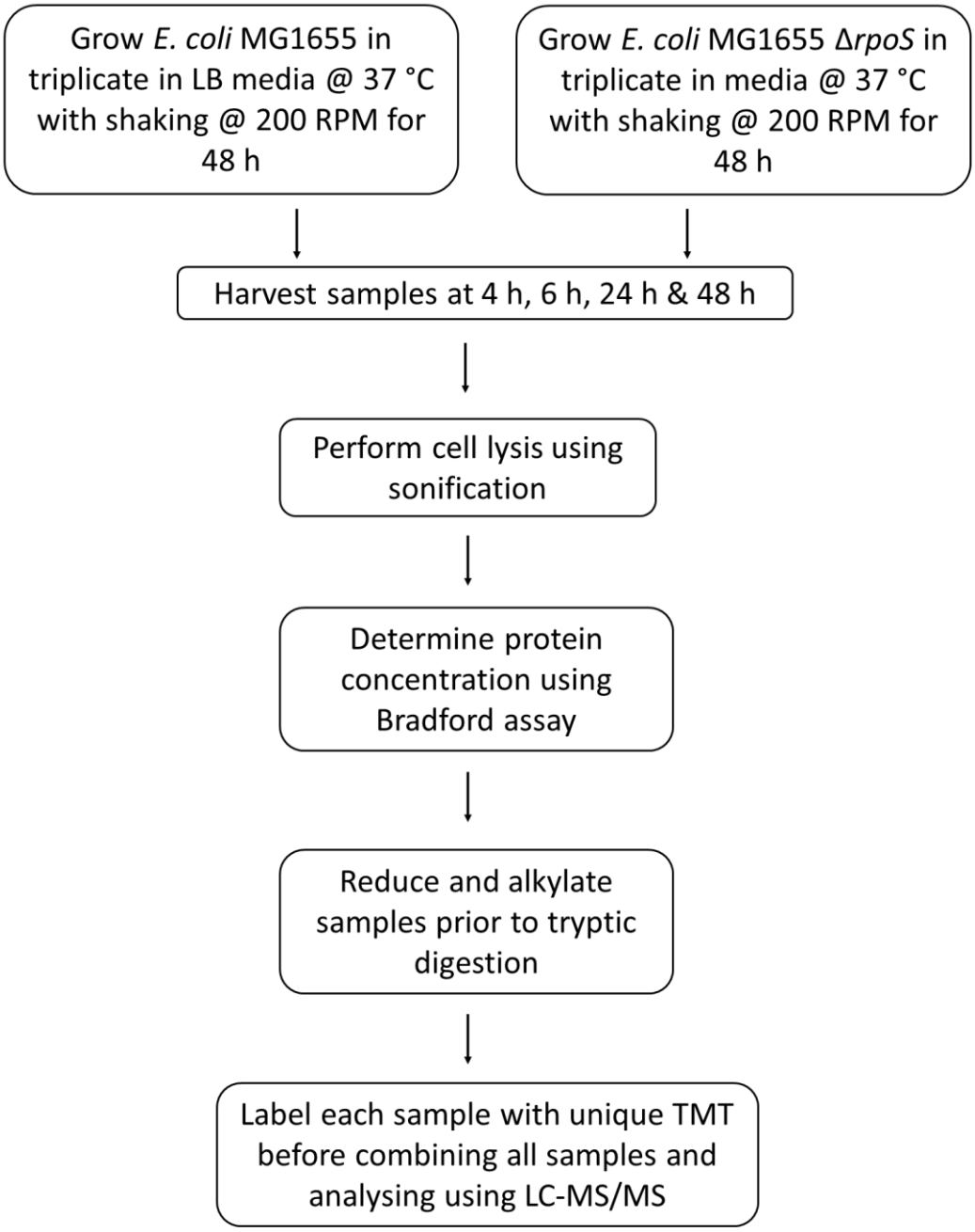


**Figure 3. Overview of Experimental Design.**
*E. coli* MG1655 and its *rpoS* mutant derivative were grown in triplicate in LB media at 37 °C with shaking at 200 RPM for 48 h. Samples were harvested during exponential (4 h), early stationary (6 h), late stationary (24 h) and prolonged stationary (48 h) phase. Samples were lysed using sonification before determining the protein concentration using Bradford assay. Samples were then reduced and alkylated prior to trypsin digestion. Each sample was labeled with a unique TMT before combining all samples. Finally, LC-MS/MS analysis was performed on all samples.

## 2.1 Bacterial Strains and Growth Conditions

*Escherichia coli* K12 MG1655 and isogenic *rpoS* mutant strain (Δ*rpoS*) were employed in triplicate in this study.

A single, independent colony of each *E. coli* strain was inoculated in LB broth (LB-Miller, 10 g peptone, 5 g yeast extract, 10 g NaCl), and grown aerobically overnight at 37 °C with shaking at 200 RPM. Overnight cultures were diluted 1:10,000 into 100 ml of fresh LB and grown under the same conditions. Sample growth was monitored using UV spectrophotometer. Samples were harvested in exponential phase (0.3 $OD_{600}$, 4 h), early stationary phase (1.5 $OD_{600}$, 6 h), late stationary phase (24 h) and prolonged stationary phase (48 h).

## 2.2 Protein Extraction

Samples for proteome analysis were collected by centrifugation at 3, 000 RPM for 15 min at 4 °C. The obtained pellet was washed in phosphate buffered saline (PBS), flash frozen in liquid nitrogen and stored at -80 °C until further processing. The cell pellets were resuspended in 0.5 ml 1× RIPA buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM $Na_2EDTA$, 1 mM EGTA, 1% NP-40, 1 % sodium deoxycholate, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM $Na_3VO_4$, 1 µg/ml leupeptin). Cell lysis was performed by sonication (10 cycles, 30 s on 30 s off, 100 % amplitude). Obtained lysates were centrifuged at 13, 000 RPM for 15 min to remove cell debris. The supernatant was collected, and the protein concentration determined using the Bradford protein assay (BioRad, USA) (Bradford, 1976).

## 2.3 Sample Preparation

TMT labeling was carried out according to the manufacturer's instructions (Thermo Fisher Scientific) with some modifications. Briefly, for each sample, aliquots containing 50 µg proteins

were reduced with 200 mM Tris (2-carboxyethyl) phosphine, TCEP, at 55 °C for 1 h. Samples were then alkylated with 375 mM iodoacetamide (IAA) for 30 min in the dark, at room temperature. Samples were allowed to precipitate for 1 h in acetone at -80 °C, centrifuged at 8, 000 ×g for 10 min at 4 °C, the acetone decanted and the pellet dried. The protein pellets were resuspended in 100 mM triethylammonium bicarbonate (TEAB) and digested by incubation with trypsin overnight at 37 °C. Isobaric tags were added to each sample and allowed to incubate at room temperature for 1 h for complete labeling. Labeled samples were pooled and desalted using peptide desalting columns before high-resolution LC-MS analysis.

## 2.4 LC-MS/MS Analysis

Labeled samples were analyzed on a Thermo Scientific Orbitrap Fusion-Lumos Tribid Mass Spectrophotometer (Thermo Fisher, San Jose, CA) coupled with a nanospray and EASY-nLC 1, 200 nano-LC system and electron transfer dissociation (ETD) mode. The labeled peptides were lyophilized and the peptide mixtures were re-dissolved in 0.1 % formic acid and loaded onto the analytical column (75 µm x 50 cm PepMax RSLC EASY-Spray column filled with 2 µm C18 beads at a pressure of 900 Bar and temperature of 60 °C). Peptides were eluted over a 240-minute gradient of 2 % to 100 % buffer B (80 % acetonitrile, 0.1 % formic acid) at a flow rate of 250 nL/min.

Once separated, the peptides were introduced by nano-electrospray into the Fusion-Lumos mass spectrophotometer. Data were acquired using the Multinotch MS3 acquisition with synchronous precursor selection (SPS) with a cycle time of 5 s. MS1 acquisition performed with a scan range of 550 m/z – 1, 800 m/z with the resolution set to 120, 000, the maximum injection time of 50 ms and automatic gain control (AGC) target set to $4 \times 10^5$. Isolation for MS2 scans was performed in the quadrupole, with an isolation window of 0.7 m/z. MS2 scans were done in the

linear ion trap with a maximum injection time of 50 ms and normalized collision energy (NCE) of 35 %. For MS3 scans, higher-energy collision dissociation (HCD) was used, with a collision energy of 30 % and scans were measured in the orbitrap with a resolution of 50, 000 and a scan range of 100 m/z-500 m/z, an AGC target of $3 \times 10^4$ with a maximum injection time of 50 ms. The dynamic exclusion, which allows more data to be obtained on more components in complex mixtures (particularly, intense components), was set to 20 s.

## 2.5 Protein Identification and Quantification

The raw acquired data were processed with Proteome Discoverer 2.0 Software (Thermo Fisher Scientific). Proteins were identified by searching MS and MS/MS data of peptides against UniProtKB *Escherichia coli* (K12) database. Proteins were identified using both Sequest HT and MS Amanda software within Proteome Discoverer. The FDR threshold was specified as <0.05. One of the main advantages of the TMT method is the ability to identify and quantify proteins from multiple complex samples at once. TMT enables multiplexing of up to 10 samples in one LC-MS/MS analysis. When more than 10 samples are being analysed, one TMT channel is dedicated as an internal standard and included in each analysis. This internal standard can then be used to normalize protein measurements within and across all analyses (Cominetti *et al.*, 2016). In the current study, equal amounts of peptides from each sample were pooled to generate the internal standard. Protein abundance was calculated based on the spectral intensity, then normalized and scaled using the total protein amount. The obtained results were exported into Microsoft Excel for data interpretation and statistical analyses.

The data were filtered to remove proteins that were not identified in at least two replicates in each growth phase in the wildtype (Mateus *et al.*, 2018). The data were log2 transformed and submitted to differential abundance analysis. Differential abundance was tested using empirical

Bayes method in the linear modeling function in *limma* package in R studio (version 3.5.1) (Becher *et al.*, 2018; D'Angelo *et al.*, 2017; Hesketh *et al.*, 2015; Mateus *et al.*, 2018; Smyth, 2005). The resulting p-values were corrected for multiple testing using the Benjamini-Hochberg method, which reduces the number of false positives and increases the chances of identifying all differentially expressed proteins (Benjamini and Hochberg, 1995).

## 2.6 Functional Enrichment Analysis

Functional enrichment analysis is a method used to assess a large set of proteins and identify classes of proteins that are statistically over-represented within particular metabolic pathways or biological functions. Functional enrichment was carried out on the EcoCyc database (Kaper *et al.*, 2004). Fisher's exact test was used to test for over-representation or under-representation of specific GO terms among the proteins within a set. The p-values obtained were further adjusted using the Benjamini-Hochberg correction method. Enriched GO terms with adjusted p-values $<0.05$ were treated as significant.

# 3.0 Results

We performed global proteome profiling at exponential, early stationary, late stationary and prolonged stationary phase using TMT in wildtype and Δ*rpoS* strains. Of the total number of proteins in *E. coli*, 1,877 proteins were identified in at least one sample, which met the minimum detection limit for identification. These proteins account for approximately 45 % of the *E. coli* proteome. The data set was filtered to remove proteins that were not quantified in at least two replicates in the wildtype. Of the total proteins identified, 1,358 (approximately 32 % of the *E. coli* proteome) proteins met this criterion. The normalized protein abundance was transformed to log2 scale prior to analysis.

To assess the overall differences in the protein profile of each strain during each growth phase, a principal component analysis (PCA) was performed. Each growth phase separated into distinct clusters in the wildtype (Fig. 4) and Δ*rpoS* strains (Fig. 5). Distinct clustering in each treatment suggests that there was a high reproducibility within triplicates. Interestingly, late stationary phase and prolonged stationary phase clustered close together in Δ*rpoS* strain suggesting that there is a similarity in the protein profile between these growth phases. The criteria used to filter the proteins used in this study included proteins that were identified in at least two replicates. As such, some proteins that were not detected in the third replicate of wildtype during late stationary phase (24 h) and the Δ*rpoS* strain in early stationary phase (6 h) resulted in a cluster containing only two replicates for the respective growth phases. Furthermore, during each of the four growth phases, both strains separated into distinct clusters, which suggests that the proteomic profile of each strain during extended growth is different (Fig. 6).

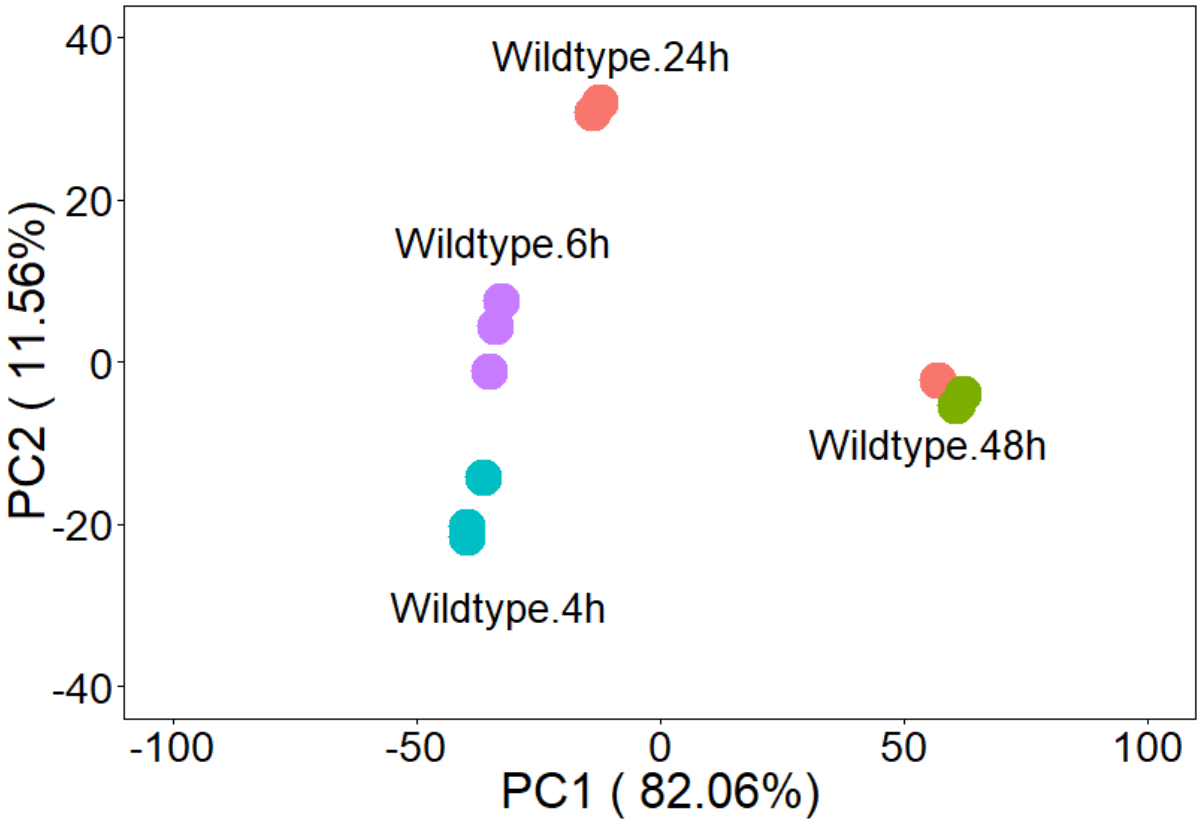


**Figure 4. Principal component analysis of log2 transformed protein abundance in wildtype.** PCA of the protein profile separated the samples collected in different growth phases. The three replicates of each growth phase distinctly clustered together.

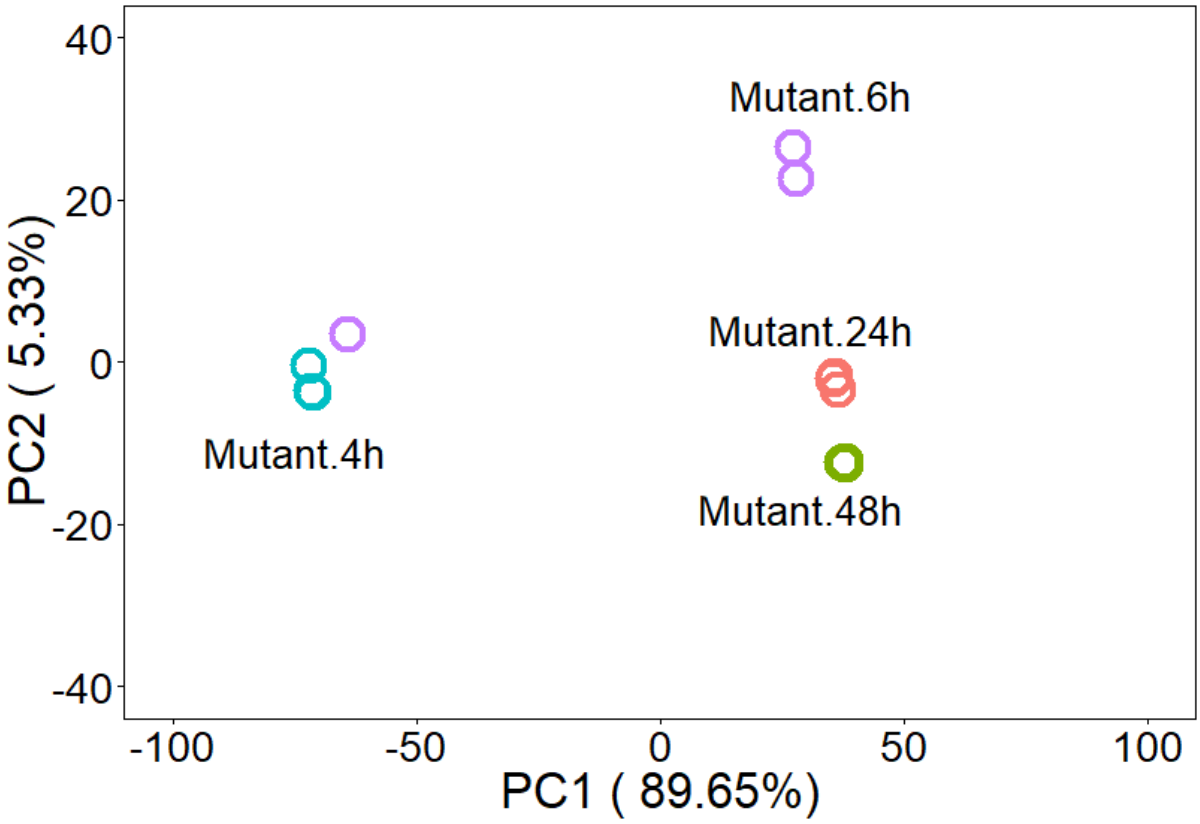


**Figure 5. Principal component analysis of log2 transformed protein abundance in the Δ*rpoS* strain**.
PCA of the protein profile separated the samples collected in different growth phases. The three replicates of each growth phase distinctly clustered together.

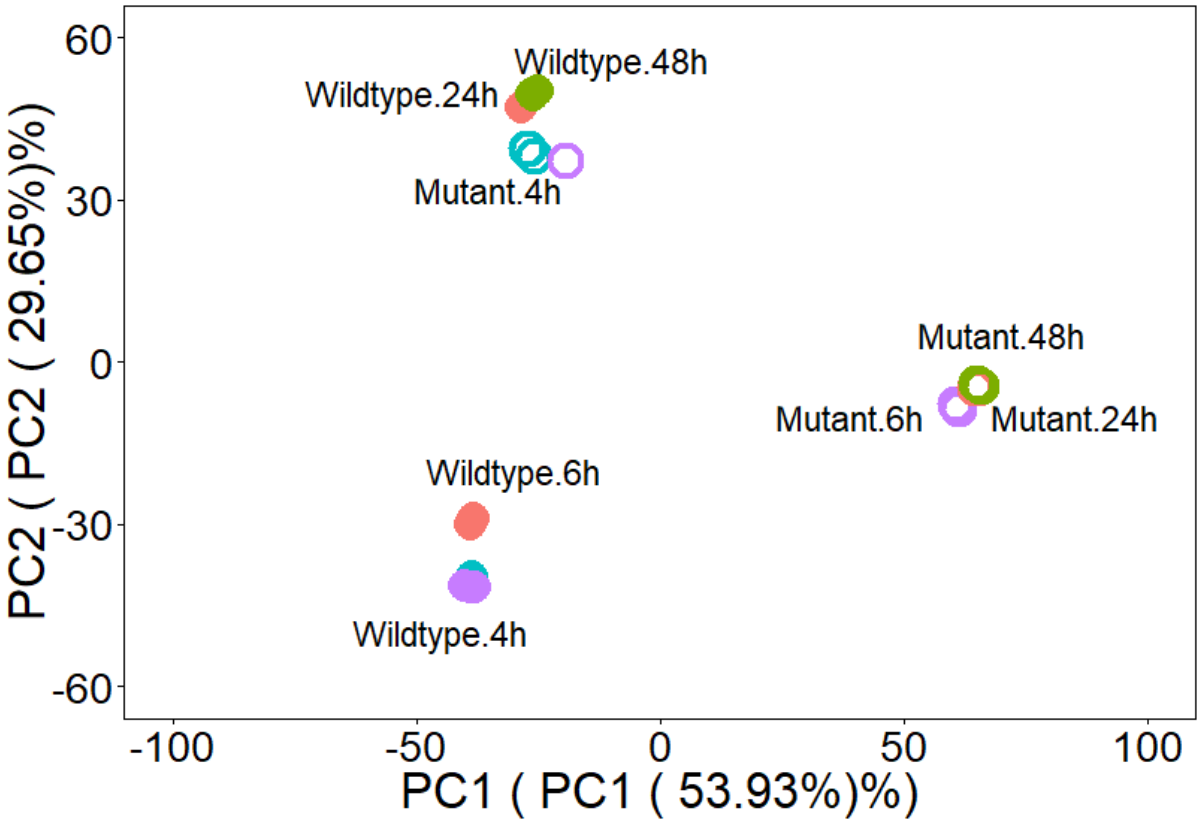


**Figure 6. Principal component analysis of log2 transformed protein abundance in wildtype and Δ*rpoS* strains during growth.**
PCA of the proteome profile separated the samples collected in different growth phases. The three replicates collected in each growth distinctly clustered together. During all four growth phases, wildtype and Δ*rpoS* strains clustered separately. Closed and open circles represent wildtype and Δ*rpoS* strains, respectively.

Pearson correlation analysis was performed which demonstrates the correlation between triplicates during each growth phase (Fig. 7 and 8).

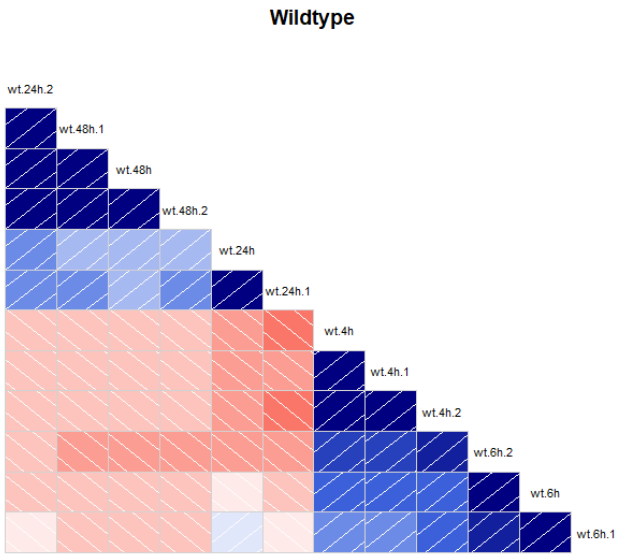


**Figure 7. Pearson correlation analysis of the proteome profile in the wildtype demonstrates that replicates during each growth phase are correlated.**

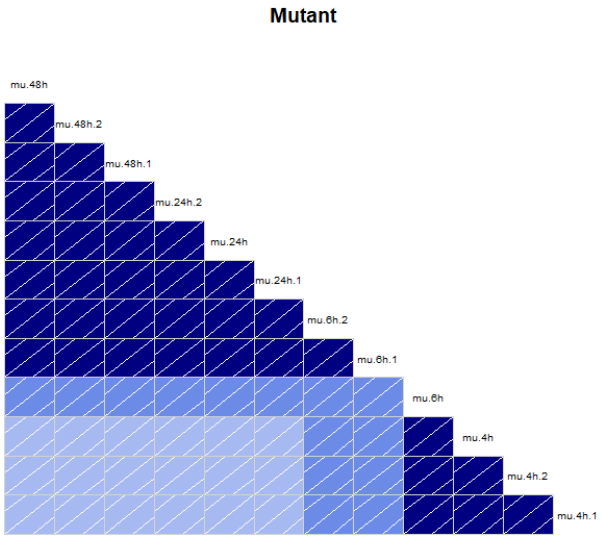


**Figure 8. Pearson correlation analysis of the protein profile in the Δ*rpoS* strain demonstrates that replicates during each growth phase are correlated.**

## 3.1 The Expression Profile of Sigma Factors in *E. coli*

To determine the protein profile of sigma factors in *E. coli* during prolonged stationary phase, we assessed their abundance. There are seven sigma factors in *E. coli,* four of which were identified and quantified in this study, namely, RpoD, RpoN, FliA (RpoF) and RpoE. The level of RpoD slightly decreased upon entry into early stationary phase but remained relatively unchanged throughout the remaining growth phases in the wildtype (Fig. 9). RpoD showed a similar expression trend in the *ΔrpoS* strain as it decreased upon entry into early stationary phase and remained relatively unchanged during late stationary phase. However, RpoD increased during prolonged stationary phase in the *ΔrpoS* strain (Fig. 10). RpoE remained relatively constant in both strains. RpoN steadily decreased in the wildtype throughout each growth phase. Although the abundance of RpoN in the Δ*rpoS* strain was similar to that of the wildtype, the abundance of RpoN markedly increased during prolonged stationary phase. Finally, FliA slightly increased during late stationary phase and then decreased during prolonged stationary phase in the wildtype. In the Δ*rpoS* strain, FliA increased upon entry into early stationary phase but steadily decreased during late and prolonged stationary growth phases.

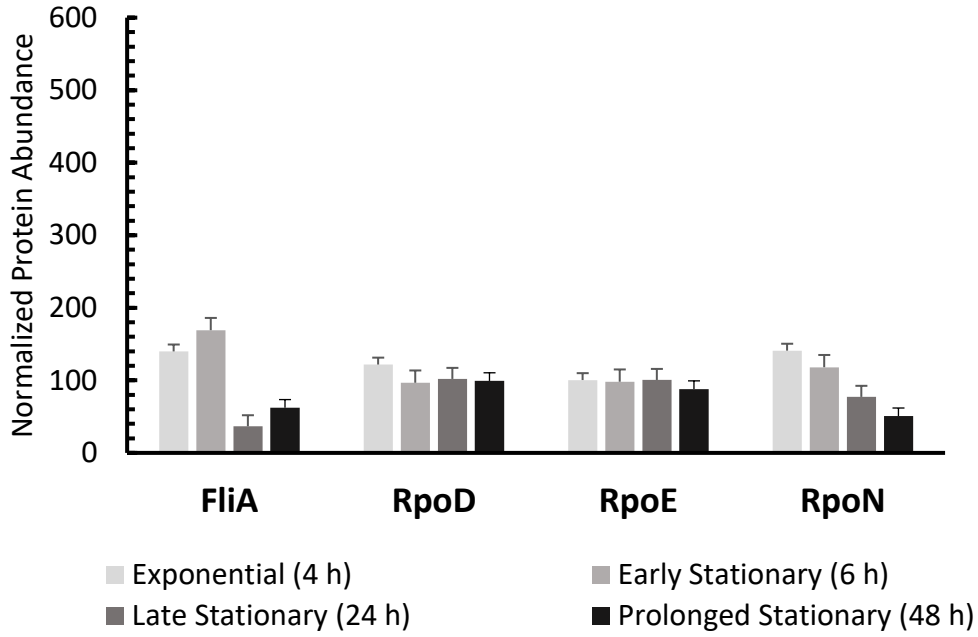


**Figure 9. Normalized protein abundance of sigma factors in *E. coli* (wildtype) during growth.**
Assessment of the abundance of sigma factors in *E. coli* across the four growth phases showed that the level of RpoN reduced while RpoD and RpoE remained relatively unchanged. FliA increased during early stationary phase and decreased thereafter. Error bars represent standard error.

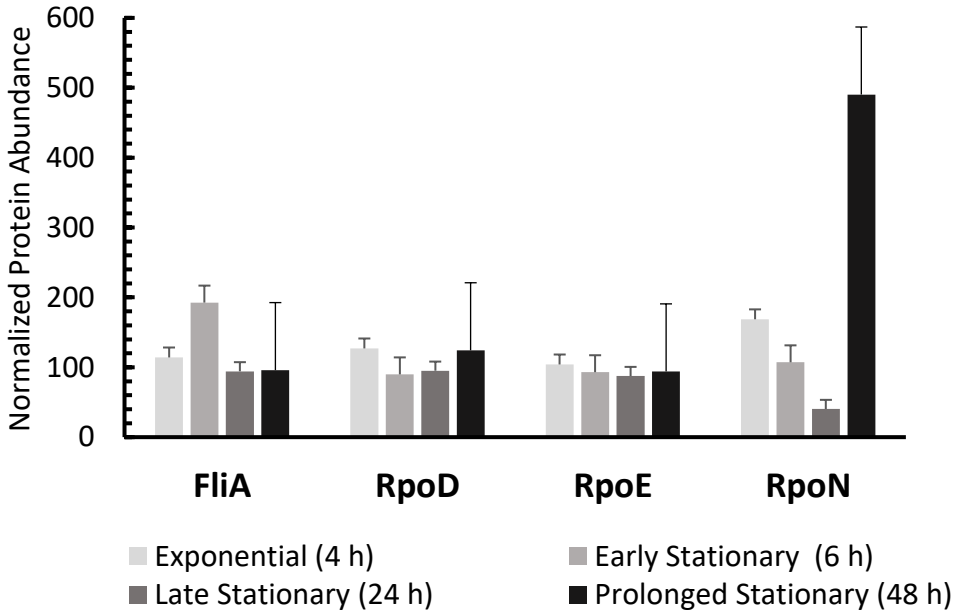


**Figure 10. Normalized protein abundance of sigma factors in *E. coli* (Δ*rpoS*) during growth.**
Assessment of the abundance of sigma factors in *E. coli* across the four growth phases showed that the expression profile of FliA, RpoD and RpoE were similar to the wildtype. However, the level of RpoN markedly increased during prolonged stationary phase. Error bars represent standard error.

## 3.2 The Expression Profile of Binding Proteins in *E. coli*

We assessed the protein abundance of DNA binding proteins in *E. coli*, namely, Dps, Fis, H-NS, Hupα, Hupβ, and StpA. Dps increased during late stationary phase and continued to increase during prolonged stationary phase in the wildtype (Fig. 11). Dps showed a similar expression trend in the Δ*rpoS* strain, however, the abundance was substantially lower. H-NS continuously increased from exponential phase to prolonged stationary phase. In the Δ*rpoS* strain, H-NS remained relatively unchanged (Fig. 12). Fis steadily decreased from exponential phase to prolonged stationary phase in the wildtype. Hupα and Hupβ and StpA all decreased during late and prolonged stationary phase in the wildtype. StpA was expressed at a higher level in the wildtype compared to the Δ*rpoS* strain during exponential phase. Though Fis showed a similar expression trend in both strains, H-NS, Hupα and Hupβ remained relatively unchanged in the Δ*rpoS* strain.

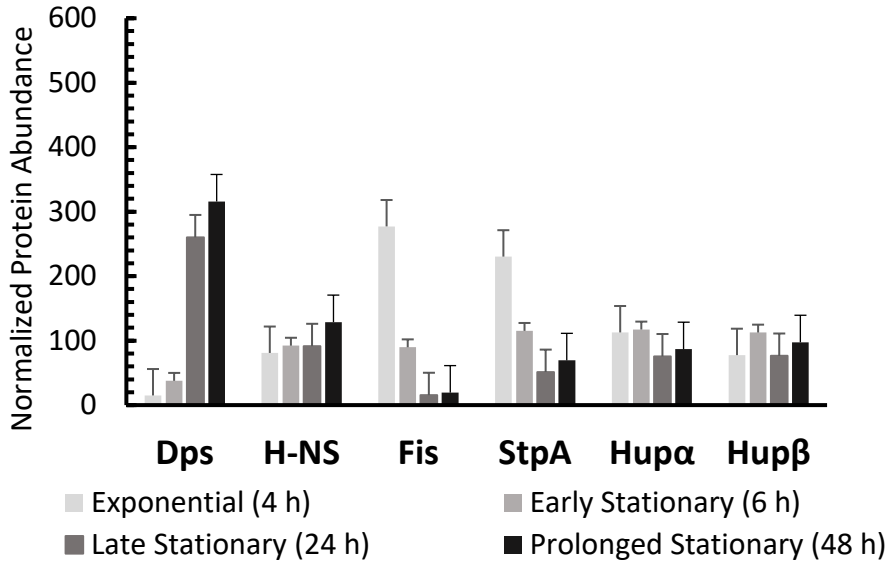


**Figure 11. Normalized protein abundance of DNA binding proteins during growth in *E. coli* (wildtype).**
Assessment of the abundance of DNA binding proteins showed an increased abundance of Dps and H-NS during prolonged stationary phase while Fis and StpA steadily decreased in abundance throughout the four growth phases. The expression of Hupα and Hupβ levels fluctuated during growth. Error bars represent standard error.

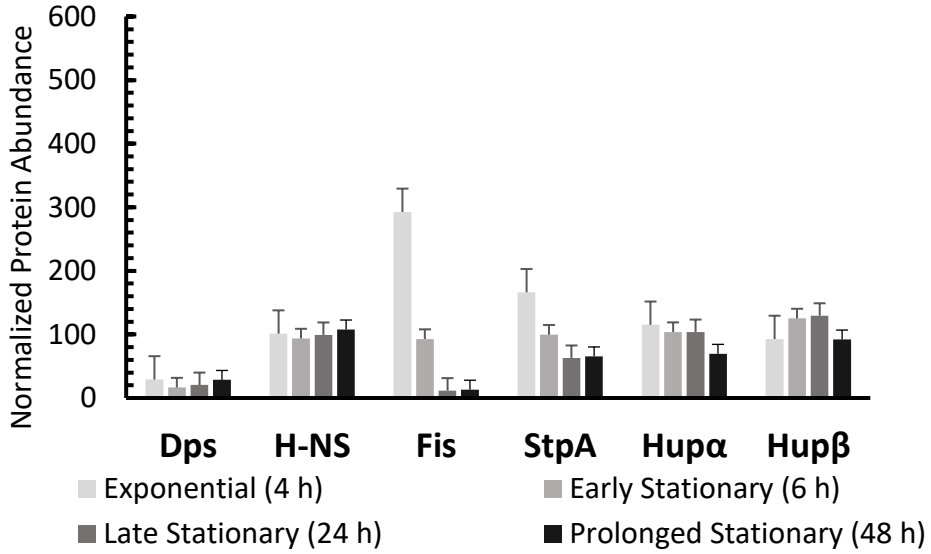


**Figure 12. Normalized protein abundance of DNA binding proteins during growth in *E. coli* (Δ*rpoS* strain).**
Assessment of the abundance of DNA binding proteins showed an increased level of abundance Dps. Fis and StpA steadily decreased in abundance throughout the four growth phases. The remaining four DNA binding factors remained relatively unchanged throughout all four growth phases. Error bars represent standard error.

## 3.3 Which Biological Processes are Enriched during Late Stationary Phase Compared to Early Stationary Phase?

To analyze whether there were functional categories of proteins over-represented in the set of differentially upregulated proteins, the EcoCyc database was used (Karp *et al.*, 2014). Biological processes including pH elevation and intracellular pH elevation were highly over-represented in the wildtype during late stationary phase compared to early stationary phase (Table 1). In contrast, biological processes such as taxis, chemotaxis and organic substance transport were over-represented during late stationary phase compared to early stationary phase in the Δ*rpoS* strain (Table 1). This suggests that in the absence of RpoS, taxis is required during late and prolonged stationary phase. The same biological processes were over-represented during prolonged stationary phase compared to early stationary phase in the wildtype, suggesting that these processes may be required during long-term growth when nutrients are limited.

**Table 1.** Enriched biological processes for upregulated proteins during late stationary phase (24 h) compared to early stationary phase (6 h) in the wildtype and Δ*rpoS* strains.
(Obtained using EcoCyc Database; $p \leq 0.05$)

| **Wildtype** | ***rpoS* mutant** |
|---|---|
| pH elevation | Taxis |
| Intracellular pH elevation | Chemotaxis |
| | Organic substance transport |

Functional enrichment also determines pathways that are statistically over-represented within a large set of proteins. During prolonged stationary phase, pathways including L-arginine degradation, detoxification, amino acid degradation and proteinogenic amino acid degradation pathways were statistically over-represented compared to early stationary phase in the wildtype

(Table 2). This indicates that these pathways are regulated by RpoS. On the other hand, the TCA cycle and super-pathway of glyoxylate bypass and TCA cycle were statistically over-represented during prolonged stationary phase compared to early stationary phase in the Δ*rpoS* strain (Table 2), indicating that enzymes implicated in the TCA cycle may be required during prolonged stationary phase in the absence of RpoS.

**Table 2.** Enriched pathways for upregulated proteins during prolonged stationary phase (48 h) compared to early stationary phase (6 h) in the wildtype and Δ*rpoS* strains.
(Obtained using EcoCyc Database; $p \leq 0.05$)

| **Wildtype** | ***rpoS* mutant** |
|---|---|
| L-arginine degradation | Super-pathway of glyoxylate bypass and TCA cycle |
| Detoxification | TCA cycle |
| Proteinogenic amino acid degradation | |
| Amino acid degradation | |

## 3.4 RpoS-dependent Protein Expression during Stationary Phase

RpoS was upregulated in early stationary phase compared to exponential phase. As a key regulator during stationary phase conditions, RpoS both positively and negatively regulates the abundance of proteins that are a part of the RpoS regulon (Makinoshima *et al.*, 2003; Patten *et al.*, 2004). In the current study, the abundance of 258, 122 and 118 proteins with at least two-fold difference between the wildtype and Δ*rpoS* strains, during early, late and prolonged stationary phase, respectively, were identified. The majority of the proteins that were expressed in an RpoS-dependent manner were upregulated during early stationary phase (Fig. 13). The most highly upregulated proteins during late and prolonged stationary phase compared to early stationary phase in the wildtype are confirmed RpoS-dependent proteins. The fact that these proteins continue to increase in abundance during late and prolonged stationary phase suggests that their role in long-term growth is not limited to early stationary phase. Furthermore, we know that the level of RpoS

decreases during late and prolonged stationary phase (Appendix 2, Fig. 24), suggesting that these proteins may be modulated by other regulators during the later stages of growth. Despite the decrease in the levels of RpoS during late and prolonged stationary phase, there are RpoS-controlled proteins that were highly abundant during late and prolonged stationary phase. These include prototypical RpoS-dependent proteins Dps, OsmY and KatE (Fig. 14).

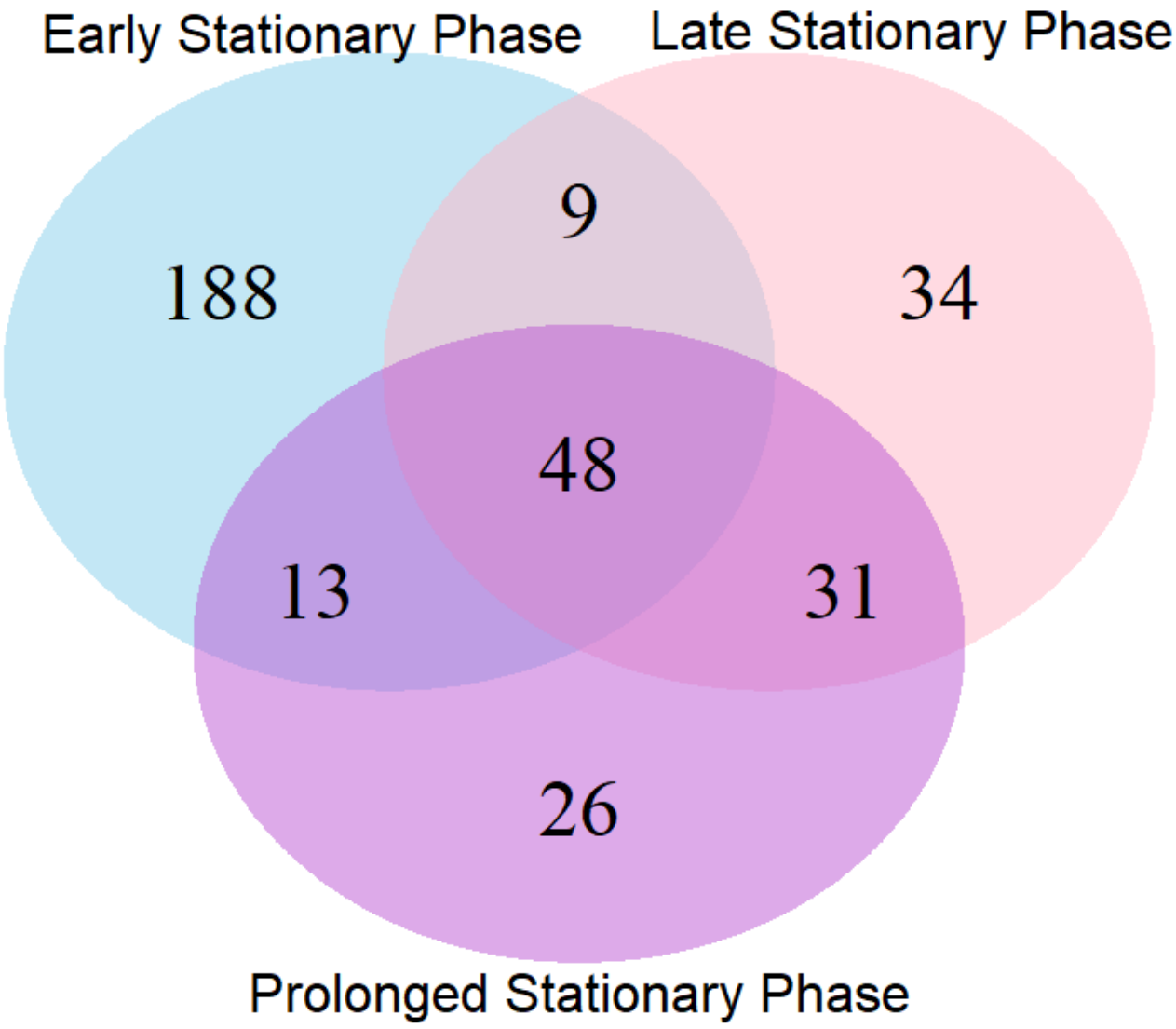


**Figure 13. Proteins expressed in an RpoS-dependent manner during early, late and prolonged stationary phase**.
Majority of the RpoS-dependent proteins were expressed during early stationary phase.

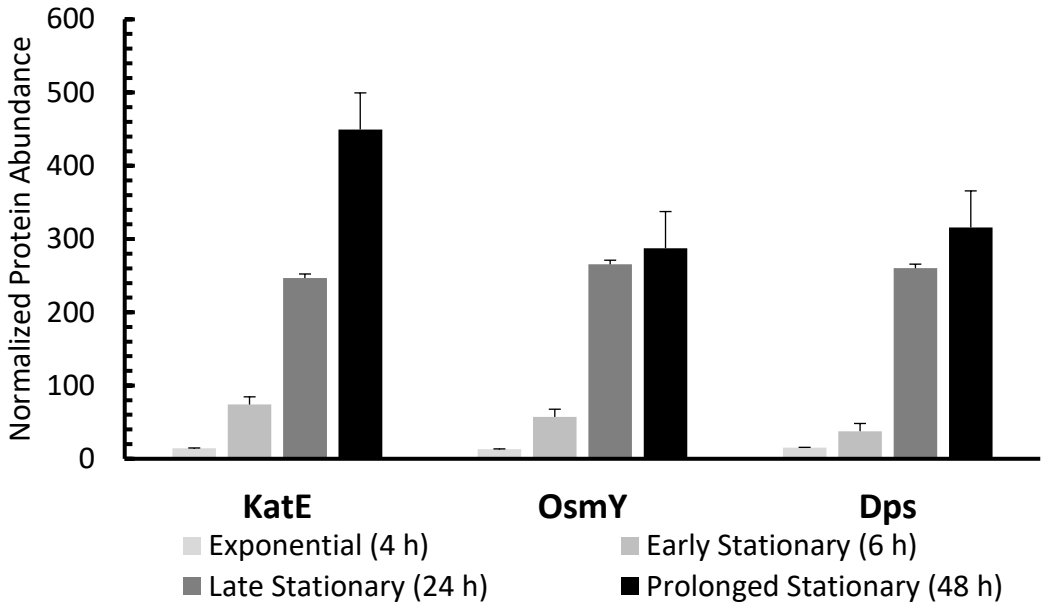


**Figure 14. Normalized protein abundance of RpoS-dependent proteins in late and prolonged stationary phase.**
The expression of known RpoS-dependent proteins (KatE, OsmY and Dps) continued to increase during late and prolonged stationary phase. Error bars represent standard error.

## 3.5 Negative Regulation by RpoS

RpoS positively regulates multiple proteins upon entry into stationary phase. However, there are also multiple proteins that are negatively regulated by RpoS. These include proteins implicated in flagellar biosynthesis, motility and chemotaxis, enzymes implicated in the tricarboxylic cycle (TCA) as well as proteins encoded by cryptic prophage genes. Twenty-six proteins belonging to the flagellar regulon were downregulated by RpoS, sixteen of which were downregulated more than 2-fold during late stationary phase compared to early stationary phase (Table 3).

**Table 3**. RpoS negatively regulates flagellar, chemotactic and motility-related protein abundance.

| Accession ID | Gene | Description | FC 24 h/6 h | FC 48 h/6 h | Class |
|---|---|---|---|---|---|
| P15070 | *fliN* | Flagellar motor switch protein FliN | 10.45* | 48.84 | II |
| P75936 | ***flgD*** | Basal-body rod modification protein FlgD | 8.61 | 5.07 | II |
| P43533 | ***flgN*** | Flagella synthesis protein FlgN | 5.61 | 2.27 | II |
| P0AEM6 | *fliA* | RNA polymerase sigma factor FliA | 4.72 | 2.04 | II |
| P0ABX5 | ***flgG*** | Flagellar basal-body rod protein FlgG | 3.37 | 1.88 | II |
| P76298 | ***flhA*** | Flagellar biosynthesis protein FlhA | 3.17 | 0.10 | II |
| P06974 | *fliM* | Flagellar motor switch protein FliM | 2.94 | 0.12 | II |
| P0ABX8 | *fliL* | Flagellar protein FliL | 2.85 | 2.7 | II |
| P0ABZ1 | *fliG* | Flagellar motor switch protein FliG | 2.84 | 3.03 | II |
| P75937 | ***flgE*** | Flagellar hook protein FlgE | 2.13 | 1.78 | II |
| P25798 | *fliF* | Flagellar M-ring protein | 1.13 | 0.71 | II |
| B1X9J2 | ***flgH*** | Flagellar L-ring protein | 0.19 | 0.07 | II |
| P0AEM9 | *fliY* | L-cystine-binding protein FliY | 0.21 | 0.70 | II |
| P0A9H9 | *cheZ* | Protein phosphatase CheZ | 16.83 | 15.78 | III |
| P0A964 | *cheW* | Chemotaxis protein CheW | 12.46 | 10.97 | III |
| P07363 | *cheA* | Chemotaxis protein CheA | 12.28 | 8.91 | III |
| P07330 | *cheB* | Chemotaxis response regulator protein-glutamate methylesterase | 7.25 | 49.49 | III |
| P07018 | *tap* | Methyl-accepting chemotaxis protein IV | 7.25 | 12.29 | III |

| Accession ID | Gene | Description | FC 24 h/6 h | FC 48 h/6 h | Class |
|---|---|---|---|---|---|
| P04949 | *fliC* | Flagellin | 3.61 | 3.25 | III |
| P07017 | *tar* | Methyl-accepting chemotaxis protein II | 1.66 | 1.86 | III |
| P0AE67 | *cheY* | Chemotaxis protein CheY | 1.52 | 1.15 | III |
| P09348 | *motA* | Motility protein A | 0.96 | 0.07 | III |
| P29744 | *flgL* | Flagellar hook-associated protein 3 | 0.94 | 3.25 | III |
| P0AF06 | *motB* | Motility protein B | 0.21 | 0.49 | III |
| P05704 | *trg* | Methyl-accepting chemotaxis protein III | 1.45 | 1.02 | III |
| P02942 | *tsr* | Methyl-accepting chemotaxis protein I | 0.89 | 0.51 | III |

[a] Fold change values are negative. [b] Fold change values are positive. Proteins with the two flagellar classes represented in the current study along with fold changes ≥2 relative to early stationary phase are highlighted in *bold*. * Significant (P<0.05); ** Highly Significant (P<0.001).

Many of the TCA cycle proteins were downregulated more than 2-fold by RpoS, including but not limited to GltA, FumA, SucABCD, AcnB, SdhABCD (Fig. 15). This means that although these proteins may be downregulated in the Δ*rpoS* strain, the downregulation is substantially higher in the wildtype strain which indicates that RpoS represses the activity of these proteins during long-term growth. Additionally, proteins encoded by cryptic prophage genes, including Icd, CP4-44 and YfkB, were downregulated in the wildtype, which suggests that RpoS also negatively regulates members of this class of proteins as well. However, FlxA and YffS were downregulated in the Δ*rpoS* strain more substantially than in the wildtype. Furthermore, GnsB was expressed in an RpoS-independent manner in this study.

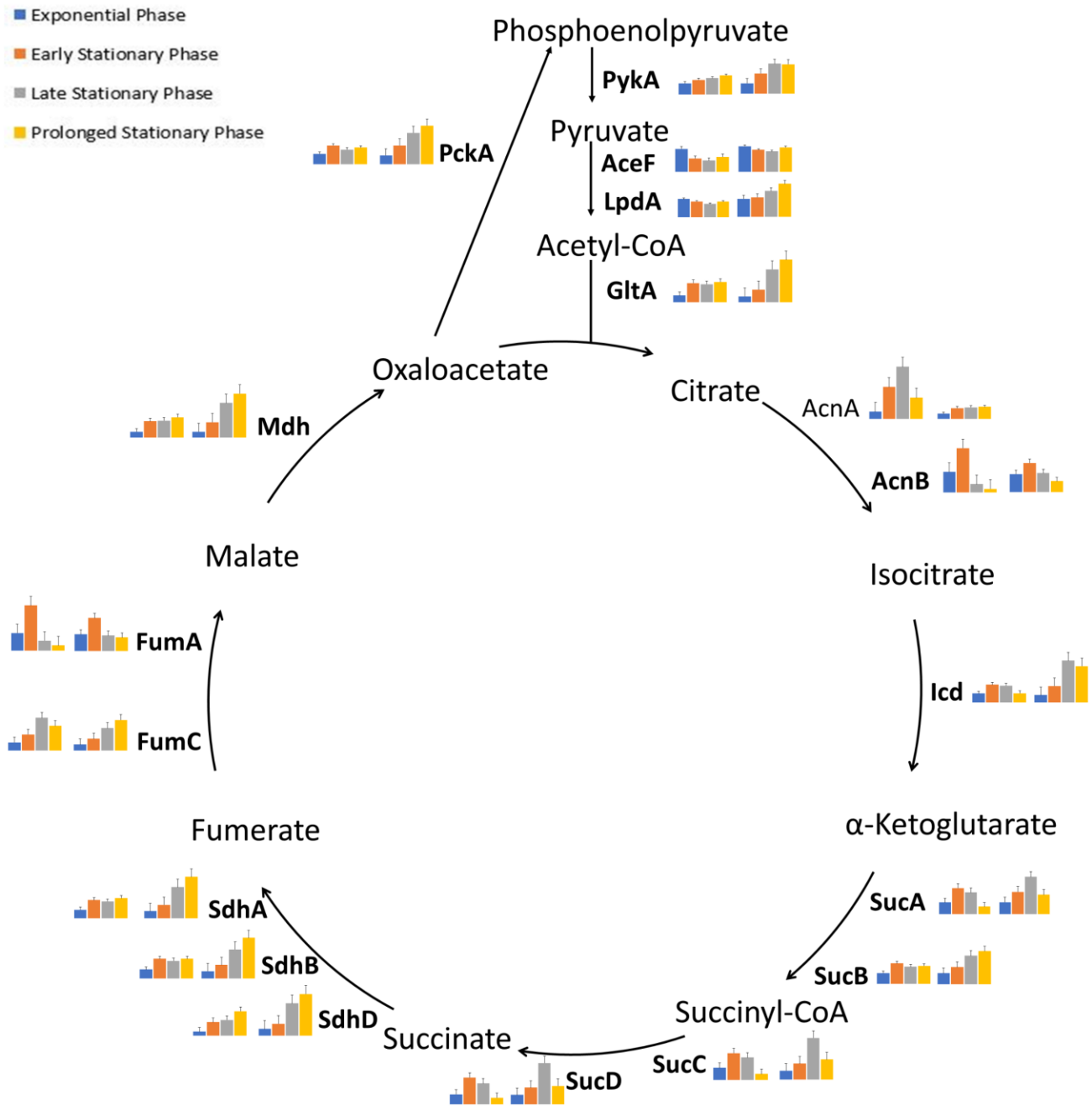


**Figure 15. The Tricarboxylic cycle (TCA cycle).**
Proteins implicated in the TCA cycle that are downregulated more than 2-fold by RpoS are highlighted in *bold*. The normalized protein abundance of each protein is shown in bar graphs where the left and right bar graphs represent the abundance in the wildtype and Δ*rpoS* strains, respectively.

Furthermore, tryptophanase was also negatively regulated by RpoS. In both wildtype and Δ*rpoS* strains, tryptophanase was a highly expressed protein upon entry into early stationary phase. TnaA levels were higher in the Δ*rpoS* strain during early stationary phase than in the wildtype. However, TnaA levels decreased in the wildtype during late and prolonged stationary phase (Fig. 16). On the other hand, TnaA levels were consistently higher in the Δ*rpoS* strain up to prolonged stationary phase (Fig. 16).

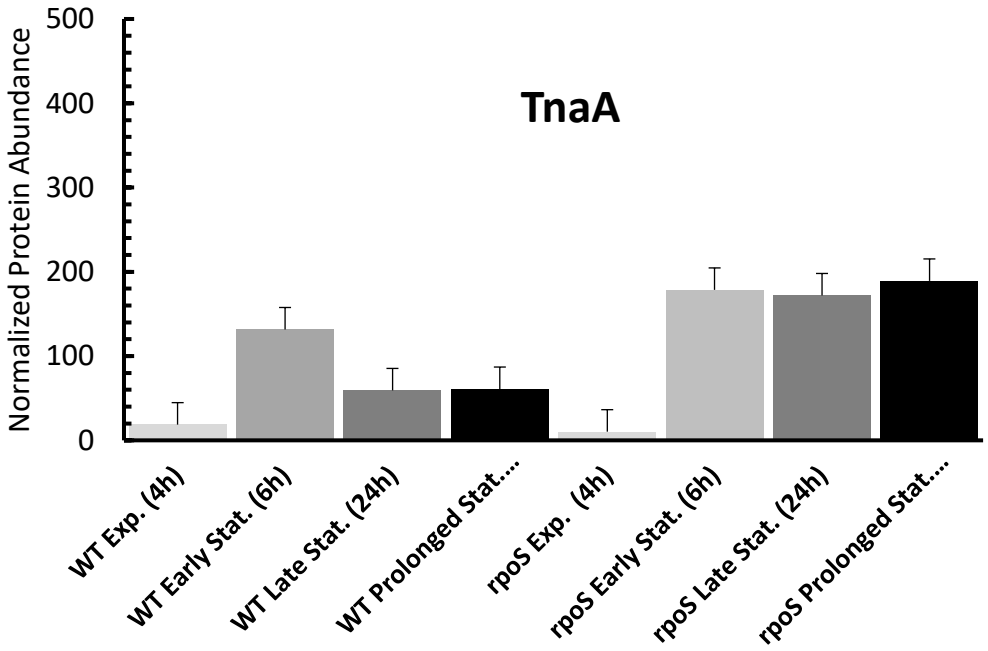


**Figure 16. Normalized protein abundance of TnaA during growth.**
Changes in the abundance of TnaA in wildtype and Δ*rpoS* strains indicated that TnaA was induced during early stationary phase (6 h) regardless of strain background. The abundance of TnaA was higher in the Δ*rpoS* strain than the wildtype during stationary phase. Error bars represent standard error.

## 3.6 RpoS-independent Protein Expression during Stationary Phase

One-hundred and five proteins were upregulated (>2-fold) during early stationary phase compared to exponential phase in the wildtype. Twenty-four of these proteins were induced in both the wildtype and Δ*rpoS* strains. However, fifteen of the twenty-four proteins were induced at

a substantially higher level in the Δ*rpoS* strain than in the wildtype. This indicates that RpoS is not required for complete induction of these proteins and that it may be repressing their full expression. Furthermore, three of the twenty-four proteins were expressed at a higher level in the wildtype than in the Δ*rpoS* strain, which indicates that RpoS is required for full induction of these proteins. Therefore, only seven of these proteins showed a similar fold change in both strains (Table 4). The protein abundance of ten of those proteins was similar between the wildtype and the Δ*rpoS* strain. To determine whether a protein was expressed as RpoS-independent, the differentially expressed proteins in wildtype were compared to the Δ*rpoS* strain. For instance, during late stationary phase, if the proteins that were most highly induced in the wildtype were similarly highly induced in the Δ*rpoS* strain, they were classified as RpoS-independent. However, proteins that were elevated during late stationary phase, that do not change in the Δ*rpoS* strain, were classified as RpoS-dependent.

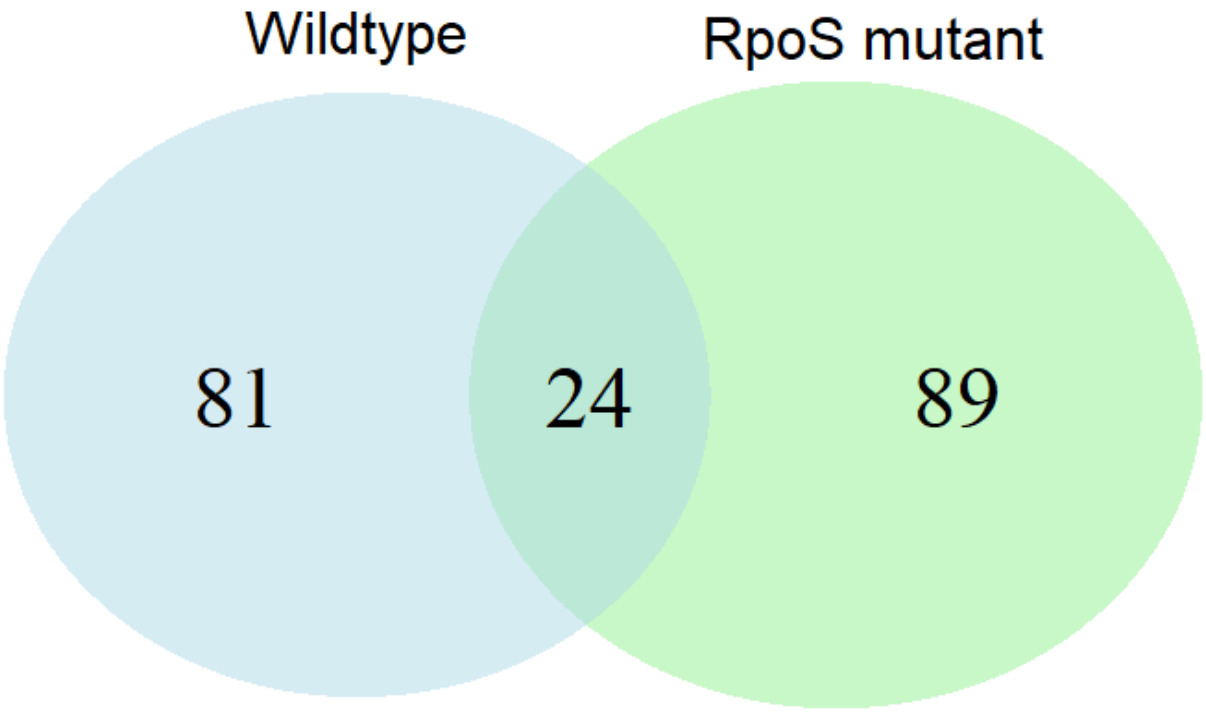


**Figure 17. Proteins upregulated (>2-fold) during early stationary phase compared to exponential phase in wildtype and Δ*rpoS* strains.**
Twenty-four proteins were upregulated in both wildtype and Δ*rpoS* strains.

**Table 4.** Proteins upregulated (>2-fold) during early stationary phase (6 h) compared to exponential phase (4 h).

| | | | **Wildtype** | **Δ*rpoS* strain** |
|---|---|---|---|---|
| **Accession ID*** | **Description** | **Gene** | **FC⁺ 6h/4h** | **FC⁺ 6h/4h** |
| C4ZZR7 | Sulfite reductase [NADPH] hemoprotein beta-component | *cysI* | 8.9 | 6.8 |
| P37330 | Malate synthase G | *glcB* | 8.1 | 2.8 |
| P0A853 | Tryptophanase | *tnaA* | 7.8 | 16.2 |
| C4ZZQ5 | Sulfate adenylyltransferase subunit 1 | *cysN* | 7.1 | 16.5 |
| P38038 | Sulfite reductase [NADPH] flavoprotein alpha-component | *cysJ* | 6.7 | 3.3 |
| P23173 | Low affinity tryptophan permease | *tnaB* | 6.7 | 16.6 |
| C4ZTE1 | Anti-adapter protein IraP | *iraP* | 6.2 | 8.2 |
| P77318 | Uncharacterized sulfatase YdeN | *ydeN* | 5.4 | 14.7 |
| P0AA31 | Putative sulfur carrier protein YedF | *yedF* | 5.4 | 24.9 |
| **P23538** | Phosphoenolpyruvate synthase | *ppsA* | 5.4 | 5.8 |
| P25889 | NAD-dependent dihydropyrimidine dehydrogenase subunit PreA | *preA* | 3.9 | 30.4 |
| **P06720** | Alpha-galactosidase | *melA* | 3.7 | 4.8 |
| P69831 | PTS system galactitol-specific EIIC component | *gatC* | 3.6 | 7.4 |
| P0ABQ2 | 2-hydroxy-3-oxopropionate reductase | *garR* | 3.5 | 12.2 |
| P31064 | UPF0394 inner membrane protein YedE | *yedE* | 3.3 | 5.2 |
| **P37188** | PTS system galactitol-specific EIIB component | *gatB* | 3.0 | 3.9 |
| **B1X775** | Glucose-1-phosphate adenylyltransferase | *glgC* | 2.8 | 3.0 |
| **P37903** | Universal stress protein F | *uspF* | 2.7 | 3.8 |

| | | | Wildtype | Δ*rpoS* strain |
|---|---|---|---|---|
| **Accession ID*** | **Description** | **Gene** | **FC[+] 6h/4h** | **FC[+] 6h/4h** |
| P15078 | Carbon starvation protein A | *cstA* | 2.7 | 15.0 |
| Q46863 | Probable deoxycholate-binding periplasmic protein YgiS | *ygiS* | 2.5 | 21.9 |
| B1XDH5 | Phosphoadenosine phosphosulfate reductase | *cysH* | 2.4 | 20.1 |
| **P61889** | Malate dehydrogenase | *mdh* | 2.3 | 2.0 |
| P0ABK5 | Cysteine synthase A | *cysK* | 2.3 | 5.0 |
| **C4ZRQ6** | UPF0325 protein YaeH | *yaeH* | 2.1 | 3.1 |

*Proteins that show a similar fold-change between the wildtype and Δ*rpoS* strains are **bolded**. [+]FC=Fold Change

Ninety-five proteins were upregulated (>2-fold) in late stationary phase compared to early stationary phase. Eighteen of these proteins were upregulated (>2-fold) in both strains (Fig. 18). However, six of these proteins were induced at a higher level in the Δ*rpoS* strain than in the wildtype. This indicates that RpoS is not required for complete induction of these proteins and that it may be repressing their full expression. Additionally, three of the eighteen proteins were more elevated in the wildtype than in the Δ*rpoS* strain suggesting that these two proteins are positively regulated by RpoS. Therefore, of the proteins that were most highly induced during late stationary phase compared to early stationary (upregulated >2-fold), 9 proteins were similarly upregulated (Table 5).

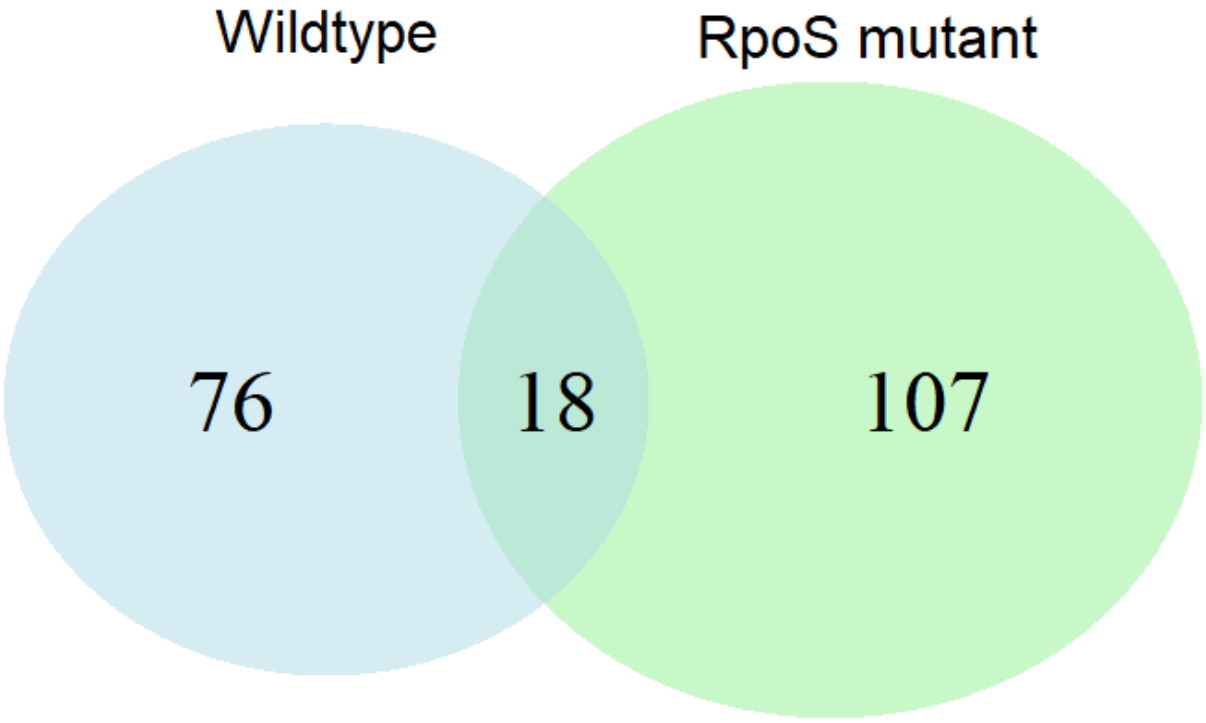


**Figure 18. Proteins upregulated (>2-fold) during late stationary phase compared to early stationary phase in wildtype and Δ*rpoS* strains.**
Eighteen proteins were upregulated in both wildtype and Δ*rpoS* strains.

**Table 5**. Proteins upregulated (>2-fold) during late stationary phase (24 h) compared to early stationary phase (6 h).

| | | | **Wildtype** | **Δ*rpoS* strain** |
|---|---|---|---|---|
| **Accession ID*** | **Description** | **Gene** | **FC⁺ 24h/6h** | **FC⁺ 24h/6h** |
| Q2M7R5 | Uncharacterized protein YibT | *yibT* | 7.6 | 3.2 |
| P0A991 | Fructose-bisphosphate aldolase class 1 | *fbaB* | 7.3 | 2.3 |
| P65292 | Uncharacterized lipoprotein YgdI | *ygdI* | 5.7 | 3.6 |
| **P76172** | Uncharacterized protein YnfD | *ynfD* | 4.8 | 3.5 |
| P0AD59 | Inhibitor of vertebrate lysozyme | *ivy* | 4.6 | 12.3 |
| **P0AC59** | Glutaredoxin 2 | *grxB* | 4.0 | 4.1 |
| P09551 | Lysine/arginine/ornithine-binding periplasmic protein | *argT* | 3.9 | 12.0 |
| P0A9G6 | Isocitrate lyase | *aceA* | 3.8 | 12.4 |
| B1XEA4 | Autoinducer 2-binding protein LsrB | *lsrB* | 3.5 | 8.2 |
| **P0AET8** | 7-alpha-hydroxysteroid dehydrogenase | *hdhA* | 2.8 | 2.6 |
| P77739 | Putative kinase YniA | *yniA* | 2.8 | 4.9 |
| **P0AF50** | Uncharacterized protein YjbR | *yjbR* | 2.8 | 2.7 |
| **P77754** | Periplasmic chaperone Spy | *spy* | 2.7 | 5.3 |
| **P33012** | DNA gyrase inhibitor | *sbmC* | 2.5 | 2.1 |
| **P76143** | 3-hydroxy-5-phosphonooxypentane-2,4-dione thiolase | *lsrF* | 2.3 | 2.8 |
| P37903 | Universal stress protein F | *uspF* | 2.3 | 4.2 |

| | | | Wildtype | Δ*rpoS* strain |
|---|---|---|---|---|
| **Accession ID*** | **Description** | **Gene** | **FC[+] 24h/6h** | **FC[+] 24h/6h** |
| **B1XD59** | UPF0253 protein YaeP | *yaeP* | 2.1 | 3.3 |
| **P0A9D2** | Glutathione S-transferase GstA | *gstA* | 2.1 | 3.7 |

*Proteins that show a similar fold-change between the wildtype and Δ*rpoS* strains are **bolded**. [+]FC=Fold Change

Finally, during prolonged stationary phase, there were 98 proteins that were upregulated (>2-fold) compared to early stationary phase. Twenty-six of these proteins were induced in both wildtype and Δ*rpoS* strains (Fig. 19). However, seven of these proteins were induced at a substantially higher level in the Δ*rpoS* strain than in the wildtype. This indicates that RpoS is not required for complete induction of these proteins and that it may be repressing their full expression. Furthermore, seven of the twenty-six proteins were more highly induced in the wildtype compared to the Δ*rpoS* strain indicating that these seven proteins are positively regulated by RpoS. As such, twelve of the twenty-six proteins were similarly upregulated during prolonged stationary phase in this study (Table 6).

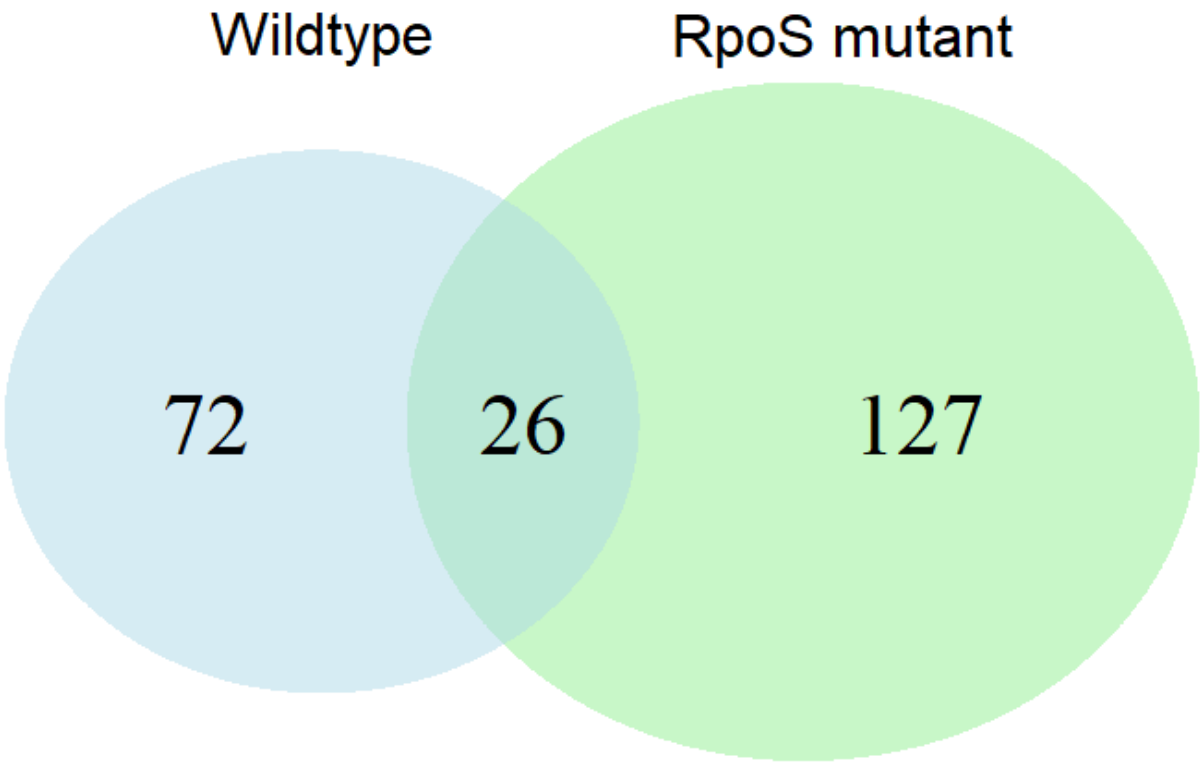


**Figure 19. Proteins upregulated (>2-fold) during prolonged stationary phase compared to early stationary phase in wildtype and Δ*rpoS* strains.**
Eighteen proteins were upregulated in both wildtype and Δ*rpoS* strains.

**Table 6.** Proteins upregulated (>2-fold) during prolonged stationary phase (48 h) compared to early stationary phase (6 h).

| | | | **Wildtype** | **Δ*rpoS* strain** |
|---|---|---|---|---|
| **Accession ID*** | **Description** | **Gene** | **FC⁺ 48h/6h** | **FC⁺ 48h/6h** |
| P65292 | Uncharacterized lipoprotein YgdI | *ygdI* | 9.1 | 5.9 |
| P0A991 | Fructose-bisphosphate aldolase class 1 | *fbaB* | 8.8 | 3.6 |
| P31658 | Protein deglycase 1 | *hchA* | 6.7 | 2.0 |
| P09551 | Lysine/arginine/ornithine-binding periplasmic protein | *argT* | 6.3 | 11.3 |
| **P0ADE6** | Uncharacterized protein YgaU | *kbp* | 6.2 | 5.8 |
| P0AD59 | Inhibitor of vertebrate lysozyme | *ivy* | 5.8 | 9.3 |
| P37685 | Aldehyde dehydrogenase B | *aldB* | 5.6 | 2.7 |
| P76113 | NADPH-dependent curcumin reductase | *curA* | 5.5 | 3.8 |
| **P0AET8** | 7-alpha-hydroxysteroid dehydrogenase | *hdhA* | 5.2 | 4.7 |
| P0ABD3 | Bacterioferritin | *bfr* | 5.1 | 8.3 |
| P0ADB1 | Osmotically-inducible putative lipoprotein OsmE | *osmE* | 5.0 | 3.3 |
| **B1XEA4** | Autoinducer 2-binding protein LsrB | *lsrB* | 4.1 | 3.0 |
| P0A915 | Outer membrane protein W | *ompW* | 3.9 | 2.0 |
| **P0AC59** | Glutaredoxin 2 | *grxB* | 3.8 | 2.5 |
| **Q46845** | Disulfide-bond oxidoreductase YghU | *yghU* | 3.4 | 3.6 |
| **P0A912** | Peptidoglycan-associated lipoprotein | *pal* | 3.1 | 2.3 |
| **P76143** | 3-hydroxy-5-phosphonooxypentane-2,4-dione thiolase | *lsrF* | 3.0 | 3.7 |
| **P25526** | Succinate-semialdehyde dehydrogenase [NADP(+)] GabD | *gabD* | 3.0 | 2.6 |

| | | | **Wildtype** | **Δ*rpoS* strain** |
|---|---|---|---|---|
| **Accession ID*** | **Description** | **Gene** | **FC[+] 48h/6h** | **FC[+] 48h/6h** |
| P02925 | Ribose import binding protein RbsB | *rbsB* | 2.7 | 5.5 |
| **P0A9D2** | Glutathione S-transferase GstA | *gstA* | 2.5 | 2.7 |
| **P22256** | 4-aminobutyrate aminotransferase GabT | *gabT* | 2.5 | 2.0 |
| **P77754** | Periplasmic chaperone Spy | *spy* | 2.5 | 3.3 |
| P0ADV7 | Probable phospholipid-binding protein MlaC | *mlaC* | 2.5 | 4.8 |
| **P0ADG7** | Inosine-5'-monophosphate dehydrogenase | *guaB* | 2.0 | 3.0 |
| **P0A9S5** | Glycerol dehydrogenase | *gldA* | 2.0 | 2.3 |
| P19926 | Glucose-1-phosphatase | *agp* | 2.0 | 5.9 |

*Proteins that show a similar fold-change between the wildtype and Δ*rpoS* strains are **bolded**. [+]FC=Fold Change

RpoS-dependent and RpoS-independent fusions exhibited a > 5-fold induction as the cells entered stationary phase (Schellhorn *et al.*, 1998). These results suggest that there are genes that are expressed in an RpoS-independent manner, that are highly induced during the transition from exponential phase to stationary phase. This indicates that RpoS-dependent genes may only account for a small proportion of the genes required for during stationary phase (Schellhorn *et al.*, 1998). To determine the RpoS-independent proteins, differentially expressed proteins in the wildtype and Δ*rpoS* strains were compared. Proteins that were more abundant during late stationary phase compared to early stationary phase in both strains, were considered RpoS-independent.

Both RpoS-dependent and independent proteins were expressed during late and prolonged stationary phase. Here we identified proteins that were upregulated (>5-fold) during stationary phase (compared to exponential phase). During the transition from exponential phase to early stationary phase, eight proteins were induced in the wildtype and Δ*rpoS* strains (Table 7). However, five of these proteins were induced at a substantially higher level in the Δ*rpoS* strain than in the wildtype. This indicates that RpoS is not required for complete induction of these proteins and that it may be repressing their full expression. On the other hand, there are three proteins that show similar fold-change in the two strains.

**Table 7. Proteins induced (>5-fold) during early stationary phase (6 h) compared to exponential phase (4 h).**

| | | | **Wildtype** | **Δ*rpoS* strain** |
|---|---|---|---|---|
| **Accession ID*** | **Gene** | **Description** | **FC[+] 6h/4h** | **FC[+] 6h/4h** |
| P0AA31 | *yedF* | Putative sulfur carrier protein YedF | 5.4 | 24.9 |
| P23173 | *tnaB* | Low affinity tryptophan permease | 6.7 | 16.6 |
| **C4ZTE1** | *iraP* | Anti-adapter protein IraP | 6.2 | 8.2 |
| P0A853 | *tnaA* | Tryptophanase | 7.8 | 16.2 |
| P77318 | *ydeN* | Uncharacterized sulfatase YdeN | 5.4 | 14.7 |
| **C4ZZR7** | *cysI* | Sulfite reductase [NADPH] hemoprotein beta-component | 8.9 | 6.8 |
| **P23538** | *ppsA* | Phosphoenolpyruvate synthase | 5.4 | 5.8 |
| C4ZZQ5 | *cysN* | Sulfate adenylyltransferase subunit 1 | 7.1 | 16.5 |

*Proteins that show a similar fold-change between the wildtype and Δ*rpoS* strains are **bolded**. [+]FC=Fold Change

Furthermore, to determine whether proteins that are induced (>5-fold) were identified during late and prolonged stationary phase, we examined the proteins that were differentially expressed during these growth phases. We identified 21 and 18 proteins that were upregulated (>5-fold) during late stationary and prolonged stationary phase, respectively (Table 8, Table 9). During late stationary phase, eight of the 21 proteins that were upregulated (>5-fold) in both strains, were induced at a higher level in the Δ*rpoS* strain, which indicates that RpoS is not required for full induction of these proteins. Additionally, four of the total number of proteins that were upregulated (>5-fold) during late stationary phase compared to exponential phase were induced substantially higher in the wildtype. This suggests that RpoS is required for the full induction of these proteins, but they still play an important role during late stationary phase when RpoS expression has decreased. The remaining nine proteins induced (>5-fold) during late stationary phase, showed similar induction between the two strains.

**Table 8. Proteins induced (>5-fold) during late stationary phase (24 h) compared to exponential phase (4 h).**

| | | | **Wildtype** | **Δ*rpoS* strain** |
|---|---|---|---|---|
| **Accession ID*** | **Gene** | **Description** | **FC$^+$ 24h/4h** | **FC$^+$ 24h/4h** |
| P09551 | *argT* | Lysine/arginine/ornithine-binding periplasmic protein | 15.5 | 6.2 |
| **P16700** | *cysP* | Thiosulfate-binding protein | 12.0 | 11.3 |
| P0AD59 | *ivy* | Inhibitor of vertebrate lysozyme | 13.0 | 6.4 |
| P76621 | *glaH* | Protein CsiD | 15.3 | 6.4 |
| P56262 | *ysgA* | Putative carboxymethylenebutenolidase | 13.3 | 5.3 |
| **P0AET8** | *hdhA* | 7-alpha-hydroxysteroid dehydrogenase | 9.6 | 7.2 |
| **P0AFX0** | *hpf* | Ribosome hibernation promoting factor | 9.0 | 7.5 |
| **P0AC59** | *grxB* | Glutaredoxin 2 | 9.5 | 7.1 |
| **P76143** | *lsrF* | 3-hydroxy-5-phosphonooxypentane-2,4-dione thiolase | 7.0 | 5.6 |
| **P0AF50** | *yjbR* | Uncharacterized protein YjbR | 7.6 | 5.3 |
| P19926 | *agp* | Glucose-1-phosphatase | 6.4 | 14.2 |
| **P0A9D2** | *gstA* | Glutathione S-transferase GstA | 6.8 | 6.8 |
| P37387 | *xylF* | D-xylose-binding periplasmic protein | 5.9 | 12.9 |

| | | | **Wildtype** | **Δ*rpoS* strain** |
|---|---|---|---|---|
| **Accession ID*** | **Gene** | **Description** | **FC[+] 24h/4h** | **FC[+] 24h/4h** |
| **P33012** | *sbmC* | DNA gyrase inhibitor | 8.6 | 5.2 |
| P0ACW6 | *ydcH* | Uncharacterized protein YdcH | 5.0 | 29.0 |
| P0A9G6 | *aceA* | Isocitrate lyase | 13.1 | 20.3 |
| P37903 | *uspF* | Universal stress protein F | 10.0 | 16.0 |
| C4ZTE1 | *iraP* | Anti-adapter protein IraP | 7.0 | 18.3 |
| P77739 | *yniA* | Putative kinase YniA | 9.5 | 14.5 |
| **P27550** | *acs* | Acetyl-coenzyme A synthetase | 10.6 | 8.7 |
| P08997 | *aceB* | Malate synthase A | 13.1 | 18.6 |

*Proteins that show a similar fold-change between the wildtype and Δ*rpoS* strains are **bolded**. [+]FC=Fold Change

Finally, many of the proteins that were induced (>5-fold) during late stationary phase were also induced (>5-fold) during prolonged stationary phase. Of the 18 proteins that were upregulated (>5-fold) in both strains, four proteins were more highly induced in the Δ*rpoS* strain, which indicates RpoS may repress these proteins during prolonged stationary phase. Additionally, five proteins were more highly induced in the wildtype, which indicates that RpoS is required for the full induction of these proteins, but they still play an important role during prolonged stationary phase in the absence of RpoS. The remaining nine proteins were similarly induced between the two strains.

**Table 9. Proteins induced (>5-fold) during prolonged stationary phase (48 h) compared to exponential phase (4 h)**.

| | | | **Wildtype** | **Δ*rpoS* strain** |
|---|---|---|---|---|
| **Accession ID*** | **Gene** | **Description** | **FC$^+$ 48h/4h** | **FC$^+$ 48h/4h** |
| B1X775 | *glgC* | Glucose-1-phosphate adenylyltransferase | 5.8 | 11.5 |
| **P06720** | *melA* | Alpha-galactosidase | 5.3 | 5.4 |
| P09551 | *argT* | Lysine/arginine/ornithine-binding periplasmic protein | 19.9 | 5.7 |
| **P0A9D2** | *gstA* | Glutathione S-transferase GstA | 7.6 | 5.4 |
| P0A9G6 | *aceA* | Isocitrate lyase | 5.5 | 17.2 |
| **P0AC44** | *sdhD* | Succinate dehydrogenase hydrophobic membrane anchor subunit | 6.7 | 6.9 |
| **P0ACW6** | *ydcH* | Uncharacterized protein YdcH | 5.8 | 18.2 |
| **P0ACX3** | *ydhR* | Putative monooxygenase YdhR | 6.0 | 5.4 |
| P0AET8 | *hdhA* | 7-alpha-hydroxysteroid dehydrogenase | 13.7 | 10.5 |
| P16700 | *cysP* | Thiosulfate-binding protein | 15.1 | 5.0 |
| P19926 | *agp* | Glucose-1-phosphatase | 7.9 | 17.3 |
| **P37387** | *xylF* | D-xylose-binding periplasmic protein | 6.6 | 7.1 |
| **P37902** | *gltI* | Glutamate/aspartate import solute-binding protein | 5.0 | 5.8 |

| | | | **Wildtype** | **Δ*rpoS* strain** |
|---|---|---|---|---|
| **Accession ID*** | **Gene** | **Description** | **FC[+] 48h/4h** | **FC[+] 48h/4h** |
| **P38038** | *cysJ* | Sulfite reductase [NADPH] flavoprotein alpha-component | 5.8 | 8.4 |
| P56262 | *ysgA* | Putative carboxymethylenebutenolidase | 14.8 | 5.5 |
| **P76143** | *lsrF* | 3-hydroxy-5-phosphonooxypentane-2,4-dione thiolase | 8.2 | 6.9 |
| P76621 | *glaH* | Protein CsiD | 15.0 | 10.5 |
| Q46845 | *yghU* | Disulfide-bond oxidoreductase YghU | 9.2 | 6.2 |

*Proteins that show a similar fold-change between the wildtype and Δ*rpoS* strains are **bolded**. [+]FC=Fold Change

### 3.7 Is the expression of all proteins dynamic during growth?

To assess which proteins were most stable during growth, a statistic (similar to the chi-square statistic) of the normalized protein abundance was calculated for all the proteins that were quantified in at least 2 replicates in the wildtype. The proteins were sorted based on this statistic from smallest to largest where the protein with the smaller values, were the least variant during growth and the larger values were differed the most across the four growth phases. The abundance of the top ten proteins during all four growth phases (least variant) are presented here (Table 10).

**Table 10**. The top ten least variant proteins during long-term growth.

| Accession ID | Gene | Exponential Phase (4 h) | Early Stationary Phase (6 h) | Late Stationary Phase (24 h) | Prolonged Stationary Phase (48 h) | Average | Standard Deviation | Variance Statistic* |
|---|---|---|---|---|---|---|---|---|
| P0A870 | *talB* | 94.1 | 97.3 | 95.9 | 101.4 | 97.2 | ± 2.68 | 0.30 |
| P0AFK0 | *pmbA* | 111.3 | 111.8 | 109.8 | 102.4 | 108.8 | ± 3.76 | 0.52 |
| C5A021 | *pepQ* | 90.1 | 88.5 | 93.9 | 97.5 | 92.5 | ± 3.48 | 0.52 |
| P21513 | *rne* | 89.5 | 88.7 | 98.1 | 93.4 | 92.4 | ± 3.73 | 0.60 |
| P39356 | *yjhU* | 98.4 | 102.5 | 98.9 | 109.6 | 102.3 | ± 4.47 | 0.78 |
| P69805 | *manZ* | 115.4 | 125.2 | 111.0 | 116.7 | 117.1 | ± 5.12 | 0.90 |
| C4ZX91 | *rodZ* | 101.6 | 103.1 | 111.5 | 99.1 | 103.8 | ± 4.66 | 0.84 |
| C4ZUK3 | *slyX* | 100.7 | 91.6 | 103.3 | 96.2 | 98.0 | ± 4.48 | 0.82 |
| P09147 | *galE* | 99.4 | 111.4 | 99.3 | 106.6 | 104.2 | ± 5.10 | 1.00 |
| P0ADT5 | *ygiC* | 102.6 | 114.0 | 100.4 | 108.1 | 106.3 | ± 5.27 | 1.05 |

*Statistic used to calculate the similarity/difference in the abundance of each protein is across the four growth phases tested.

# 4.0 Discussion

Gene expression during extended growth has been extensively studied in *E. coli*, however, there is limited information regarding protein expression during prolonged periods of growth. Additionally, RpoS is a key regulator during stationary phase, however, there are stationary phase genes whose expression is elevated independent of RpoS (Schellhorn *et al.*, 1998). This suggests that RpoS-dependent genes only account for a small set of genes that are induced during stationary phase. This study highlights the global protein expression profile in the wildtype and Δ*rpoS* strains using TMT during extended growth. Furthermore, the use of *rpoS*$^{+}$ and *rpoS*$^{-}$ strains allowed us to identify proteins that were negatively or positively regulated by RpoS and those that were expressed in an RpoS-independent manner. Here we confirm that there are stationary phase proteins that are elevated (>5-fold) independent of RpoS. Additionally, we explore the proteomic profile of sigma factors and DNA binding proteins in *E. coli.* Proteins that were regulated by RpoS, positively or negatively, were also identified.

## 4.1 The Expression Profile of Sigma Factors in *E. coli*

In *E. coli*, there are seven known sigma factors, each of which modulates a set of genes in response to external conditions (Ishihama, 2000). These include RpoD, RpoS, RpoN, FecI, FliA, RpoE and RpoH. In this work, we identified 5 of the 7 sigma factors, namely, RpoD, RpoN, RpoS, FliA and RpoE. RpoD slightly decreased upon entry into stationary phase and remained relatively unchanged during stationary phase. As expected, RpoD, the housekeeping sigma factor, was consistently expressed during all growth stages in both strains. RpoS is a major regulator during stationary phase and many stress conditions and is therefore induced upon entry into stationary phase. RpoS has the lowest affinity for RNAP core enzyme (Kusano *et al.*, 1996), yet it out-

competes RpoD, the primary sigma factor. Many factors improve the ability of RpoS to bind to RNAP. One such factor is the anti-sigma factor Rsd, which increases in stationary phase, and enables RpoS to out-compete RpoD for the enzyme base of RNAP by reducing the affinity of RpoD to RNAP (Jishage *et al.*, 2002). Consistent with previous findings, Rsd was upregulated 3.4-fold upon entry into early stationary phase compared to exponential phase and remained relatively unchanged during late stationary phase. This is consistent with its role in decreasing the affinity of RpoD for the sigma subunit of RNAP, allowing RpoS to bind and direct transcription of stationary phase genes. During late stationary phase, the abundance of RpoS decreased indicating that the major role of RpoS is during early stationary phase. Additionally, the abundance of Rsd decreased during prolonged stationary phase.

FliA (RpoF) is responsible for flagellar synthesis and chemotaxis in *E. coli*. FliA increased upon entry into early stationary phase, however, during late and prolonged stationary phase, FliA decreased substantially in the wildtype. There was a 4.8-fold and 2.0-fold decrease in abundance of FliA during late and prolonged stationary phase, respectively, compared to early stationary phase. FliA was similarly expressed in the Δ*rpoS* strain, however, though FliA levels decreased during late and prolonged stationary phase, the decrease was not as substantial as in the wildtype. This is consistent with previous findings, derived from microarray and immunoblot analyses, that show that FliA decreases during stationary phase in both wildtype and Δ*rpoS* strain, but the decrease in wildtype is more substantial than that of Δ*rpoS* strain (Makinoshima *et al.*, 2003; Patten *et al.*, 2004). RpoS negatively regulates FliA during late and prolonged stationary phase, which would explain the difference between the two strains (Makinoshima *et al.*, 2003; Patten *et al.*, 2004). RpoE increases in response to heat shock but remains constant under most other stress

conditions (Jishage *et al.,* 1996). In this work, RpoE remained relatively unchanged during all tested growth phases in both strains.

Finally, RpoN steadily decreased throughout all four growth phases in the wildtype and Δ*rpoS* strains. However, in the Δ*rpoS* strain, RpoN markedly increased during prolonged stationary phase suggesting that in the absence of RpoS, RpoN is required during long-term growth. RpoN is primarily responsible for the transcription of genes implicated in nitrogen utilization (Ishihama, 2000). RpoN and RpoS negatively affect the expression of each other (Dong *et al.,* 2011). As such, the levels of RpoN in the Δ*rpoS* strain were consistently higher than those in the wildtype strain. Consistent with previous findings (Dong *et al.,* 2011), the level of RpoN was higher in exponential phase than early stationary phase, however, the expression of RpoN during late and prolonged stationary phase was not examined. In the current study, the level of RpoN was also higher in exponential phase than in early stationary phase and late stationary phase. However, RpoN increased (5.8-fold) during prolonged stationary compared to early stationary phase. The determination that RpoN expression was unaffected by RpoS, only considered expression during early stationary phase. The current data suggests that in the absence of RpoS, RpoN may play an important role during long-term growth conditions and may be affected by RpoS during extended growth.

## 4.2 The Expression Profile of DNA Binding Proteins in *E. coli*

DNA protection during starvation, Dps, was highly abundant during late stationary phase and prolonged stationary phase. Dps was upregulated by 7.9-fold and 9.6-fold in late stationary phase and prolonged stationary phase, respectively, compared to early stationary phase. However, Dps showed no significant change in the Δ*rpoS* strain confirming its dependence on RpoS for induction during stationary phase. Dps is one of the most abundant proteins during stationary

growth phase in *E. coli* and like many other proteins induced during stationary phase, it is induced in an RpoS-dependent manner (Almiron *et al.*, 1992; Nair and Finkel, 2004). Dps is crucial for survival in starvation conditions as well as multiple other stress conditions (Karas *et al.*, 2015; Nair and Finkel, 2004). The production of Dps is at it's highest between 6-7 d during extended incubation (Farrell and Finkel, 2003). However, the levels of Dps increased by approximately 4-fold between 30 h and 4 d of stationary phase growth suggesting that it may be required to maintain the integrity of the cell earlier in stationary phase (Soufi *et al.*, 2015). This finding is consistent with our current finding, which suggests that Dps may play an important role in protecting from cellular damage as early as 24 h during stationary growth phase. On the other hand, Fis expression decreased dramatically and was among the low abundance proteins during stationary phase. Fis was downregulated by 2.7-fold, 6.6-fold and 4.8-fold during early stationary phase compared to exponential phase and late stationary phase and prolonged stationary, compared to early stationary phase, respectively. The decrease in the level of Fis confirms that its expression is growth-phase dependent (Bradley *et al.*, 2007; Xu and Johnson, 1995). The expression profile of Fis in both strains was similar and therefore, Fis is RpoS-independent.

H-NS, a global regulator for gene expression, is a DNA binding protein that has been implicated in transcriptional repression by binding to A-T rich double-stranded DNA and inhibiting transcription (Chib and Mahadevan, 2012; Yamada *et al.*, 1990). Additionally, H-NS has been implicated in the protection of cells against stress (Erol *et al.*, 2006). In the current study, H-NS steadily increased throughout all four growth phases with the greatest increase occurring during prolonged stationary phase. This suggests that H-NS plays an important role during prolonged stationary phase, which is consistent with previous reports (Chib and Mahadevan, 2012). The binding regions for StpA overlap with those of H-NS in *E. coli* (Ueda *et al.*, 2013). In

the absence of H-NS, StpA plays a key role in maintaining DNA topology (Uyar *et al.*, 2009; Zhang *et al.*, 1996). Therefore, it is a "molecular backup" of H-NS in *E. coli* (Sonden and Uhlin, 1996; Zhang *et al.*, 1996). StpA decreased during early stationary phase, continued to decrease during late stationary phase and increased slightly during prolonged stationary phase. The decrease in early stationary phase is consistent with previous reports (Talukder and Ishihama, 2015). The abundance profile of H-NS and StpA were similar in both strains indicating that the expression of these proteins is RpoS-independent.

In most bacteria, HU is a homodimer, however, it is encoded by two paralogs in *E. coli, hupA* and *hupB* (Kano *et al.*, 1988; Kano *et al.*, 1986), which encode HUα and HUβ, respectively. Consistent with previous findings (Williams and Foster, 2007), HUα was expressed at a higher level in exponential phase than HUβ, while both HUα and HUβ were both upregulated in early stationary phase. As such, the heterodimer HUαβ will be the most abundant of the three forms of HU during stationary phase (Rouviere-Yaniv and Kjeldgaard, 1979; Williams and Foster, 2007). HUαβ is necessary for survival during nutrient-limited conditions in *E. coli* (Claret and Rouviere-Yaniv, 1997).

## 4.3 Amino Acid Metabolism during Growth

During prolonged stationary phase, enzymes implicated in catabolic processes were upregulated (>2-fold) compared to early stationary phase and expressed in an RpoS-dependent manner. Amino acid metabolism encompasses a class of proteins that were statistically over-represented during prolonged stationary phase in the wildtype. These processes degrade amino acids to utilize their products as a source of carbon, nitrogen and energy. For many bacteria, carbon catabolism is the source of energy generation. *E. coli* can utilize many different carbon sources; however, its preferred carbon source is glucose, which supports more rapid growth compared to

other carbon sources (Bren *et al.*, 2016). During exponential phase, glucose is the sole source of carbon for *E. coli.* As glucose is consumed, acetate is produced as a fermentation product under aerobic conditions. Once glucose is depleted and the cells enter early stationary phase, acetate is utilized as the source of carbon (Akesson *et al.*, 1999). Moreover, once acetate is used up, the cell switches to the use of amino acids as a source of carbon and nitrogen during stationary phase. The cells first consume amino acids that can easily be utilized including L-serine, L-tryptophan, L-glutamate, L-alanine, L-glycine and L-aspartate. Once these amino acids are depleted and then other amino acids that are more difficult to utilize are consumed including L-arginine, L-glutamine, L-asparagine, L-cysteine and L-lysine. Consistently, in this work AstC and AstD, encoded by members of the *ast* operon (*astC* and *astD,* respectively), which are implicated in the L-arginine degradation pathway, were upregulated during prolonged stationary phase. These proteins steadily increased from exponential phase to prolonged stationary phase in both strains. However, the protein levels in the wildtype were substantially higher than in the Δ*rpoS* strain. This indicates that RpoS may play a role in the regulation of members of this operon.

## 4.4 RpoS-dependent Protein Expression during Stationary Phase

Many RpoS-dependent proteins peaked during late stationary phase, but the level of expression was sustained during prolonged stationary phase. This is contrary to the expression profile of RpoS since RpoS peaks upon entry into early stationary phase and declines during late stationary and prolonged stationary phase. RpoS is induced during the transition from exponential phase to early stationary phase. As such, proteins that are regulated by RpoS at the gene level are expected to be induced at this time. We identified over 200 proteins that were at least two-fold higher in abundance in the wildtype strain than in the Δ*rpoS* strain. This number decreased as the cells entered late and prolonged stationary phase. This suggests that RpoS activity is reduced after

early stationary phase adaptation of the cells. This is consistent with previous transcriptomic studies that indicate a decrease in RpoS activity during late and prolonged stationary(Patten et al., 2004). This decrease could be due to a decrease in the activity of the sigma factor or a decrease in the levels present. Our data indicate that the reason for the reduction in the abundance of RpoS-dependent proteins is because of the reduced levels of RpoS during later and prolonged stationary phase. Despite this, there are RpoS-controlled proteins that were highly abundant during late and prolonged stationary phase. These include prototypical RpoS-dependent proteins Dps, OsmY and KatE (Fig. 13). Interestingly, the transcript abundance, determined using RT-qPCR, of *katE* peaked upon entry into early stationary phase and decreased during late and prolonged stationary phase (Sharma, 2016). The transcript abundance of *dps* and *osmY* both peaked during late stationary phase and decreased during prolonged stationary phase (Sharma, 2016). The expression of these proteins in this work indicate that their role may not be limited to early stationary phase and their regulation may not be limited to RpoS. This idea is consistent with the observation that some RpoS-controlled promoters such as t*alA, elaB* and *hdeA* have continued activity during extended incubation (Sharma, 2016). TalA was upregulated 2.9-fold, 7.1-fold and 8.6-fold in early, late and prolonged stationary phase, respectively. TalA is encoded by the gene *talA*, which is a part of a two-member operon, *talA-tktB.* The second member of this operon, *tktB*, which encodes the protein TktB, was also upregulated 1.5-fold, 5.5-fold and 6.0-fold in early, late and prolonged stationary phase, respectively. Both TalA and TktB were maintained at a low-level abundance during all four growth phases in the Δ*rpoS* strain, therefore, the expression of these proteins in the wildtype increased in an RpoS-dependent manner. On the other hand, the protein abundance of a paralogous system, *talB-tktA*, was not upregulated during stationary phase. TalB, which is encoded by *talB*, remained relatively unchanged during all four growth phases in the wildtype, however,

the abundance of TalB continuously increased during stationary phase in the Δ*rpoS* strain. TktA, which is encoded by *tktA,* decreased during stationary phase in the wildtype but similar to TalB, the abundance of TktA steadily increased during stationary phase in the Δ*rpoS* strain. This suggests that *talB-tktA* may play an important role in extended periods of nutrient limitation but only in the absence of RpoS.

## 4.5 Negative Regulation by RpoS

Genes that are a part of the RpoS regulon are induced upon entry into stationary phase. However, RpoS can also play an important role as a repressor (Lévi-Meyrueis *et al.*, 2015). RpoS directs transcription of select genes in response to environmental change by binding to the sigma subunit of RNAP and directing it to the promoter region of stationary phase genes. RpoS competes with the housekeeping sigma factor, RpoD, and the other alternative sigma factors for the core of the RNAP enzyme. Furthermore, the activity of anti-sigma factors plays a key role in this process. For example, Rsd (an RpoS-controlled protein) interacts with RpoD, thus limiting its ability to bind RNAP, which inhibits the activity of RpoD (Jishage and Ishihama, 1999). Overexpression of RpoS attenuates the expression of genes that are controlled by RpoD, which is consistent with the ability of RpoS to suppress the activity of genes due to sigma factor competition (Farewell *et al.*, 1998; Nystrom, 2004). However, gene repression by RpoS is not solely the result of the competition between sigma factors. Negative regulation by RpoS may also depend on the negative effects of RNAP with RpoS at the DNA level of the promoter (Lévi-Meyrueis *et al.*, 2015). For instance, in sigma factor competition, RpoS is only required to bind to RNAP, the ability to bind to DNA is not necessary. An RpoS variant was constructed that was able to bind to RNAP but unable to bind to promoter DNA (Lévi-Meyrueis et al., 2015). During stationary phase, RpoS bound to RNAP but the expected gene repression by RpoS was not detected, suggesting that sigma

factor competition is not the sole factor responsible for gene repression by RpoS. Furthermore, it is possible that RpoS may activate negative effectors of particular genes, which are then repressed when RpoS increases in activity. Additionally, RpoS can also act as a repressor by blocking the promoter from RNAP (also a DNA binding protein), which interferes with transcription (Bendtsen et al., 2011; Grainger et al., 2008; Palmer et al., 2009; Zafar et al., 2014). Here we demonstrate that RpoS negatively affects proteins implicated in metabolism and flagella biosynthesis as well as proteins encoded by cryptic phage genes (Patten *et al.*, 2004), which may be the result of sigma factor competition, the induction of a negative regulator, or the negative effects of the RpoS bound sigma factor subunit of RNAP at the promoter DNA level.

### 4.5.1 Downregulation of Members of the Flagella Regulon by RpoS

There are many proteins that were downregulated during late and prolonged stationary phase compared to early stationary phase. These include 26 flagellar proteins, sixteen of which were downregulated more than 2-fold, during late stationary phase. In *E. coli*, the genes implicated in flagellar biosynthesis, and chemotaxis (receptors and signal transductors) are all a part of the same regulon: the flagellar regulon (Chilcott and Hughes, 2000). Flagellar proteins can be divided into three classes: I, II, III, which are temporally regulated and hierarchically expressed (Kutsukake and Iino, 1994; Macnab, 2003). The *flhDC* operon is the only member of class I and is, therefore, a master regulator of flagellar and motility proteins, directly activating class II proteins (Kutsukake and Iino, 1994; Macnab, 1992). Although neither of the two members of class I flagellar proteins were quantitated in the current study, members of class II operons under the control of *flhDC* (*flgAMN, flgBCDEFGHIJ, flhBAE, fliAZY, fliE, fliFGHIJK, and fliLMNOPQR*) (Macnab, 1992) were quantitated here. These are important regulatory and structural proteins. The flagellar synthesis and chemotaxis sigma factor, FliA, recognizes the promoter region for flagellar

synthesis and chemotactic genes and is a member of class II (Shimada *et al.*, 2017). FliA controls at least 30 genes implicated in flagellar synthesis and motility (Ide and Kutsukake, 1997). Many of the genes regulated by FliA are class III operons (*flgLK, fliDST, flgMN, fliC, tar-tap-cheRBYZ, motAB-cheAW*) (Macnab, 1992) including its own operon (*fliZY*) (Macnab, 1992), however, there are also class II operons that are controlled by FliA (*fliLMNOPQR*) (Fitzgerald *et al.*, 2014; Macnab, 1992). As previously discussed, FliA is negatively regulated by RpoS in late and prolonged stationary phase. Thus, we would expect that these proteins would also be downregulated during stationary phase. Current results confirm that proteins that are regulated by FliA were downregulated during stationary phase. Furthermore, this confirms that these proteins are negatively regulated by RpoS (Dong *et al.*, 2011; Dudin *et al.*, 2014; Makinoshima *et al.*, 2003; Patten *et al.*, 2004) (Table 3).

Motility and chemotaxis are important mechanisms in many global stress response networks (Amsler *et al.*, 1993). Flagellar synthesis has high metabolic costs to the cell (Macnab, 1992), therefore, synthesis is low in lag and exponential phases where the cells are primarily non-motile (Amsler *et al.*, 1993). The cell's need to expend energy on flagellar synthesis and motility is minimal due to the high nutrient and low toxic metabolic waste environment. However, as growth progresses and the cells enter post-exponential phase, the nutrients begin to become depleted and metabolic waste accumulates, and as such, the flagellar biosynthesis process is triggered (Adler and Templeton, 1967). However, when cells enter stationary phase, though the conditions have become increasingly stressful, flagellar synthesis decreases (Amsler *et al.*, 1993). When cells enter stationary phase, RpoS is the master regulator of many of the proteins expressed. As discussed previously, RpoS negatively regulates FliA, which in turn regulates many proteins implicated in flagellar synthesis. Thus, downregulation of these proteins during stationary phase

is expected. These results support the negative role of RpoS in regulating flagellar biosynthesis (Dong *et al.*, 2011; Makinoshima *et al.*, 2003; Patten *et al.*, 2004).

### 4.5.2 The Tricarboxylic Cycle (TCA) Enzyme Expression

During stationary phase, when nutrients are limited, RpoS decreases the expression of genes that are required for energy metabolism including enzymes that catalyzes each stage in the tricarboxylic cycle (TCA) (Patten *et al.*, 2004). For example, *acnB*, which encodes aconitase B, decreased during stationary phase (Gruer *et al.*, 1997) and its expression level was 2.4-fold higher in the Δ*rpoS* strain (Cunningham et al., 1997). Additionally, *sdhA*, which encodes a subunit of succinate dehydrogenase, is inhibited by RpoS (Xu and Johnson, 1995). The majority of the genes encoding enzymes of the TCA cycle were at least two-fold downregulated by RpoS (including but not limited to *gltA, fumA, sucABCD, acnB, sdhABCD*) (Patten *et al.*, 2004). This is consistent with our findings that many of the TCA cycle proteins were downregulated more than 2-fold by RpoS, including but not limited to GltA, FumA, SucABCD, AcnB, SdhABCD. This means that although these proteins may be downregulated in the Δ*rpoS* strain, the downregulation is substantially higher in the wildtype strain which indicates that RpoS represses the activity of these proteins during long-term growth. Furthermore, this confirms that other regulators (likely other sigma factors) play a key role in modulating this metabolic pathway and are able to do so more efficiently in the absence of RpoS – sigma factor competition (Farewell *et al.*, 1998). Two examples are the regulation of *icdA* by RpoD and Cra, and the regulation of *acnA* by RpoS and SoxRS (Gruer and Guest, 1994; Shimizu, 2013). Some of the proteins in the TCA cycle that are downregulated in the wildtype strain are upregulated in the Δ*rpoS* strain (Rahman *et al.*, 2006; Rahman and Shimizu, 2008), supporting the idea that enzymes that are a part of the TCA cycle are negatively regulated by RpoS but are required in the absence of RpoS during long-term growth.

### 4.5.3 Expression of Proteins Encoded by Prophage Genes

Cryptic prophages are those that become inactive as it relates to cell lysis, phage particle formation and plaque formation as a result of being trapped in the chromosome (Wang *et al.*, 2010). *E. coli* contains nine cryptic prophage elements namely, CP4-6, DLP12, e14, rac, Qin, CP4-44, CPS-53, CPZ-55 and CP4-57. *E. coli* growth was tested in nutrient-rich media where the deletion of each of the cryptic prophage elements resulted in a growth disadvantage (Wang *et al.*, 2010). This indicates that prophages aid in the ability of *E. coli* to grow rapidly and obtain nutrients during stationary phase (Wang *et al.*, 2010). Here we examined the effect of extended growth of the expression of proteins encoded by cryptic prophage genes.

GnsB and FlxA are both a part of the Qin prophage. FlxA was downregulated by 3-fold during late and prolonged stationary phase compared to early stationary phase in the wildtype. However, during early stationary phase, it was more substantially downregulated in the Δ*rpoS* strain than in the wildtype. GnsB steadily increased up to prolonged stationary phase in the wildtype. Similarly, GnsB steadily increased in abundance up to late stationary phase but decreased during prolonged stationary phase in the Δ*rpoS* strain. This indicates that RpoS may positively regulate the Qin prophage proteins identified in this study during prolonged stationary phase. In the earlier phases of growth, the Qin prophage proteins were expressed in an RpoS-independent manner. Isocitrate dehydrogenase enzyme (Icd) contributes to the resistance of the cell against hydrogen peroxide and is located within the e14 cryptic prophage (Hill *et al.*, 1989). Icd was more abundant in all four phases of growth in the Δ*rpoS* strain suggesting that RpoS negatively regulates this protein. YkfB is an uncharacterized protein that is a part of the CP4-6 prophage. YkfB continuously decreased in abundance during early and late stationary phase compared to exponential phase. The cryptic prophage gene *flu* encodes the CP4-44 prophage (self-

recognizing antigen 43 autotransporter). The abundance of this protein was consistently higher in the wildtype than in the Δ*rpoS* strain. Additionally, during prolonged stationary phase, CP4-44 was upregulated compared to early stationary phase. This suggests that The CP4-44 prophage is positively regulated by RpoS. Ag43 is an important element that promotes aggregation of cells which in turn promote resistance to oxidative stress (Laganenka *et al.*, 2016). It is also an important element in biofilm formation (Laganenka *et al.*, 2016).

### 4.5.4 Tryptophanase (TnaA) Expression during Stationary Phase

TnaA was identified as a protein that is highly abundant during early stationary phase in both wildtype and Δ*rpoS* strains but the expression level in the Δ*rpoS* strain was higher than that of the wildtype. During entry into early stationary phase, the upregulation of TnaA results in the conversion of exogenous tryptophan into indole, pyruvate and ammonia (Gong *et al.*, 2001). Indole is produced by *E. coli* and other Gram-negative bacteria. It is a signaling molecule that alters gene expression in response to an environmental signal. Transcriptionally, regulation of TnaA by RpoS can either be negative or positive (Dong and Schellhorn, 2009a; Lacour and Landini, 2004; Patten et al., 2004). The induction of TnaA in the absence of RpoS suggests that RpoS is not required for complete induction of TnaA. Therefore, other regulators, such as tryptophan and cAMP-CRP may serve as the primary regulators for TnaA (Chiang *et al.*, 2011). At the proteomic level examined here, TnaA is negatively regulated by RpoS during late and prolonged stationary phase. TnaB was also induced during early stationary phase and negatively regulated by RpoS during late and prolonged stationary phase. Additionally, in the *ΔrpoS* strain, the expression of TnaB like that of TnaA was induced at a higher level during early stationary phase and maintained at a higher level during late and prolonged stationary phase. TnaA is encoded by *tnaA*, a member of the *tnaC-tnaA-tnaB* operon. TnaA is required to convert tryptophan to indole, which occurs during early

stationary phase. Once this is complete, it is not required to maintain the enzyme at such a high level. RpoS serves to minimize the metabolic burden associated with TnaA once tryptophan has been converted to indole. In the absence of RpoS, the high level of TnaA is not only higher during early stationary phase but also sustained during late and prolonged stationary phases of growth. This sustained expression could potentially alter indole signaling; however, this has not been tested.

## 4.6 Is the expression of all proteins dynamic during growth?

Protein expression is a dynamic process that is influenced by external conditions. It is well known that the protein expression of many proteins changes in response to varying environmental conditions. In many cases, differential expression occurs in response to an extracellular signal. Here we assessed whether there are proteins that remain stable across the four tested growth phases. Identification of such proteins can be useful in molecular biology techniques such as immunoblot analyses. A control protein provides a baseline for comparison when assessing changes in protein expression under different conditions. A loading control can be used to normalize differences in protein expression between different samples by immunoblot analyses. RpoB is often used as a control in experiments where bacteria are grown to mid-exponential phase (Zere *et al.*, 2015) and up to 14 d (Odermatt *et al.*, 2018) suggesting that RpoB can serve as an effective control for protein expression studies. However, in this work, the expression of RpoB continuously decreased throughout all four growth phases, which suggests that RpoB cannot serve as a reliable and suitable control for protein expression studies in *E. coli*. Furthermore, glyceraldehyde -3-phosphate dehydrogenase (GAPDH) has been used as an internal loading control in exponentially growing *E. coli* (*gapA*) cells (Wu *et al.*, 2012). Therefore, though this protein may be a suitable loading control during exponential phase, its expression beyond this stage cannot be predicted. In this work, GapA, like RpoB, continuously decreased throughout the first three growth phases (exponential, early stationary and late stationary phase) but slightly increased during prolonged stationary phase. This suggests that GapA cannot serve as a reliable internal control for extended growth in *E. coli* as the expression of this protein was not sufficiently stable during long-term growth. Therefore, we assessed our results to identify potential control proteins by using a statistic that determines the difference between normalized protein abundance

in each growth phase of the of the proteins identified and quantified across all four growth phases in the wildtype.

The top ten (least variant) proteins include transaldolase B (TalB), PmbA, Xaa-Pro dipeptidase (prolidase), Ribonuclease E, YjhU, PTS system mannose-specific EIID component, RodZ, SlyX, UDP-glucose 4-epimerase and YgiC. TalB is encoded by the gene *talB*, which is a member of the *talB-tktA* operon. TalB is an enzyme that is implicated in the nonoxidative branch of the pentose-phosphate pathway where it catalyzes the reversible conversion of glyceralde-3-phosphate and sedoheptulose-7-phosphate to fructose-6-phosphate and erythrose-4-phosphate. A *talB* deletion does not affect growth on glucose minimal media containing glucose (Schorken et al., 2001). Previously, *E. coli* cells grown in minimal media supplemented with glucose were harvested at 6 h, 8 h and 12 h (Rahman et al., 2008). The changing expression of genes implicated in metabolic pathways, such as the pentose-phosphate pathway, were examined. The expression of *talB* continuously decreased from 6 h to 12 h of growth. In this work, we show that the expression of TalB was stable during all four growth phases. UDP-glucose 4-epimerase is implicated in the metabolism of galactose and is constitutively expressed in *Erwinia stewartii* (Dolph et al., 1988). The function of SlyX protein is unknown, however, the expression of *slyX* decreased in the transition from mid-exponential phase (6 h) to stationary phase (24 h) in *Yersinia pestis* (Chauvaux et al., 2007). PepQ prolidase catalyzes the hydrolysis of dipeptide substrates that contain a proline residue at the C-terminus (Park et al., 2004). RNase E plays a key role in the breakdown of mRNAs and the processing of rRNAs (Coburn and Mackie, 1999). PmbA plays a role in secretion and maturation of the antibiotic MccB17 (Rodriguez-Sainz et al., 1990). The protein YgiC may play a role in ligase formation. YjhU is an uncharacterized transcriptional regulator in *E. coli.* RodZ maintains the rod-shape of *E. coli* and ManZ is a part of the enzyme II

ManXYZ phosphotransferase system which is implicated in mannose transport. The expression of these proteins during long term growth is understudied. As such, their suitability to act as a control because of their constitutive expression throughout each growth phase needs to be assessed further.

## 4.7 Limitations of Isobaric Labeling

The use of isobaric tags, including TMT, is probably one of the most exhaustive approaches for relative quantitation, not only because of its multiplexing capabilities but also, the use of an internal standard to which all results can be normalized (Li *et al.*, 2017). As such, TMT is a widely used labeling approach for relative quantitation of proteins in complex mixtures. However, the limitations include its high cost, a lengthy protocol and a limit to the number of tags available for multiplexing. Furthermore, as in many other quantification techniques, isobaric labeling is biased toward identifying and quantifying a larger portion of the more abundant proteins because their precursor ions have a higher signal (Lund *et al.*, 2007; Seshi, 2006). These include transcription factors, heat shock proteins, and ribosomal proteins. During LC-MS/MS analysis, a peptide with a higher signal intensity will more likely be selected for fragmentation (Rauniyar and Yates, 2014). Finally, another disadvantage with the isobaric tag approach is the inaccuracies which can result from the relatively low-mass tags, especially in a complex matrix. This issue has been found to affect low as well as high-resolution instruments (Christoforou and Lilley, 2011; Ting *et al.*, 2011). However, the addition of a third stage of mass selection ($MS^3$) on an Orbitrap eliminates these interferences but it reduces the sensitivity by approximately 12% (Li *et al.*, 2017; Ting *et al.*, 2011). The reduction in the number of quantified proteins is justified as this results in greater accuracy and precision and therefore, increases the quality of the data obtained (Ting *et al.*, 2011).

In summary, these findings are consistent with previous studies that indicate that RpoS is a major regulator during early stationary. RpoS acts as a positive regulator by inducing proteins

such as those implicated in acid stress response (GadA, GadB, HdeA, HdeB) during early stationary phase. However, RpoS can also act as a negative regulator by repressing proteins such as those involved in the TCA cycle, flagella synthesis, motility and chemotaxis as well as some proteins encoded by cryptic prophage genes. During prolonged stationary phase, the abundance of RpoS decreased, resembling the abundance of RpoS during exponential phase. Despite this, there are many RpoS-controlled proteins that show continued expression during prolonged stationary phase in the wildtype. This indicates that other global regulators play a key role in regulating the expression of these proteins during long-term growth. Finally, we determined that there are some proteins whose expression was not dynamic during the four growth phases tested. Further testing could lead to the identification of a suitable loading control for immunoblot analyses done with proteins expressed in *E. coli.* Altogether, these results further the understanding of the changes that occur in *E. coli* during starvation period. These results can be used to further understand how *E. coli* survives in the external environment.

## 4.8 Future directions

The work performed in this study identified and characterized proteins that are preferentially expressed during prolonged stationary phase. TNSeq could be used to verify whether proteins preferentially expressed during prolonged stationary phase have are essential for the survival of *E. coli*. In this work, we identified proteins that were negatively regulated by RpoS during stationary phase, however, the mechanism of regulation is unclear. Chromatin-Immunoprecipitation-sequencing (Chip-Seq) could be used to determine whether RpoS directly inhibits the expression of these repressed proteins. We also identified proteins that remained stable during the four growth phases tested that could be used when analysing the differences in protein

expression during prolonged stationary phase in *E. coli*. We would need to verify whether these proteins can be used as a loading control. To do this, we would develop antibodies for these proteins and use immunoblot analyses to confirm whether they are suitable loading controls. Furthermore, the data acquired through this study can be combined with that of previous transcriptomic studies to determine the overlap between preferentially expressed genes and preferentially expressed proteins during stationary phase. Integration of this data can be used to target specific proteins such as sigma factors in *E. coli* or the proteins that increase significantly during stationary phase.

# 5.0 Chapter 2

# 5.0 Introduction

Most *E. coli* strains are harmless and live commensally within the human and animal gastrointestinal tract (Kaper *et al.*, 2004). While the role of RpoS in regulating stress conditions is well-known in non-pathogenic *E. coli* K12 strains, its effect on transcriptome expression is not well understood to the same extent in pathogenic *E. coli* strains. *E. coli* strains evolved pathogenicity by acquiring virulence factors through plasmids, bacteriophages, transposons and pathogenicity islands (Lim *et al.*, 2010) from foreign DNA (Hayashi *et al.*, 2001). Pathogenic *E. coli* strains are classified by their virulence and associated disease. Foodborne illnesses, often characterized by extreme diarrhea, are one of the leading causes of death globally (Liu *et al.*, 2016). While there are many causative agents, 66 % can be attributed to bacteria alone (Addis and Sisay, 2015) of which, pathogenic *E. coli* is a major contributor. Enterohemorrhagic *E. coli* (EHEC) is responsible for lethal cases of food poisoning worldwide (Karmali *et al.*, 2010). The most common serotype of EHEC responsible for multiple outbreaks of severe diarrhea is *E. coli* O157:H7.

*E. coli* O157:H7 may encounter many stress conditions during transmission and infection such as nutrient limitation, heat exposure, acid stress, and host defense upon entry into a human host (Dong and Schellhorn, 2010). Therefore, it is critical for this pathogen to adapt to survive and successfully infect a host. RpoS is a key regulator in response to stress conditions in *E. coli*. However, RpoS dependent gene expression differs based on the stress condition as well as the strain background (Dong and Schellhorn, 2009).

The *E. coli* strain O157:H7 EDL933 is responsible for serious health problems. To understand its survival in the environment, researchers studied EDL933 – specific gene regulation

(Dong and Schellhorn, 2009). Genes that are regulated by RpoS and specific to EDL933 were identified (Dong and Schellhorn, 2009). In addition to stress response, genes that are a part of metabolic pathways, transcription, and virulence were discovered to be RpoS dependent. These genes include those responsible for curli production, which is important for colonization, and the ehxCABD operon, which encodes enterohemolysin (Dong and Schellhorn, 2010; Mundy *et al.*, 2005). In addition, a microarray study was conducted to reveal the differences in gene expression in response to heat shock in EDL933 and K12 strains. Approximately 30 genes specific to EDL933 were identified (Carruthers and Minion, 2009. Of those 30 genes, only 4 of the top 25 heat shock response genes are RpoS-dependent (Dong and Schellhorn, 2009) suggesting other factors contribute to EDL933 heat stress response. This also reinforces the findings that RpoS regulation differs based on strain background and stress conditions (Dong *et al.*, 2008; Dong and Schellhorn, 2009). The reference strain *E. coli* K12 MG1655 and the pathogenic strain, *E. coli* O157:H7 differ in genome size as the *E. coli* O157:H7 strain is 5.5 Mb, instead of 4.6 Mb (Hayashi *et al.*, 2001). In addition to this size difference, both strains only share 4.1 Mb DNA, which may have a considerable impact on gene regulation (Dong and Schellhorn, 2009). Further research into the regulation of gene expression in response to stress can elucidate the factors, other than RpoS, that control the stress response.

### 5.1.1 *E. coli* - Long-term survival

Similar to other pathogenic bacteria, *E. coli* has a biphasic lifestyle, consists of host-associated and host-independent phases (van Elsas *et al.*, 2011). Once ingested, *E. coli* O157:H7 survives the acidity in the host's stomach and passes through to the intestine, where it establishes a niche for itself in the colon. Once excreted, the bacterium is forced to quickly adapt to a

contrasting environment to survive. During this time, *E. coli* O157:H7 lives in soil and water, which in turn serve as reservoirs for the disease (Ferens and Hovde, 2011).

To determine whether pathogens can survive long-term on seeds, seeds with *E. coli* O157:H7 were placed in storage for two years. *E. coli* O157:H7 was recovered from seeds that had been stored for two years (Van der Linden *et al.*, 2013). Depending on the recovery method used, approximately 4-14 % of *E. coli* O157:H7 were recovered from the seeds (Van der Linden *et al.*, 2013). Additionally, the pathogen was able to grow on the germinating seeds and therefore capable of causing subsequent infections (Van der Linden *et al.*, 2013). This cycle from host to the environment, which serves as a reservoir, allows *E. coli* O157:H7 to persist and cause subsequent outbreaks. Therefore, it is important to study the genetic factors that enable the rapid adaptation of *E. coli* O157:H7 to its changing environment, thus enabling survival.

### 5.1.2 Identification of Essential Genes during Long-Term Survival

For a complete understanding of how a specific genotype contributes to the expression of a phenotype, the genetic factors that regulate any given process need to be determined. As a result, techniques that screen for mutant genotypes that resulted in a phenotype was developed. However, this was very labour intensive as it would require thousands of mutants. As a result, a technique called Signature-Tagged mutagenesis (STM) was developed to identify novel bacterial virulence factors without the need of mutant genotypes (Hensel et al., 1995). In STM, mutants are created by random transposon insertions. These insertions contain a unique ‘tag’ sequence. Once a genome is sequenced, it is searched for any tags used in the experiment. Any gene that it disrupts is also located. This allows for the identification of genes that are important for virulence. However, high throughput versions of STM use genomic microarrays. Nevertheless, the development of next-generation sequencing has introduced other methods that are more accurate than STM. Transposon

mutagenesis coupled with massively parallel (TNSeq) sequencing eliminates the limitations of screening using thousands of mutant genotypes and demonstrates improved accuracy compared to STM (Lin et al., 2014).

### 5.1.3 TNSeq

TNSeq combines an experimental approach with a computational approach to highlight information about the roles of specific bacterial genes under a growth condition. TNSeq can be applied to determine the frequency of transposon insertions in a library under one condition, or the changes in mutant abundance between conditions (control and extended growth period). This allows for the identification of essential genes and the fitness contributions of non-essential genes on a genome-wide scale. TNSeq can be used to determine the fitness of a single gene or to map genetic interactions in microorganisms. It does not depend on gene knockouts, which allows for high throughput gene screening.

In TNSeq, a mutant library is constructed by mating a bacterial strain containing a transposon (donor) with the bacterial strain of interest (recipient). A mariner-based vector is commonly used for transposition (Dong *et al.*, 2013; Skurnik *et al.*, 2013) since the DNA encoding the transposon is efficiently delivered to the recipient demonstrating appropriate conditions for transposon mutagenesis in bacterial species (Rubin *et al.*, 1999). Following library construction, the resulting transposon insertion sites, which would include the surrounding sites of the genome for identification of those genes, is extracted. Furthermore, the DNA is digested and the short sequence containing the transposon insertion is PCR amplified and sequenced (Fig. 20).

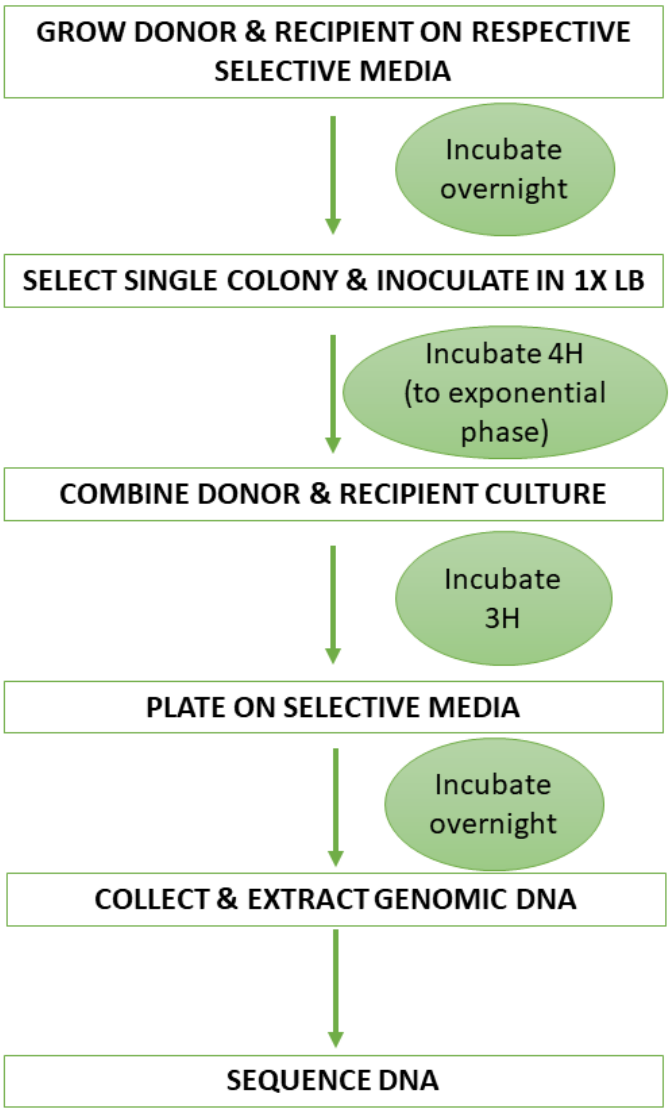


**Figure 20. Overview of TNSeq Experiment**.
In TNSeq, a mutant library is constructed by mating a bacterial strain containing the transposon (donor) with the strain of interest (recipient). Once the library has been created, DNA can be extracted, digested (restriction enzyme that recognizes the transposon), amplified by polymerase chain reaction (PCR) and sequenced.

TNSeq has been used to study fitness conditions of various bacteria. For example, TNSeq was used to successfully identify genes that are required for basal growth in *Streptococcus pneumoniae* (van Opijnen *et al.*, 2009). Additionally, 97 high confidence interactions for carbohydrate transport and transcriptional regulation in *S. pneumoniae* were identified (van Opijnen *et al.*, 2009). TNSeq enabled the identification of genes that contribute to the growth of *Enterococcus faecium* in human serum (Zhang *et al.*, 2017). Initially, RNA-seq was employed to compare the transcriptome of *E. faecium* during exponential growth in rich medium and in human serum. This identified 27.8 % of genes that were differentially expressed in the two conditions. TNSeq was then used to identify genes that are specifically required for growth in human serum. The results demonstrated that genes for de novo nucleotide biosynthesis and a gene encoding phosphotransferase system subunit are required for growth in human serum (Zhang *et al.*, 2017). When these gene mutants were grown in human serum they demonstrated impaired growth, which supports the TNSeq findings (Zhang *et al.*, 2017). TNSeq was used to analyse the effect of growth arrest, organic carbon and oxygen-depleted conditions on *Pseudomonas aeruginosa* (Basta *et al.*, 2017). These conditions were chosen as they are common causes of growth arrest in *P. aeruginosa* (Basta *et al.*, 2017). TNSeq revealed essential activities for a pathogen experiencing these conditions (Fig. 21).

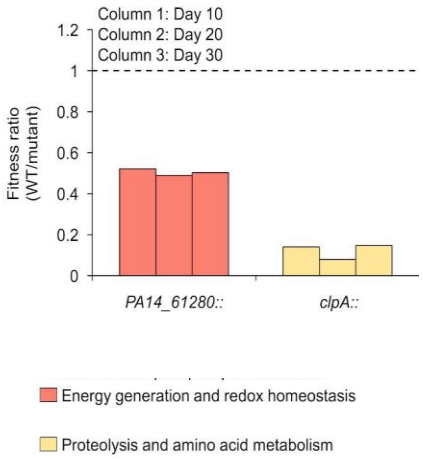


**Figure 21. Essential Processes for *Pseudomonas aeruginosa* in energy limited conditions.** TNSeq revealed that energy generation and redox homeostasis, as well as proteolysis and amino acid metabolism, are essential processes for Pseudomonas aeruginosa in energy-limited conditions. Adapted from (Basta *et al.*, 2017).

Finally, a comprehensive analysis of the genetic fitness of *P. aeruginosa in vivo* and *in vitro* was conducted using TNSeq (Skurnik *et al.*, 2013). To do this, a transposon library containing ~ 300,000 individual insertions was constructed. The transposon insertions were grown *in vitro* (which was used as a baseline) and compared to the survival of transposon insertions six days after inoculation into a murine host. Changes in transposon insertions were used to quantify *in vivo* fitness resulting from the loss of a gene (Skurnik *et al.*, 2013). Multiple gene losses were identified with transposon inserts interrupting many genes implicated in processes such as virulence and energy utilization (Skurnik *et al.*, 2013). Additionally, new candidates for virulence factors were also identified. Overall, the genes that were essential for survival in *P. aeruginosa's* host environment were identified (Skurnik *et al.*, 2013).

# 6.0 Rationale

The role of RpoS has been extensively characterized in *E. coli* K12 MG1655 highlighting the importance of this sigma factor in survival. RpoS also plays a critical role in controlling virulence factors as well as some stress response genes in *E. coli* O157:H7. However, some of the

genes identified as RpoS-dependent are of unknown function. Furthermore, RpoS-independent genes have also been identified in response to stress conditions indicating that other factors play a role in the *E. coli's* survival. The gene expression profiles derived from previous studies have greatly expanded our knowledge of the role of RpoS in gene regulation and their functional processes. Nonetheless, other factors that are responsible for stress response remain ambiguous.

This study aims to identify the genetic factors that are required for the survival of *E. coli* strain O157:H7 strain EDL933 during long-term growth, expanding on our current knowledge of this organism. Genome-enabled experimental and computational approaches have been developed to link genes to cellular function and phenotype (Zomer *et al.*, 2012). This study will utilize transposon mutagenesis sequencing (TNSeq), to highlight information about gene expression during stationary phase of growth. Monitoring the frequency of the transposon insertions in a library under one growth condition, or changes in mutant abundance between conditions allow for the identification of essential genes and the fitness contributions of non-essential genes on a genome-wide scale. TNSeq is useful for identifying genes required for fitness in a specific condition as well as identifying processes required for survival in those conditions (Skurnik *et al.*, 2013). Therefore, in this work, we aim to optimize the use of TNSeq to identify the genes responsible for *E. coli* O157:H7 ability to survive in stress conditions. Furthermore, the number of transposon insertions in each gene during exponential phase will be compared to the number of transposon insertions in each gene following seven days of growth in LB.

# 6.0 Materials and Methods

## 6.1 Generating Antibiotic Resistant Strains

*E. coli* O157:H7 EDL933 and *E. coli* MG1655 do not contain biomarkers. For selectivity in conjugation, a streptomycin resistant strain was generated. EDL933 and MG1655 were grown overnight on LB agar. A single isolated colony from each strain was selected and inoculated in 50 ml LB, incubated at 37 °C on a shaker at 200 RPM. Once the cultures reached saturation ($OD_{600}$ 1.7-1.9), 3 ml of each culture was poured onto LB agar containing 100 µg/ml of streptomycin. The plates were left at room temperature for approximately 10 min to allow the liquid to absorb into the agar. The excess liquid was removed, and the plates were incubated at 37 °C for 48 h.

## 6.2 Library Construction

*E. coli* O157: H7 and wildtype strains used selected for this study were EDL933 and K12 MG1655 respectively. *E. coli* SM10 λ pir bearing pSAM-DGM was used as a donor for conjugation. The donor strain was grown overnight on LB (LB-Miller, 10 g of peptone, 5 g of yeast extract, and 10 g of NaCl) containing 15 µg/ml of gentamicin, 50 µg/ml of kanamycin and 100 µg/ml of ampicillin. The recipient strains were also grown overnight on LB agar containing 10 µg/ml of streptomycin. The cells were collected using 1 ml LB, centrifuged and resuspended in 1 ml LB. The donor strain was mixed with each recipient in a 1:10 ratio, vortexed and 400 µl of the mixture were spotted onto LB agar plates. The plates were left at room temperature for approximately 15-20 min to allow the liquid to absorb into the agar. Mating then continued for 3 h incubated at 37 °C. Each conjugation reaction was scraped off, suspended in 1 ml LB and 200 µl aliquots were plated on LB plates containing streptomycin (100 µg/ml) and gentamycin (15 µg/ml). After 18 h of incubation at 37 °C, the plates were washed with LB containing 20 % glycerol, the colonies scraped off and the mutant libraries collected and stored at – 80 °C. Genomic

DNA was extracted according to manufacturer's instructions using Norgen Biotek's DNA isolation kit with an additional step of RNase treatment. The DNA yields were within the expected range (~5-10 µg).

### 6.3 Restriction Enzyme Digestion with *MmeI*

*MmeI* is a type-IIS restriction modification enzyme that cuts the double-stranded DNA 20bp away from the recognition site, therefore including the gene interrupted by the transposon insertion. The transposon inserted in the EDL933 and MG1655 genome contains *MmeI* restriction sites on both ends of the transposon, allowing the *MmeI* enzyme fragments of the genes on both sides of the transposon insertion to be identified through sequencing. This feature allows the high throughput sequencing to identify the gene interrupted as well as the insertion site for each transposon insertion. All DNA samples were digested with MmeI (New England Biolabs) according to the manufacturer's instructions. Each DNA sample was incubated at 37 °C for one hour and then heat inactivated for 20 min at 80 °C. Each sample was then purified following manufacturer's instructions (Nucloespin Gel and PCR Clean-up Kit, Takara Bio USA), before being eluted to a final volume of 20 µl.

### 6.4 Gel Extraction of the Transposon and Adjacent Genomic DNA

Digested DNA samples were run on a 1% agarose gel and the 1.0 – 1.5 kb band was excised, and gel extracted (Nucleospin Gel and PCR Clean-up Kit, Takara Bio USA). This allowed recovery of all transposons containing adjacent genomic DNA. To prepare the sequencing libraries, digested DNA was ligated to adapters. The adaptors (Table 11) were ligated to 1µg of the gel extracted DNA samples with T4 DNA Ligase (New England Biolabs) in a 20 µL overnight reaction. The reaction was incubated at 16 °C overnight (~16 h), heat-inactivated at 65 °C for 10 min, purified (Nucleospin Gel and PCR Clean-up Kit, Takara Bio USA) and quantified using the

Nanodrop. Ligated products were PCR amplified with primers containing Illuminia compatible index sequences.

## 6.5 PCR Amplification

All extracted transposon (with adapters) were PCR amplified (Table 11) using the HotStar HiFidelity PCR reagents (Qiagen). This PCR step amplifies the fragments containing the P7 Illumina sequence, the end of the transposon, the genomic DNA fragments and the P5 adapter which generates the libraries for Illumina sequencing. The PCR product of each sample was visualised on a 1 % agarose gel and the presence of a 180 bp product was confirmed. The samples were purified (Nucleospin Gel and PCR clean-up Kit, Takara Bio USA) and quantified using the Nanodrop prior to sequencing. The samples were sequenced on Illumina MiSeq platform at the McMaster Genome Facility.

## 6.6 Data Analysis

The read pool generated from sequencing was trimmed to retain only the genomic sequence using Cutadapt. Reads were then mapped to their respective genome by the software CLC Genomics Workbench (7.5.1). Transposon insertions per gene is represented as the normalized RPKM value (Mortazavi et al., 2008), which show the relative abundance of the transposon insertions in a given gene in the total pool.

**Table 11**. Primers and adapters used in this study.

| Name | Sequence |
|---|---|
| **Adapters** | |
| LIB_AdaptT | 5’ - TTCCCTACACGACGCTCTTCCGATCTN N - 3’ |
| LIB_AdaptB | 5’- AGATCGGAAGAGCGTCGTGTAG GG AA - 3’ |
| **Primers** | |
| LIB_PCR_5_15 | 5’ – AATGATACGGCGACCACCGA GATCTACAC TTCTAGCT ACACTCTTTCCCTACACGACGCTCTTCCGATCT – 3’ |
| LIB_PCR_5_16 | 5’-AATGATACGGCGACCACCGA GATCTACACCCTAGAGTACACTCTTTCCCTACACGACGCTCTTCCGATCT- 3’ |
| LIB_PCR_7_01 | 5’- CAAGCAGAAGACGGCATACGAGATTCGCCTTAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTAGACCGGGGA CTTATCATCCAACCTGT – 3’ |
| LIB_PCR_7_02 | 5’-CAAGCAGAAGACGGCATACGAGATCTAGTACGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTAGACCGGGGA CTTATCATCCAACCTGT - 3’ |
| LIB_PCR_7_03 | 5’-CAAGCAGAAGACGGCATACGAGATTTCTGCCTGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTAG ACCGGGGACTTATCATCCAACCTGT - 3’ |

Overview of Data Analysis

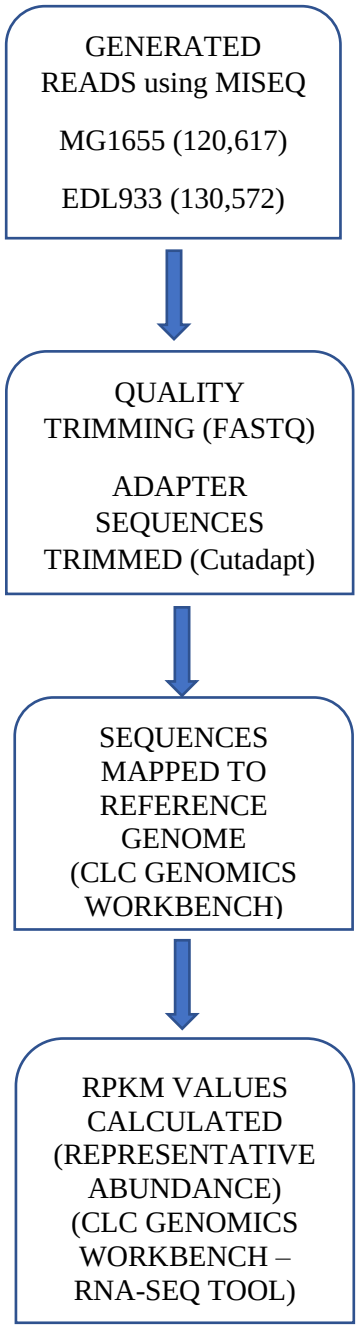


**Figure 22. Overview of TNSeq data analyses performed in this study.**
All samples were subject to sequencing, which produced raw sequence data files. Each dataset was quality trimmed using FASTQC, after which the sequencing adapters were removed using Cutadapt. Each data set was mapped to their respective reference genome and analyzed using the RNA-seq tool in CLC Genomics Workbench. This analysis generated RPKM values, which represent the abundance of transposon insertions in each gene.

# 7.0 Results

TNSeq is used to identify genes that are essential under different conditions. Essential genes are those that do not tolerate transposon insertions as the disruption would result in a high fitness cost (Carey et al., 2018). To determine the number and location of transposon insertions, we analyzed the pool of read generated using DNA sequencing technologies (MiSeq). A pool 130,572 and 120,617 paired-end reads for EDL933 and MG1655 strains, respectively. The sequences were trimmed to retain quality reads and to remove sequencing adapters using Cutadapt (Martin M., 2011). CLC Genomics Workbench (7.5.1) was used to map the reads to their respective genome (*E. coli* O157:H7 str EDL933 NZ_CP008957.1 & *E. coli* str. MG1655 NC_000913.3) using the default settings of the RNA-seq function, and to calculate RPKM values (Gutierrez *et al.*, 2015) (Fig. 22). The RPKM values represent the abundance of transposon insertions in each gene, with the lower RPKM values representing fewer insertions (Dong *et al.*, 2013) (See Table 12 and Table 13).

**Table 12.** Genes containing transposon insertions in EDL933.
The normalized RPKM (Reads Per Kilobase per Million) values represent the number of insertions in each gene. The average RPKM value is 75007.1.

| Name | Description | Region | Gene length | RPKM | Unique gene reads | Total gene reads |
|---|---|---|---|---|---|---|
| *glpK* | Glycerol kinase | Complement (4984532..4986061) | 1530 | 24379.2 | 42 | 42 |
| *glpA* | Anaerobic glycerol-3-phosphate dehydrogenase subunit A | 3153288..3154916 | 1629 | 6542.2 | 12 | 12 |
| Z1153 | Hypothetical protein | 1085324..1085464 | 141 | 6298.6 | 0 | 1 |
| Z3025 | Hypothetical protein | 2710471..2710770 | 300 | 5920.7 | 0 | 2 |
| Z3135 | Putative Invasin | 2789871..2797853 | 7983 | 5562.4 | 50 | 50 |
| Z1352 | putative endolysin of cryptic prophage CP-933M | 1270754..1271104 | 351 | 2530.2 | 0 | 1 |
| *dut* | deoxyuridine 5'-triphosphate nucleotidohydrolase | 4626774..4627229 | 456 | 1947.6 | 1 | 1 |
| *ssrA* | RNA 3B Macromolecule synthesis 2C modification: Ribosomal and stable RNAs | 3545346..3545811 | 466 | 1905.8 | 1 | 1 |
| Z3601 | Putative major fimbrial subunit | complement(3251489..3252052) | 564 | 1574.6 | 1 | 1 |
| *yggV* | putative ribosomal protein | 3905499..3906092 | 594 | 1495.1 | 1 | 1 |
| *phoR* | positive and negative sensor protein for pho regulon | 479872..481167 | 1296 | 1370.5 | 2 | 2 |
| Z2049 | Unknown protein encoded by prophage CP-933O | 1857865..1858530 | 666 | 1333.5 | 1 | 1 |
| Z3757 | Orf, hypothetical protein | 3402073..3403536 | 1464 | 1213.3 | 2 | 2 |
| *yafS* | Hypothetical protein | 238349..239089 | 741 | 1198.5 | 1 | 1 |
| *sdhA* | succinate dehydrogenase, flavoprotein subunit | 834206..835972 | 1767 | 1005.2 | 2 | 2 |
| *fimH* | minor fimbrial subunit, D-mannose specific adhesin | 5433702..5434604 | 903 | 983.5 | 1 | 1 |
| Z2419 | Orf, hypothetical protein | complement(2168661..2169644) | 984 | 902.5 | 1 | 1 |

| Name | Description | Region | Gene length | RPKM | Unique gene reads | Total gene reads |
|---|---|---|---|---|---|---|
| Z4276 | Putative oxidoreductase | complement(3884098..3885087) | 990 | 897.1 | 1 | 1 |
| *hipA* | persistence to inhibition of murein or DNA biosynthesis, DNA-binding regulator | 1972050..1973072 | 1023 | 868.1 | 1 | 1 |
| *ybdH* | putative oxidoreductase | complement(713640..714728) | 1089 | 815.5 | 1 | 1 |
| *aceA* | isocitrate lyase | 5094238..5095557 | 1320 | 672.8 | 1 | 1 |
| *qseC* | quorum sensing *Escherichia coli* regulator C | 3977227..3978576 | 1350 | 657.9 | 1 | 1 |
| *atpA* | membrane-bound ATP synthase, F1 sector, alpha-subunit | complement(4780741..4782282) | 1542 | 575.9 | 1 | 1 |
| *trpD* | anthranilate synthase component II, glutamine amidotransferase and phosphoribosylanthranilate transferase | 2264773..2266368 | 1596 | 556.5 | 1 | 1 |
| *yhjW* | orf, hypothetical protein | complement(4517834..4519558) | 1725 | 514.8 | 1 | 1 |
| *ytfM* | orf, hypothetical protein | 5327848..5329581 | 1734 | 512.2 | 1 | 1 |
| *thiC* | thiamin biosynthesis, pyrimidine moiety | complement(5071775..5073670) | 1896 | 468.4 | 1 | 1 |
| *aas* | 2-acyl-glycerophospho-ethanolamine acyltransferase; acyl-acyl-carrier protein synthetase | complement(3763553..3765712) | 2160 | 411.2 | 1 | 1 |
| *mutS* | methyl-directed mismatch repair | 3648075..3650636 | 2562 | 346.6 | 1 | 1 |
| *rrlB* | | 5046586..5049490 | 2905 | 305.7 | 0 | 1 |
| *lacZ* | beta-D-galactosidase | complement(420009..423083) | 3075 | 288.8 | 1 | 1 |
| *ybdE* | putative inner membrane component for iron transport | 681031..684168 | 3138 | 283.0 | 1 | 1 |
| *mfd* | transcription-repair coupling factor; mutation frequency decline | complement(1614739..1618185) | 3447 | 257.6 | 1 | 1 |
| *narZ* | cryptic nitrate reductase 2, alpha subunit | 2026647..2030387 | 3741 | 237.4 | 1 | 1 |
| Z5932 | Putative invasin | complement(5446463..5451565) | 5103 | 174.0 | 1 | 1 |

**Table 13.** Genes containing transposon insertions in MG1655.
The normalized RPKM (Reads Per Kilobase per Million) values represent the number of insertions in each gene. The average RPKM value is 686347.8.

| Name | Description | Region | Gene length | RPKM | Unique gene reads | Total gene reads |
|---|---|---|---|---|---|---|
| *glpK* | Glycerol kinase | Complement (4115714..4117222) | 1509 | 620662.7 | 5154 | 5154 |
| *yedL* | Putative acetyltransferase | 2010600..2011079 | 480 | 24986.4 | 66 | 66 |
| *phoR* | sensory histidine kinase in two-component regulatory system with PhoB | 417889..419184 | 1296 | 15003.0 | 107 | 107 |
| *glpA* | Anaerobic glycerol-3-phosp | 2352647..2354275 | 1629 | 9482.0 | 85 | 85 |
| *yiaO* | 2,3-diketo-L-gulonate-binding periplasmic protein | 3746094..3747080 | 987 | 4050.5 | 22 | 22 |
| *pabB* | Aminodeoxychorismate synthase, subunit I | 1894805..1896166 | 1362 | 2001.3 | 15 | 15 |
| *fliS* | Flagellar protein potentiates polymerization | 2005303..2005713 | 411 | 1326.4 | 3 | 3 |
| *paaX* | Transcriptional repressor of phenylacetic acid degradation paa operon, phenylacetyl-CoA inducer | 1463539..1464489 | 951 | 1146.5 | 6 | 6 |
| *kptA* | RNA 2'-phosphotransferase | 4560930..4561484 | 555 | 982.3 | 3 | 3 |
| *yjdN* | Metalloprotein superfamily protein | complement(4325298..4325741) | 444 | 818.6 | 2 | 2 |
| *qseC* | Quorum sensing sensory histidine kinase in two-component regulatory system with QseB | 3170484..3171833 | 1350 | 403.8 | 3 | 3 |
| *elaA* | GNAT family putative N-acetyltransferase | complement(2381082..2381543) | 462 | 393.3 | 1 | 1 |
| *yahI* | Uncharacterized protein | 340165..341115 | 951 | 382.2 | 2 | 2 |
| *ypfG* | DUF1176 family protein | complement(2581734..2582777) | 1044 | 348.1 | 2 | 2 |
| *arcB* | Aerobic respiration control sensor histidine protein kinase, cognate to two-component response regulators ArcA and RssB | complement(3350689..3353025) | 2337 | 311.0 | 4 | 4 |
| *casD* | CRISP RNA (crRNA) containing Cascade antiviral complex protein | complement(2880374..2881048) | 675 | 269.2 | 1 | 1 |

| Name | Description | Region | Gene length | RPKM | Unique gene reads | Total gene reads |
|---|---|---|---|---|---|---|
| *glpT* | Sn-glycerol-3-phosphate transporter | complement(2351016..2352374) | 1359 | 267.4 | 2 | 2 |
| *argB* | Acetylglutamate kinase | 4156013..4156789 | 777 | 233.9 | 1 | 1 |
| *yafT* | Lipoprotein | 237335..238120 | 786 | 231.2 | 1 | 1 |
| *bax* | Putative glucosaminidase | complement(3736353..3737177) | 825 | 220.3 | 1 | 1 |
| *kduI* | Hexuronate isomerase | complement(2983288..2984124) | 837 | 217.1 | 1 | 1 |
| *tamA* | Translocation and assembly module for autotransporter export, outer membrane subunit | 4442382..4444115 | 1734 | 209.6 | 2 | 2 |
| *prkB* | Putative phosphoribulokinase | 3484490..3485359 | 870 | 208.9 | 1 | 1 |
| *sdhA* | Succinate dehydrogenase, flavoprotein subunit | 755907..757673 | 1767 | 205.7 | 2 | 2 |
| *eamA* | Cysteine and O-acetyl-L-serine efflux system | complement(1620238..1621137) | 900 | 201.9 | 1 | 1 |
| *gspK* | General secretory pathway component, cryptic | 3462924..3463907 | 984 | 184.7 | 1 | 1 |
| *fbp* | Fructose-1,6-bisphosphatase I | complement(4454611..4455609) | 999 | 181.9 | 1 | 1 |
| *malE* | Maltose transporter subunit | complement(4245229..4246419) | 1191 | 152.6 | 1 | 1 |
| *fabB* | 3-oxoacyl-[acyl-carrier-protein] synthase I | complement(2440385..2441605) | 1221 | 148.8 | 1 | 1 |
| *mdtM* | Multidrug efflux system protein | complement(4567287..4568519) | 1233 | 147.4 | 1 | 1 |
| *lacY* | Lactose permease | complement(361926..363179) | 1254 | 144.9 | 1 | 1 |
| *ytfL* | UPF0053 family inner membrane protein | complement(4439872..4441215) | 1344 | 135.2 | 1 | 1 |
| *phoA* | Bacterial alkaline phosphatase | 401747..403162 | 1416 | 128.3 | 1 | 1 |
| *asnB* | Asparagine synthetase B | complement(697513..699177) | 1665 | 109.1 | 1 | 1 |
| *btuB* | Vitamin B12/cobalamin outer membrane transporter | 4163639..4165483 | 1845 | 98.5 | 1 | 1 |
| *cyoB* | Cytochrome o ubiquinol oxidase subunit I | complement(448650..450641) | 1992 | 91.2 | 1 | 1 |
| *glgP* | Glycogen phosphorylase | complement(3564134..3566581) | 2448 | 74.2 | 1 | 1 |
| *rrlG* | 23rRNA encoded by *rrnG* operon | complement(2726281..2729184) | 2904 | 62.6 | 0 | 1 |
| *rrlC* | 23rRNA encoded by *rrnC* operon | 3943704..3946607 | 2904 | 62.6 | 0 | 1 |
| *rrlA* | 23rRNA encoded by *rrnA* operon | 4037519..4040423 | 2905 | 62.6 | 0 | 1 |

# 8.0 Discussion

Analysis of data derived from TNSeq experiment suggests that this is a useful and robust technique that can be used to identify genes that are critical for survival during long-term growth. The data presented here represent two isolated samples that were used to optimize the experiment. Here, glycerol kinase had the highest RPKM value in both strains, which indicates a large number of insertions within the gene sequence. This suggests that *glpK* may not be crucial for survival in either strain. On the other hand, *mutS* had a low RPKM which indicates fewer insertions. This suggests that *mutS* may be important during stationary phase growth. This is consistent with previous proteomic findings that show that MutS is upregulated during stationary phase growth (Han, 2017). In interpreting TNSeq, it is important that the RPKM value of each gene is compared to its RPKM value during another growth phase. The difference in the RPKM value of a certain gene under each condition indicate its significance for survival. Therefore, samples from each growth phase need to be submitted for sequencing to conclude that a gene or group of genes are enabling survival in *E. coli* during prolonged growth.

# Appendix 1

**Table 14. Average normalized protein abundance of proteins upregulated (>2-fold) during early stationary phase (6 h) compared to exponential phase (4 h).**

| | | | Wildtype | | | | RpoS | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Accession ID** | **Gene** | **RNA regulation[+]** | **4 h** | **6 h** | **24 h** | **48 h** | **4 h** | **6 h** | **24 h** | **48 h** | **RpoS-dependent** | **Reference** |
| P76621 | *glaH* | RpoS, ppGpp, CRP-cAMP, Lrp-Leu, H-NS, GlaR | 16.0 | 230.8 | 245.3 | 236.7 | 13.3 | 40.1 | 78.3 | 129.7 | RpoS-dependent, stationary phase specific | (Loewen and Hengge-Aronis, 1994; Loewen *et al.*, 1998; Marschall *et al.*, 1998) |
| P16700 | *cysP* | RpoD, CysB, H-NS | 13.6 | 137.6 | 150.9 | 201.0 | 16.5 | 19.2 | 172.8 | 79.2 | | |
| P25738 | *msyB* | RpoS, ppGpp | 6.3 | 55.8 | 253.0 | 328.6 | 26.6 | 17.0 | 13.7 | 30.7 | RpoS-dependent | (Lacour and Landini, 2004) |
| P0ACY3 | *yeaG* | RpoN, ppGpp, NtrC | 31.6 | 274.3 | 213.1 | 115.0 | 33.8 | 20.7 | 18.2 | 278.2 | RpoS-dependent, Growth Phase - dependent | (Patten *et al.*, 2004; Weber *et al.*, 2005) |
| P33219 | *yebF* | ppGpp | 28.5 | 235.8 | 255.5 | 178.7 | 21.4 | 1.8 | N.D. | N.D. | RpoS-dependent | (Weber *et al.*, 2005) |
| C4ZZR7 | *cysI* | RpoD, CysB, IHF | 25.7 | 219.4 | 123.2 | 86.6 | 17.1 | 105.9 | 116.9 | 87.3 | | |
| P75694 | *yahO* | ppGpp | 38.0 | 293.1 | 306.6 | 91.9 | 36.2 | 15.2 | N.D. | N.D. | RpoS-dependent | (Ibanez-Ruiz *et al.*, 2000) |
| P37330 | *glcB* | RpoD, GlcC, IHF, PdhR, ArcA | 26.0 | 193.2 | 108.2 | 119.0 | 17.5 | 58.3 | 118.1 | 149.4 | | |
| P0A853 | *tnaA* | RpoD, L-trp, CRP-cAMP, TorR | 18.7 | 131.6 | 59.3 | 60.9 | 10.3 | 178.5 | 172.0 | 189.2 | RpoS-dependent | (Lacour and Landini, 2004) |
| P08997 | *aceB* | RpoD, IHF, Cra, ArcA, CRP-cAMP, IclR-pyruvate | 17.4 | 124.5 | 216.5 | 45.1 | 11.3 | 30.7 | 245.9 | 124.3 | RpoS-dependent | (Dong and Schellhorn, 2009b) |
| C4ZZA4 | *astC* | ArgR,NtrC,RpoS, RpoN, RpoD | 13.8 | 90.7 | 274.1 | 251.4 | 34.3 | 29.1 | 56.0 | 73.1 | RpoS-dependent | (Dong and Schellhorn, 2009b) |

| | | | Wildtype | | | | RpoS | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Accession ID** | **Gene** | **RNA regulation⁺** | **4 h** | **6 h** | **24 h** | **48 h** | **4 h** | **6 h** | **24 h** | **48 h** | **RpoS-dependent** | **Reference** |
| P39325 | *ytfQ* | AraC | 14.5 | 103.0 | 250.7 | 250.8 | 26.4 | 23.6 | 63.7 | 43.9 | RpoS-dependent | (Patten *et al.*, 2004) |
| P33014 | *yeeD* | | 48.1 | 307.5 | 18.7 | 43.6 | 95.0 | 178.6 | 126.5 | 54.6 | | |
| P23847 | *dppA* | RpoD, IHF, GcvB, FNR | 17.8 | 117.6 | 208.8 | 262.7 | 21.9 | 12.6 | 57.1 | 65.4 | RpoS-dependent | (Dong and Schellhorn, 2009b) |
| P77674 | *prr* | RpoS, Nac | 13.4 | 91.7 | 269.1 | 315.1 | 30.0 | 19.2 | 18.5 | 35.6 | | |
| C4ZZQ5 | *cysN* | RpoD, CysB | 51.2 | 351.5 | 71.3 | 12.1 | 12.8 | 190.6 | 47.2 | 26.8 | | |
| P0ADB7 | *ecnB* | RpoS, ppGpp, MicA, OmpR | 8.1 | 52.1 | 247.6 | 358.7 | 18.0 | 10.4 | 11.2 | 22.3 | RpoS-dependent | (Dong and Schellhorn, 2009b) |
| P38038 | *cysJ* | RpoD, CysB, IHF | 24.2 | 156.8 | 112.9 | 129.4 | 19.8 | 72.5 | 130.7 | 153.1 | | |
| P68191 | *sra* | RpoS, ppGpp, Fis, IHF, OmpR | 64.4 | 411.5 | 177.6 | 91.1 | 32.3 | 60.2 | 81.2 | 90.6 | RpoS-dependent | (Izutsu *et al.*, 2001) |
| P23173 | *tnaB* | RpoD, L-trp, CRP-cAMP, TorR | 15.7 | 90.7 | 71.2 | 64.9 | 7.2 | 127.4 | 202.3 | 212.3 | RpoS-dependent | (Dong and Schellhorn, 2009b) |
| P22256 | *gabT* | RpoS, RpoD, Nac, ppGpp, CRP-cAMP, Lrp, H-NS, GlaR | 15.9 | 100.5 | 246.3 | 292.9 | 18.5 | 10.8 | 21.0 | 30.0 | | |
| P0AEF8 | *dppB* | RpoD, IHF, Spf RNA, GcvB, FNR | 24.1 | 146.6 | 224.2 | 260.5 | 15.1 | 5.0 | N.D. | N.D. | RpoS-dependent | (Dong and Schellhorn, 2009b) |
| P27550 | *acs* | RpoD, RpoS, CRP-cAMP, Fis, IHF | 23.6 | 145.4 | 230.5 | 66.4 | 20.2 | 26.9 | 162.2 | 102.4 | RpoS-dependent, stationary phase specific | (Shin *et al.*, 1997) |
| P56262 | *ysgA* | RpoD, ppGpp, GcvB, FNR | 17.7 | 101.0 | 218.8 | 252.4 | 18.3 | 31.9 | 92.1 | 93.7 | RpoS-dependent | (Weber *et al.*, 2005) |
| C4ZTE1 | *iraP* | RpoD, DksA-ppGpp, ppGpp, CsgD | 17.1 | 93.1 | 103.6 | 59.0 | 11.0 | 87.6 | 231.4 | 131.5 | | |
| P07117 | *putP* | RpoD, PutA, CRP-cAMP | 24.0 | 141.3 | 66.2 | 64.1 | 39.8 | 60.0 | 208.0 | 228.8 | | |
| P0AEH5 | *elaB* | RpoS, ppGpp | 11.9 | 60.0 | 269.6 | 356.4 | 19.2 | 11.5 | 8.3 | 21.2 | RpoS-dependent | (Patten *et al.*, 2004) |

| | | | Wildtype | | | | RpoS | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Accession ID** | **Gene** | **RNA regulation⁺** | **4 h** | **6 h** | **24 h** | **48 h** | **4 h** | **6 h** | **24 h** | **48 h** | **RpoS-dependent** | **Reference** |
| P0ADB1 | *osmE* | RpoS, RpoD, IHF, Fis | 14.5 | 77.2 | 289.0 | 351.8 | 17.2 | 12.4 | 28.4 | 43.3 | RpoS-dependent, stationary phase specific | (Loewen and Henggearonis, 1994; Loewen *et al.*, 1998) |
| P33362 | *yehZ* | ppGpp, RpoH, RpoS | 17.9 | 95.1 | 252.6 | 275.2 | 19.0 | 5.1 | N.D. | N.D. | RpoS-dependent | (Dong and Schellhorn, 2009b) |
| P77318 | *ydeN* | RpoD, GadX, AraC, NagC | 25.0 | 125.7 | 68.5 | 77.6 | 12.2 | 183.9 | 160.1 | 181.6 | | |
| P21179 | *katE* | RpoS, ppGpp, Fur, Fis | 14.2 | 74.1 | 246.7 | 449.6 | 24.7 | 4.5 | N.D. | N.D. | RpoS-dependent, stationary phase specific | (Loewen and Henggearonis, 1994; Loewen *et al.*, 1998) |
| P0AA31 | *yedF* | | 37.8 | 183.1 | 33.0 | N.D. | N.D. | 118.1 | 184.3 | 135.7 | | |
| P23538 | *ppsA* | RpoD, Cra | 30.9 | 156.4 | 120.5 | 52.2 | 27.7 | 151.8 | 129.6 | 95.0 | | |
| P0AAN5 | *yaiA* | RpoD, ppGpp, TyrR, TrpR | 17.5 | 84.9 | 293.3 | 293.1 | 17.9 | N.D. | N.D. | N.D. | RpoS-dependent | (Weber *et al.*, 2005) |
| B1X9C5 | *ECDH10B_1076* | | 12.3 | 59.6 | 262.1 | 291.3 | 19.0 | 24.4 | 37.7 | 26.1 | | |
| P21156 | *cysD* | RpoD, CysB | 57.7 | 303.1 | 47.6 | N.D. | N.D. | N.D. | N.D. | N.D. | | |
| P76217 | *astD* | ArgR,NtrC,RpoS, RpoN, RpoD | 11.3 | 55.7 | 264.1 | 287.4 | 23.3 | 28.5 | 69.1 | 66.5 | RpoS-dependent | (Dong and Schellhorn, 2009b) |
| P21367 | *ycaC* | BaeR, Nac, FNR | 12.3 | 55.8 | 343.3 | 342.8 | 15.4 | 1.3 | N.D. | N.D. | RpoS-dependent | (Patten *et al.*, 2004) |
| P76172 | *ynfD* | ppGpp | 12.2 | 57.8 | 264.5 | 324.5 | 25.6 | 18.1 | 61.1 | 25.9 | RpoS-dependent | (Traxler *et al.*, 2008) |
| P09546 | *putA* | RpoD, MarA, BasR, PutA | 43.1 | 199.2 | 58.7 | 44.1 | 33.0 | 86.0 | 175.5 | 174.3 | | |
| P0AFH8 | *osmY* | RpoS, RpoD, FliZ, Fis, IHF, Lrp, CRP-cAMP | 12.9 | 57.2 | 265.5 | 287.6 | 30.6 | 12.2 | 7.6 | 15.9 | RpoS-dependent, stationary phase specific | (Loewen and Henggearonis, 1994; Loewen *et al.*, 1998) |
| P25526 | *gabD* | RpoS, RpoD, Nac, ppGpp, CRP-cAMP, Lrp, H-NS, GlaR | 21.1 | 93.3 | 237.2 | 303.3 | 17.7 | 20.9 | 25.9 | 64.1 | RpoS-dependent | (Weber *et al.*, 2005) |

| | | | Wildtype | | | | RpoS | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Accession ID** | **Gene** | **RNA regulation⁺** | **4 h** | **6 h** | **24 h** | **48 h** | **4 h** | **6 h** | **24 h** | **48 h** | **RpoS-dependent** | **Reference** |
| P25516 | *acnA* | RpoD, RpoS, ppGpp, Rob, MarA, SoxS, CRP-cAMP, Cra, ArcA, FNR | 37.3 | 165.6 | 271.3 | 110.9 | 27.7 | 56.2 | 60.9 | 63.1 | RpoS-dependent, stationary phase specific | (Cunningham et al., 1997) |
| C5A020 | *fadB* | RpoD, Fis, FadR, ArcA | 31.2 | 131.3 | 172.7 | 57.1 | 41.3 | 88.6 | 150.1 | 154.8 | RpoS-dependent | (Dong and Schellhorn, 2009b) |
| P0AG78 | *sbp* | | 53.2 | 227.1 | 179.8 | 203.1 | 52.4 | 14.8 | N.D. | N.D. | | |
| P0AEM9 | *fliY* | CysB, GcvB | 30.9 | 133.9 | 178.6 | 231.4 | 45.4 | 26.9 | 144.7 | 71.9 | | |
| P37387 | *xylF* | XylR, CRP-cAMP, Fis, Spf RNA | 19.8 | 82.3 | 108.3 | 119.8 | 23.7 | 28.4 | 290.1 | 151.9 | RpoS-dependent | (Dong and Schellhorn, 2009b) |
| P25889 | *preA* | RpoD, CRP-cAMP | 50.1 | 198.1 | 27.8 | N.D. | N.D. | 206.6 | 71.3 | 100.6 | | |
| P0ABD3 | *bfr* | RyhB | 17.7 | 71.1 | 260.9 | 328.9 | 15.5 | 5.7 | 7.6 | 44.5 | RpoS-dependent, early stationary phase specific | (Lacour and Landini, 2004) |
| P0ADU5 | *ygiW* | | 16.2 | 66.3 | 365.4 | 146.3 | 15.3 | 8.9 | 4.9 | 7.8 | RpoS-dependent, early stationary phase induction | (Lacour and Landini, 2004) |
| P09551 | *argT* | RpoD, DksA, Fis, DksA-ppGpp, ppGpp | 13.5 | 53.5 | 212.5 | 305.0 | 19.5 | 9.3 | 107.9 | 100.4 | | |
| P0AAG0 | *dppD* | RpoD, IHF, GcvB, FNR | 33.7 | 136.9 | 205.7 | 210.8 | 49.0 | 34.4 | 38.3 | 161.2 | RpoS-dependent | (Dong and Schellhorn, 2009b) |
| B1X658 | *otsA* | RpoS | 35.5 | 139.0 | 297.5 | 117.7 | 14.6 | 31.3 | 49.7 | 77.3 | RpoS-dependent, stationary phase specific | (Loewen and Henggearonis, 1994; Loewen *et al.*, 1998) |
| P06720 | *melA* | RpoD, MelR, CRP-cAMP | 32.7 | 128.3 | 119.8 | 164.1 | 22.6 | 100.5 | 124.5 | 111.2 | | |
| P69831 | *gatC* | RpoD, CRP-cAMP, GatR, ArcA | 29.1 | 110.8 | 8.7 | 8.2 | 24.5 | 167.3 | 228.2 | 243.9 | | |

| | | | Wildtype | | | | RpoS | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Accession ID** | **Gene** | **RNA regulation⁺** | **4 h** | **6 h** | **24 h** | **48 h** | **4 h** | **6 h** | **24 h** | **48 h** | **RpoS-dependent** | **Reference** |
| P64581 | *yqjD* | | 15.5 | 59.9 | 224.1 | 291.7 | 19.8 | 18.3 | 16.9 | 28.6 | RpoS-dependent | (Patten *et al.*, 2004) |
| P68206 | *yjbJ* | ppGpp, FliZ | 11.0 | 41.9 | 322.5 | 338.8 | 18.8 | 6.8 | 5.4 | 10.5 | RpoS-dependent, early stationary phase induction | (Lacour and Landini, 2004) |
| P0ABQ2 | *garR* | RpoD, CdaR, FNR, Fur, H-NS | 40.9 | 148.4 | 76.5 | 54.4 | 18.8 | 219.8 | 128.6 | 110.7 | RpoS-dependent | (Dong and Schellhorn, 2009b) |
| C5A0T6 | *rsd* | RpoS, RpoD, SlyA, SdiA, RcdA, McbR, ArcA | 35.6 | 127.2 | 134.0 | 116.1 | 29.0 | 76.5 | 144.8 | 106.0 | | |
| P31064 | *yedE* | | 40.6 | 146.2 | 18.8 | 25.4 | 31.4 | 155.7 | 147.4 | 181.1 | | |
| B1X6A8 | *ECDH10B_2097* | | 28.7 | 91.6 | 434.3 | 153.5 | 54.5 | 17.8 | N.D. | N.D. | | |
| P65292 | *ygdI* | | 13.6 | 47.4 | 251.1 | 383.8 | 16.4 | 5.2 | 18.2 | 27.5 | RpoS-dependent | (Dong and Schellhorn, 2009b) |
| P0AC44 | *sdhD* | RpoD, CRP-cAMP, Fur, RyhB, FNR, ArcA | 21.2 | 70.0 | 80.8 | 127.0 | 34.5 | 60.9 | 167.8 | 216.3 | | |
| P32177 | *fdhD* | Nac | 24.6 | 86.2 | 73.2 | 123.1 | 22.3 | 53.9 | N.D. | N.D. | | |
| P0AFX0 | *hpf* | RpoE, RpoD | 18.9 | 65.2 | 158.1 | 162.2 | 24.9 | 57.4 | 171.9 | 79.8 | | |
| P37188 | *gatB* | RpoD, CRP-cAMP, GatR, ArcA | 92.2 | 306.3 | 28.5 | 9.7 | 60.7 | 244.0 | 50.7 | 33.0 | | |
| P76193 | *ynhG* | ppGpp | 35.8 | 121.1 | 160.9 | N.D. | N.D. | N.D. | N.D. | N.D. | RpoS-dependent | (Patten *et al.*, 2004) |
| P07003 | *poxB* | RpoD, RpoS, ppGpp, Cra, MarA, SoxS | 27.6 | 91.1 | 392.7 | 181.7 | 15.3 | 17.7 | 15.5 | 26.9 | RpoS-dependent, stationary phase specific | (Loewen *et al.*, 1998) |
| P0A867 | *talA* | RpoS, CreB, DksA, ppGpp | 13.6 | 45.0 | 282.4 | 340.2 | 22.4 | 10.5 | 9.2 | 18.3 | RpoS-dependent, early stationary phase | (Lacour and Landini, 2004) |
| P76227 | *ynjH* | FliA | 46.4 | 148.7 | 116.9 | 156.5 | 63.0 | 29.4 | 109.3 | 70.7 | | |

| | | | Wildtype | | | | RpoS | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Accession ID** | **Gene** | **RNA regulation⁺** | **4 h** | **6 h** | **24 h** | **48 h** | **4 h** | **6 h** | **24 h** | **48 h** | **RpoS-dependent** | **Reference** |
| P23843 | *oppA* | FliA, GcvB, Fur, Lrp, ArcA, ModE, spermidine | 39.9 | 134.7 | 25.3 | 22.6 | 33.6 | 51.7 | 282.0 | 234.8 | | |
| P04846 | *nlpA* | CsgD, GcvB | 43.6 | 151.2 | 119.4 | 113.4 | 86.5 | 72.2 | 151.8 | 190.5 | | |
| P0A9G6 | *aceA* | RpoD, IHF, Cra, ArcA, CRP-cAMP, IclR-pyruvate | 17.3 | 58.5 | 221.6 | 91.4 | 9.1 | 19.7 | 224.9 | 175.3 | RpoS-dependent | (Dong and Schellhorn, 2009b) |
| B1X775 | *glgC* | RpoD, ppGpp, CRP-cAMP, carbon storage regulator | 23.4 | 75.0 | 110.8 | 124.2 | 19.8 | 67.2 | 155.6 | 216.5 | RpoS-dependent, stationary phase specific | (Loewen *et al.*, 1998) |
| P75691 | *yahK* | | 14.1 | 46.0 | 273.0 | 315.1 | 30.2 | 17.5 | 18.8 | 46.5 | RpoS-dependent | (Traxler *et al.*, 2008) |
| P0AG84 | *yghA* | ppGpp | 13.6 | 42.5 | 266.7 | 315.0 | 18.8 | 10.4 | 4.7 | 13.6 | RpoS-dependent | (Weber *et al.*, 2005) |
| P77717 | *ybaY* | | 14.9 | 48.1 | 274.2 | 345.1 | 20.1 | 10.2 | 9.9 | 19.0 | RpoS-dependent | (Patten *et al.*, 2004) |
| P37903 | *uspF* | | 15.2 | 48.2 | 138.5 | 62.4 | 17.2 | 68.2 | 287.2 | 122.8 | RpoS-dependent, stationary phase specific | (Farewell *et al.*, 1998) |
| P15078 | *cstA* | RpoD, CRP-cAMP | 35.2 | 108.0 | 55.5 | N.D. | N.D. | 36.5 | 184.2 | 256.1 | RpoS-dependent | (Dubey *et al.*, 2003) |
| P0AAT9 | *ybeL* | ppGpp | 20.4 | 64.4 | 242.5 | 272.2 | 22.1 | 24.2 | 64.8 | 48.9 | RpoS-dependent | (Weber *et al.*, 2005) |
| Q46863 | *ygiS* | | 32.2 | 97.8 | 59.3 | N.D. | N.D. | 86.1 | 204.2 | 162.8 | RpoS-dependent, early stationary phase induction | (Lacour and Landini, 2004) |
| P23857 | *pspE* | | 112.2 | 336.5 | 56.0 | 36.0 | 110.9 | 68.4 | 66.1 | 41.5 | RpoS-dependent, stationary phase specific | (Loewen *et al.*, 1998) |
| B1XDH5 | *cysH* | HypT | 58.4 | 182.8 | 50.5 | N.D. | N.D. | 69.7 | 151.2 | 139.5 | | |
| P77499 | *sufC* | RpoD, ppGpp, OxyR, IHF, IscR, NsrR, Fur | 39.3 | 113.5 | 158.4 | 137.6 | 51.3 | 29.3 | N.D. | N.D. | RpoS-dependent | (Dong and Schellhorn, 2009b) |

| | | | Wildtype | | | | RpoS | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Accession ID** | **Gene** | **RNA regulation⁺** | **4 h** | **6 h** | **24 h** | **48 h** | **4 h** | **6 h** | **24 h** | **48 h** | **RpoS-dependent** | **Reference** |
| C4ZTN8 | *treA* | RpoS, ppGpp | 61.8 | 183.9 | 103.2 | N.D. | N.D. | N.D. | N.D. | N.D. | RpoS-dependent, stationary phase specific | (Loewen and Henggearonis, 1994; Loewen *et al.*, 1998) |
| P0AG80 | *ugpB* | RpoD, CRP-cAMP, PhoB | 36.5 | 107.4 | 223.4 | 243.4 | 33.9 | 17.8 | 38.9 | 36.8 | RpoS-dependent | (Weber *et al.*, 2005) |
| P37685 | *aldB* | RpoS, CRP-cAMP, Fis | 19.7 | 60.8 | 309.6 | 306.0 | 18.4 | 9.8 | 23.2 | 32.0 | RpoS-dependent, stationary phase specific | (Loewen *et al.*, 1998) |
| P0A991 | *fbaB* | RpoS, ppGpp, Cra | 16.2 | 48.2 | 308.5 | 369.0 | 20.8 | 12.9 | 37.6 | 48.9 | RpoS-dependent, early stationary phase induction | (Lacour and Landini, 2004) |
| B1XA76 | *dadA* | RpoD, CRP-cAMP, Lrp | 81.1 | 236.1 | 69.4 | 27.1 | 71.6 | 161.8 | 53.9 | 82.7 | | |
| P61889 | *mdh* | RpoD, MpdA, CRP-cAMP, ArcA, FlhDC | 29.5 | 84.2 | 87.0 | 105.1 | 29.1 | 79.2 | 178.8 | 228.0 | | |
| P0ABK5 | *cysK* | RpoD, CysB | 41.2 | 123.3 | 107.5 | 155.2 | 18.4 | 86.0 | 162.9 | 172.7 | | |
| P25553 | *aldA* | RpoD, SoxS, Rob, MarA, CRP-cAMP, DnaA, ArcA, FNR | 30.5 | 86.6 | 109.0 | 97.3 | 26.0 | 50.3 | 181.4 | 231.7 | | |
| P76113 | *curA* | ppGpp, Nac | 18.6 | 53.5 | 219.7 | 272.1 | 29.6 | 24.7 | 49.4 | 97.6 | | |
| P0AD59 | *ivy* | Nac | 18.2 | 50.5 | 226.0 | 270.4 | 21.0 | 11.2 | 122.2 | 89.1 | | |
| P0ABH7 | *gltA* | RpoD, CRP-cAMP, IHF, ArcA | 35.9 | 99.4 | 93.5 | 106.2 | 30.1 | 66.0 | 170.6 | 221.8 | | |
| P0AA53 | *qmcA* | | 38.6 | 136.2 | 125.0 | 142.1 | 96.6 | 84.1 | 168.9 | 228.3 | | |
| C4ZRQ6 | *yaeH* | | 42.5 | 118.3 | 201.7 | 83.8 | 27.0 | 94.4 | 158.7 | 77.2 | | |
| P76440 | *preT* | RpoD, CRP-cAMP | 60.3 | 163.4 | 44.8 | 34.9 | 198.2 | 163.6 | 87.3 | 153.2 | | |
| P37313 | *dppF* | RpoD, IHF, GcvB, FNR | 51.1 | 136.2 | 171.8 | 167.2 | 25.3 | 9.4 | N.D. | N.D. | RpoS-dependent | (Dong and Schellhorn, 2009b) |
| P0AET8 | *hdhA* | | 16.2 | 44.8 | 147.8 | 218.5 | 19.4 | 41.5 | 127.6 | 188.0 | RpoS-dependent | (Weber *et al.*, 2005) |

| | | | Wildtype | | | | RpoS | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Accession ID** | **Gene** | **RNA regulation⁺** | **4 h** | **6 h** | **24 h** | **48 h** | **4 h** | **6 h** | **24 h** | **48 h** | **RpoS-dependent** | **Reference** |
| P42620 | *yqjG* | | 23.7 | 64.1 | 210.4 | 254.5 | 58.4 | 14.8 | N.D. | N.D. | RpoS-dependent | (Traxler *et al.*, 2008) |
| P0AEU0 | *hisJ* | RpoN, RpoD, H-NS,NtrC, ArgR, ppGpp | 33.2 | 89.8 | 120.2 | 156.1 | 70.4 | 24.1 | 159.1 | 81.7 | | |
| P0AGE9 | *sucD* | RpoS, RpoD, CRP-cAMP, Fur, RyhB, IHF, ArcA, FNR | 51.4 | 138.8 | 108.6 | 34.1 | 49.2 | 86.2 | 214.5 | 95.4 | | |
| P29013 | *ycgB* | ppGpp | 58.8 | 155.5 | 182.9 | 122.0 | 87.1 | 28.0 | N.D. | N.D. | RpoS-dependent | (Ibanez-Ruiz *et al.*, 2000) |

N.D. = Not Detected. ⁺Regulation at the transcriptome level as determined by RNA sequencing methodologies and Microarray analyses.

**Table 15. The average normalized abundance of proteins upregulated (>2-fold) during late stationary phase (24 h) compared to early stationary phase (6 h).**

| | | | Wildtype | | | | RpoS | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Accession ID** | **Gene** | **RNA regulation[+]** | **4 h** | **6 h** | **24 h** | **48 h** | **4 h** | **6 h** | **24 h** | **48 h** | **RpoS-dependent** | **Reference** |
| P69908 | *gadA* | RpoD, RpoS, ppGpp, AdiY, GadX, GadE-RcsB, ArcA, RcsB, Fis, H-NS, GadW, TorR, CRP-cAMP, FNR | 7.9 | 7.6 | 225.9 | 340.5 | 17.7 | 8.8 | 4.6 | 11.9 | RpoS-dependent, stationary phase specific | (Castanie-Cornet *et al.*, 1999; De Biase *et al.*, 1999) |
| P0AES9 | *hdeA* | RpoS, RpoD, RcsB, PhoP, GadE, TorR, FliZ, GadW, GadX, Lrp, H-NS, MarA | 5.2 | 10.9 | 282.3 | 387.2 | 15.4 | 4.8 | 6.9 | 9.3 | RpoS-dependent, stationary phase specific | (Loewen *et al.*, 1998) |
| P63235 | *gadC* | RpoD, RpoS, AdiY, ppGpp, RcsB, GadX, GadE, FliZ, GadW, Fis, CRP-cAMP | 6.5 | 12.2 | 315.4 | 363.0 | 21.4 | 7.4 | 2.2 | 7.4 | RpoS-dependent | (Weber *et al.*, 2005) |
| P69910 | *gadB* | RpoD, RpoS, ppGpp, AdiY, GadX, GadE, RcsB, Fis, GadW, CRP-Camp | 11.9 | 16.9 | 286.5 | 350.4 | 24.9 | 14.1 | 9.3 | 13.2 | RpoS-dependent, stationary phase specific | (Castanie-Cornet *et al.*, 1999; De Biase *et al.*, 1999) |
| P37194 | *slp* | RpoD, YdeO, GadE-RcsB, GadX, H-NS, GadW, MarA | 7.2 | 18.6 | 284.8 | 321.0 | 18.2 | 9.6 | 8.8 | 13.5 | RpoS-dependent | (Patten *et al.*, 2004) |
| P0AET2 | *hdeB* | RpoD, RpoS, ppGpp, RcsB, PhoP, GadE, TorR, FliZ, GadW, GadX, Lrp, H-NS, MarA | 14.4 | 19.4 | 270.4 | 329.3 | 30.4 | 12.3 | 7.3 | 11.4 | RpoS-dependent, stationary phase specific | (Loewen *et al.*, 1998) |
| P0AAV6 | *ybgS* | ppGpp | 19.0 | 30.7 | 297.8 | 413.6 | 26.0 | 8.3 | 8.6 | 11.5 | RpoS-dependent | (Patten *et al.*, 2004) |
| P37636 | *mdtE* | RpoS, RpoD, ppGpp, GadX, GadW, GadE, EvgA, YdeO, FliZ, H-NS, CRP-cAMP | 38.5 | 32.6 | 300.1 | 331.1 | 31.0 | 10.1 | N.D. | N.D. | RpoS-dependent | (Kobayashi *et al.*, 2006) |
| P76402 | *yegP* | ppGpp | 15.2 | 34.3 | 284.7 | 302.2 | 74.6 | 14.2 | 7.6 | 15.4 | RpoS-dependent, stationary phase specific | (Patten *et al.*, 2004) |

| | | | Wildtype | | | | RpoS | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Accession ID** | **Gene** | **RNA regulation⁺** | **4 h** | **6 h** | **24 h** | **48 h** | **4 h** | **6 h** | **24 h** | **48 h** | **RpoS-dependent** | **Reference** |
| P68206 | *yjbJ* | ppGpp, FliZ | 11.0 | 41.9 | 322.5 | 338.8 | 18.8 | 6.8 | 5.4 | 10.5 | RpoS-dependent, early stationary phase induction | (Lacour and Landini, 2004) |
| P0A786 | *pyrB* | UTP, ppGpp | 27.2 | 31.4 | 243.5 | 295.8 | 41.5 | 38.1 | 42.8 | 80.4 | RpoS-dependent | (Patten *et al.*, 2004) |
| P77454 | *glsA1* | | 27.3 | 44.9 | 328.8 | 158.2 | 89.9 | 20.8 | N.D. | N.D. | RpoS-dependent, stationary phase specific | (Loewen and Henggearonis, 1994; Loewen *et al.*, 1998) |
| C4ZXY4 | *dps* | RpoS, RpoD, ppGpp, OxyR, IHF, MntR, H-NS, Fis | 15.0 | 37.7 | 260.2 | 315.8 | 29.1 | 16.5 | 20.5 | 28.5 | RpoS-dependent, stationary phase specific | (Loewen and Henggearonis, 1994; Loewen *et al.*, 1998) |
| Q2M7R5 | *yibT* | | 33.4 | 36.4 | 247.4 | 255.7 | 21.6 | 23.7 | 81.0 | 52.7 | RpoS-dependent* | (Lago *et al.*, 2017) |
| P0AGD1 | *sodC* | RpoS, ppGpp | 20.9 | 40.9 | 270.6 | 303.3 | 27.6 | 2.9 | N.D. | N.D. | RpoS-dependent, stationary phase specific | (Gort et al., 1999a) |
| P0AB80 | *ilvE* | RpoD, ppGpp, IHF, Lrp, Lrp-Leu | 26.9 | 28.2 | 201.2 | 126.8 | 95.3 | 83.4 | 78.3 | 93.9 | | |
| P0A991 | *fbaB* | RpoS, ppGpp, Cra | 16.2 | 48.2 | 308.5 | 369.0 | 20.8 | 12.9 | 37.6 | 48.9 | RpoS-dependent, early stationary phase induction | (Lacour and Landini, 2004) |
| C4ZRB7 | *pyrI* | UTP, ppGpp | 33.5 | 36.4 | 238.1 | 285.6 | 31.5 | 32.6 | 43.1 | 65.7 | | |
| P31130 | *ydeI* | RpoD, RcdA | 26.3 | 41.5 | 271.5 | 327.9 | 23.7 | 14.6 | 19.1 | 25.7 | RpoS-dependent | (Patten *et al.*, 2004) |
| Q46857 | *dkgA* | RpoD, RpoS, YqhC, ppGpp | 19.7 | 43.0 | 272.9 | 313.1 | 20.5 | 17.3 | 36.8 | 41.7 | RpoS-dependent | (Lelong *et al.*, 2007) |
| P0AG84 | *yghA* | ppGpp | 13.6 | 42.5 | 266.7 | 315.0 | 18.8 | 10.4 | 4.7 | 13.6 | RpoS-dependent | (Weber *et al.*, 2005) |
| P0A867 | *talA* | RpoS, CreB, DksA, ppGpp | 13.6 | 45.0 | 282.4 | 340.2 | 22.4 | 10.5 | 9.2 | 18.3 | RpoS-dependent, early stationary phase | (Lacour and Landini, 2004) |
| P21367 | *ycaC* | BaeR, Nac, FNR | 12.3 | 55.8 | 343.3 | 342.8 | 15.4 | 1.3 | N.D. | N.D. | RpoS-dependent | (Patten *et al.*, 2004) |
| P33224 | *aidB* | RpoS, RpoD, ppGpp, Ada, Lrp | 22.7 | 46.7 | 292.0 | 186.5 | 109.9 | 23.6 | N.D. | N.D. | RpoS-dependent, stationary phase specific | (Loewen and Henggearonis, 1994; Loewen *et al.*, 1998) |
| P31660 | *prpC* | RpoN, CRP-cAMP, PrpR, Cra | 55.5 | 43.8 | 264.4 | 116.4 | 74.6 | 25.6 | N.D. | N.D. | | |
| P75691 | *yahK* | | 14.1 | 46.0 | 273.0 | 315.1 | 30.2 | 17.5 | 18.8 | 46.5 | RpoS-dependent | (Traxler *et al.*, 2008) |
| P77717 | *ybaY* | | 14.9 | 48.1 | 274.2 | 345.1 | 20.1 | 10.2 | 9.9 | 19.0 | RpoS-dependent | (Patten *et al.*, 2004) |

| | | | Wildtype | | | | RpoS | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Accession ID** | **Gene** | **RNA regulation⁺** | **4 h** | **6 h** | **24 h** | **48 h** | **4 h** | **6 h** | **24 h** | **48 h** | **RpoS-dependent** | **Reference** |
| B1XBC2 | *argE* | RpoD, ArgR | 30.7 | 39.6 | 230.2 | 74.8 | 85.0 | 108.0 | 92.8 | 99.0 | | |
| P76145 | *tam* | RpoS, ppGpp | 28.1 | 30.2 | 192.6 | 352.4 | 31.4 | 23.4 | N.D. | N.D. | RpoS-dependent, stationary phase specific | (Cai and Clarke, 1999) |
| P33368 | *yohF* | | 23.8 | 47.7 | 264.6 | 309.5 | 13.9 | 4.2 | N.D. | N.D. | RpoS-dependent, stationary phase specific | (Loewen *et al.*, 1998) |
| P0ADU5 | *ygiW* | | 16.2 | 66.3 | 365.4 | 146.3 | 15.3 | 8.9 | 4.9 | 7.8 | RpoS-dependent | (Lacour and Landini, 2004) |
| P37645 | *yhjG* | RpoS | 31.3 | 65.9 | 353.9 | 183.4 | 24.6 | N.D. | N.D. | N.D. | RpoS-dependent | (Weber *et al.*, 2005) |
| P65292 | *ygdI* | | 13.6 | 47.4 | 251.1 | 383.8 | 16.4 | 5.2 | 18.2 | 27.5 | RpoS-dependent | (Dong and Schellhorn, 2009b) |
| P31658 | *hchA* | RpoD, RpoS, ppGpp, H-NS | 17.4 | 44.9 | 232.5 | 268.4 | 23.8 | 20.8 | 54.0 | 57.0 | RpoS-dependent | (Patten *et al.*, 2004) |
| P37685 | *aldB* | RpoS, CRP-cAMP, Fis | 19.7 | 60.8 | 309.6 | 306.0 | 18.4 | 9.8 | 23.2 | 32.0 | RpoS-dependent, stationary phase specific | (Loewen *et al.*, 1998) |
| P33570 | *tktB* | RpoS, PhoB | 20.3 | 50.1 | 252.7 | 272.1 | 25.5 | 22.5 | 14.8 | 32.2 | RpoS-dependent | (Patten *et al.*, 2004) |
| P06999 | *pfkB* | RpoS | 31.5 | 42.2 | 222.5 | 276.4 | 27.0 | 42.5 | 58.7 | 59.5 | RpoS-dependent | (Lacour and Landini, 2004) |
| B1X6A8 | *ECDH10B_2097* | | 28.7 | 91.6 | 434.3 | 153.5 | 54.5 | 17.8 | N.D. | N.D. | | |
| P0ADB7 | *ecnB* | RpoS, ppGpp, MicA, OmpR | 8.1 | 52.1 | 247.6 | 358.7 | 18.0 | 10.4 | 11.2 | 22.3 | RpoS-dependent | (Dong and Schellhorn, 2009b) |
| P16681 | *yjdN* | | 33.6 | 53.9 | 261.4 | 237.4 | 35.0 | 16.9 | N.D. | N.D. | | |
| P76217 | *astD* | ArgR,NtrC,RpoS, RpoN, RpoD | 11.3 | 55.7 | 264.1 | 287.4 | 23.3 | 28.5 | 69.1 | 66.5 | RpoS-dependent | (Dong and Schellhorn, 2009b) |
| P0AFH8 | *osmY* | RpoS, RpoD, FliZ, Fis, IHF, Lrp, CRP-cAMP | 12.9 | 57.2 | 265.5 | 287.6 | 30.6 | 12.2 | 7.6 | 15.9 | RpoS-dependent, stationary phase specific | (Loewen and Henggearonis, 1994; Loewen *et al.*, 1998) |
| P21363 | *yciE* | RpoD | 37.3 | 54.5 | 256.9 | 186.1 | 68.0 | 20.3 | N.D. | N.D. | RpoS-dependent | (Dong and Schellhorn, 2009b) |
| P25738 | *msyB* | RpoS, ppGpp | 6.3 | 55.8 | 253.0 | 328.6 | 26.6 | 17.0 | 13.7 | 30.7 | RpoS-dependent | (Lacour and Landini, 2004) |
| P76172 | *ynfD* | ppGpp | 12.2 | 57.8 | 264.5 | 324.5 | 25.6 | 18.1 | 61.1 | 25.9 | RpoS-dependent | (Traxler *et al.*, 2008) |
| P0AEH5 | *elaB* | RpoS, ppGpp | 11.9 | 60.0 | 269.6 | 356.4 | 19.2 | 11.5 | 8.3 | 21.2 | RpoS-dependent | (Patten *et al.*, 2004) |

| | | | Wildtype | | | | RpoS | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Accession ID** | **Gene** | **RNA regulation⁺** | **4 h** | **6 h** | **24 h** | **48 h** | **4 h** | **6 h** | **24 h** | **48 h** | **RpoS-dependent** | **Reference** |
| P0C0L2 | *osmC* | RpoD, RpoS, ppGpp, RcsB, Lrp, H-NS, YaaB | 29.7 | 57.7 | 262.9 | 305.0 | 28.2 | 17.8 | 25.1 | 25.8 | RpoS-dependent | (Gordia and Gutierrez, 1996) |
| B1X9C5 | *ECDH10B_1076* | | 12.3 | 59.6 | 262.1 | 291.3 | 19.0 | 24.4 | 37.7 | 26.1 | | |
| P0AD59 | *ivy* | Nac | 18.2 | 50.5 | 226.0 | 270.4 | 21.0 | 11.2 | 122.2 | 89.1 | | |
| P45470 | *yhbO* | ppGpp | 22.4 | 52.3 | 237.4 | 371.0 | 17.9 | 2.4 | N.D. | N.D. | RpoS-dependent | (Weber *et al.*, 2005) |
| P07003 | *poxB* | RpoD, RpoS, ppGpp, Cra, MarA, SoxS | 27.6 | 91.1 | 392.7 | 181.7 | 15.3 | 17.7 | 15.5 | 26.9 | RpoS-dependent, stationary phase specific | (Loewen *et al.*, 1998) |
| P76113 | *curA* | ppGpp, Nac | 18.6 | 53.5 | 219.7 | 272.1 | 29.6 | 24.7 | 49.4 | 97.6 | | |
| P77667 | *sufA* | RpoD, ppGpp, OxyR, IHF, IscR, NsrR, Fur | 34.1 | 57.7 | 239.6 | 365.1 | 33.1 | 5.5 | N.D. | N.D. | RpoS-dependent | (Patten *et al.*, 2004) |
| P0AC59 | *grxB* | RpoD, ppGpp | 23.9 | 52.3 | 209.2 | 202.2 | 21.9 | 34.6 | 141.2 | 103.8 | | |
| P75818 | *ybjP* | RpoS, ppGpp | 32.0 | 49.8 | 201.5 | 227.9 | 61.5 | 28.3 | 43.0 | 53.2 | RpoS-dependent | (Traxler *et al.*, 2008) |
| P09551 | *argT* | RpoD, DksA, Fis, DksA-ppGpp, ppGpp | 13.5 | 53.5 | 212.5 | 305.0 | 19.5 | 9.3 | 107.9 | 100.4 | | |
| P0A9G6 | *aceA* | RpoD, IHF, Cra, ArcA, CRP-cAMP, IclR-pyruvate | 17.3 | 58.5 | 221.6 | 91.4 | 9.1 | 19.7 | 224.9 | 175.3 | RpoS-dependent | (Dong and Schellhorn, 2009b) |
| P0ADB1 | *osmE* | RpoS, RpoD, IHF, Fis | 14.5 | 77.2 | 289.0 | 351.8 | 17.2 | 12.4 | 28.4 | 43.3 | RpoS-dependent, stationary phase specific | (Loewen and Henggearonis, 1994; Loewen *et al.*, 1998) |
| P0AAT9 | *ybeL* | ppGpp | 20.4 | 64.4 | 242.5 | 272.2 | 22.1 | 24.2 | 64.8 | 48.9 | RpoS-dependent | (Weber *et al.*, 2005) |
| P0ABD3 | *bfr* | RyhB | 17.7 | 71.1 | 260.9 | 328.9 | 15.5 | 5.7 | 7.6 | 44.5 | RpoS-dependent, early stationary phase specific | (Lacour and Landini, 2004) |
| P64581 | *yqjD* | | 15.5 | 59.9 | 224.1 | 291.7 | 19.8 | 18.3 | 16.9 | 28.6 | RpoS-dependent | (Patten *et al.*, 2004) |
| P39451 | *adhP* | ppGpp | 25.9 | 69.5 | 262.8 | 290.5 | 41.2 | 12.6 | N.D. | N.D. | RpoS-dependent | (Weber *et al.*, 2005) |
| B1XEA4 | *lsrB* | RpoS, CRP-cAMP, LsrR | 39.1 | 61.7 | 211.8 | 236.7 | 52.0 | 18.9 | 118.6 | 54.4 | RpoS-dependent | (Dong and Schellhorn, 2009b) |
| B1X8V5 | *cbpA* | RpoD, RpoS, ppGpp, Fis | 27.3 | 59.2 | 214.8 | 285.5 | 24.4 | 32.2 | 61.1 | 85.8 | RpoS-dependent, stationary phase specific | (Loewen *et al.*, 1998) |
| P64451 | *ydcL* | ppGpp | 35.5 | 64.0 | 230.0 | 302.3 | 33.8 | 35.0 | 46.9 | 77.7 | RpoS-dependent | (Traxler *et al.*, 2008) |

| | | | Wildtype | | | | RpoS | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Accession ID** | **Gene** | **RNA regulation⁺** | **4 h** | **6 h** | **24 h** | **48 h** | **4 h** | **6 h** | **24 h** | **48 h** | **RpoS-dependent** | **Reference** |
| P0AAN5 | *yaiA* | RpoD, ppGpp, TyrR, TrpR | 17.5 | 84.9 | 293.3 | 293.1 | 17.9 | N.D. | N.D. | N.D. | RpoS-dependent | (Weber *et al.*, 2005) |
| P21179 | *katE* | RpoS, ppGpp, Fur, Fis | 14.2 | 74.1 | 246.7 | 449.6 | 24.7 | 4.5 | N.D. | N.D. | RpoS-dependent, stationary phase specific | (Loewen and Henggearonis, 1994; Loewen *et al.*, 1998) |
| P42620 | *yqjG* | | 23.7 | 64.1 | 210.4 | 254.5 | 58.4 | 14.8 | N.D. | N.D. | RpoS-dependent | (Traxler *et al.*, 2008) |
| P0AET8 | *hdhA* | | 16.2 | 44.8 | 147.8 | 218.5 | 19.4 | 41.5 | 127.6 | 188.0 | RpoS-dependent | (Weber *et al.*, 2005) |
| P77739 | *yniA* | ppGpp | 25.4 | 68.8 | 218.7 | 89.4 | 17.9 | 55.9 | 254.0 | 106.6 | RpoS-dependent | (Dong and Schellhorn, 2009b) |
| P0AF50 | *yjbR* | GadE | 29.1 | 62.6 | 200.9 | 216.7 | 25.1 | 41.1 | 122.8 | 76.1 | | |
| P37339 | *lhgD* | RpoS, ppGpp, CRP-cAMP, Lrp-Leu, H-NS, GlaR | 220.1 | 164.4 | 934.0 | 458.1 | 100.2 | 32.5 | N.D. | N.D. | | |
| P0ADQ7 | *ygaM* | | 28.9 | 64.8 | 207.1 | 256.6 | 64.7 | 31.0 | 22.2 | 33.8 | RpoS-dependent | (Weber *et al.*, 2005) |
| P77674 | *prr* | RpoS, Nac | 13.4 | 91.7 | 269.1 | 315.1 | 30.0 | 19.2 | 18.5 | 35.6 | | |
| P77754 | *spy* | RpoD, BaeR, CpxR | 46.5 | 66.7 | 210.2 | 198.7 | 34.6 | 26.5 | 130.5 | 92.2 | | |
| P27250 | *ahr* | | 34.0 | 84.8 | 258.8 | 338.6 | 44.1 | 8.7 | N.D. | N.D. | | |
| C4ZZA4 | *astC* | ArgR,NtrC,RpoS, RpoN, RpoD | 13.8 | 90.7 | 274.1 | 251.4 | 34.3 | 29.1 | 56.0 | 73.1 | RpoS-dependent | (Dong and Schellhorn, 2009b) |
| P12994 | *ybhB* | | 39.6 | 109.6 | 317.4 | 174.0 | 23.0 | 33.5 | 57.6 | 23.5 | | |
| P0AFM4 | *psiF* | RpoD, PhoB | 38.9 | 98.9 | 302.8 | 202.9 | 54.1 | 10.6 | N.D. | N.D. | RpoS-dependent, early stationary phase specific | (Lacour and Landini, 2004) |
| Q46845 | *yghU* | | 25.1 | 58.6 | 176.9 | 212.9 | 25.6 | 38.9 | 102.2 | 146.3 | | |
| P33012 | *sbmC* | CRP-cAMP, LexA | 33.5 | 86.2 | 255.4 | 185.8 | 22.0 | 39.6 | 108.3 | 62.5 | | |
| P0AB14 | *yccJ* | RpoS, ppGpp, CsgD | 48.5 | 78.8 | 231.7 | 315.2 | 15.6 | 18.2 | 34.6 | 30.7 | RpoS-dependent | (Weber *et al.*, 2005) |
| P0A6X7 | *ihfA* | RpoS, IHF | 47.2 | 64.1 | 189.5 | 197.0 | 45.6 | 61.7 | 101.1 | 65.8 | | |
| P22255 | *cysQ* | Nac, ppGpp | 43.9 | 69.8 | 205.5 | 222.7 | 51.1 | 42.6 | 71.6 | 49.6 | RpoS-dependent | (Traxler *et al.*, 2008) |
| P76143 | *lsrF* | RpoS, CRP-cAMP, LsrR | 28.1 | 58.6 | 162.4 | 188.8 | 26.7 | 44.4 | 141.6 | 167.6 | RpoS-dependent | (Dong and Schellhorn, 2009b) |
| P37903 | *uspF* | | 15.2 | 48.2 | 138.5 | 62.4 | 17.2 | 68.2 | 287.2 | 122.8 | RpoS-dependent, stationary phase specific | (Farewell *et al.*, 1998) |

| | | | Wildtype | | | | RpoS | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Accession ID** | **Gene** | **RNA regulation⁺** | **4 h** | **6 h** | **24 h** | **48 h** | **4 h** | **6 h** | **24 h** | **48 h** | **RpoS-dependent** | **Reference** |
| P75874 | *yccU* | ppGpp | 44.7 | 83.0 | 232.2 | 196.7 | 48.5 | 39.7 | 82.4 | 54.1 | RpoS-dependent | (Traxler *et al.*, 2008) |
| C4ZXT8 | *pgl* | | 47.5 | 80.2 | 224.9 | 186.8 | 55.2 | 50.6 | 55.2 | 50.0 | | |
| P0ABE2 | *bolA* | RpoD, RpoS, ppGpp, H-NS, OmpR | 46.9 | 90.1 | 251.5 | 171.4 | 66.5 | 39.7 | 29.6 | 45.7 | RpoS-dependent, stationary phase specific | (Loewen and Henggearonis, 1994; Loewen *et al.*, 1998) |
| B1XD59 | *yaeP* | CRP-cAMP | 40.9 | 68.5 | 189.8 | 184.6 | 34.7 | 44.7 | 154.8 | 105.2 | | |
| P76108 | *ydcS* | RpoS, Nac | 53.6 | 90.7 | 244.8 | 304.9 | 42.2 | 7.7 | N.D. | N.D. | RpoS-dependent | (Schellhorn *et al.*, 1998) |
| P0A9D2 | *gstA* | | 24.2 | 55.8 | 151.1 | 169.2 | 28.5 | 46.5 | 177.5 | 146.0 | | |
| P0ADE6 | *kbp* | RpoS, CpxR, ppGpp | 16.2 | 42.8 | 119.0 | 306.2 | 35.9 | 14.2 | 22.5 | 77.9 | | |
| P33362 | *yehZ* | ppGpp, RpoH, RpoS | 17.9 | 95.1 | 252.6 | 275.2 | 19.0 | 5.1 | N.D. | N.D. | RpoS-dependent | (Dong and Schellhorn, 2009b) |

N.D. = Not Detected. ⁺Regulation at the transcriptome level as determined by RNA sequencing methodologies and Microarray analyses.

**Table 16. The average normalized abundance of proteins upregulated (>2-fold) during prolonged stationary phase (48 h) compared to early stationary phase (6 h).**

| | | | Wildtype | | | | RpoS | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Accession ID** | **Gene** | **RNA regulation[+]** | **4 h** | **6 h** | **24 h** | **48 h** | **4 h** | **6 h** | **24 h** | **48 h** | **RpoS-dependent** | **Reference** |
| **P69908** | *gadA* | RpoD, RpoS, ppGpp, AdiY, GadX, GadE-RcsB, ArcA, RcsB, Fis, H-NS, GadW, TorR, CRP-cAMP, FNR | 7.9 | 7.6 | 225.9 | 340.5 | 17.7 | 8.8 | 4.6 | 11.9 | RpoS-dependent, stationary phase specific | (Castanie-Cornet *et al.*, 1999; De Biase *et al.*, 1999) |
| **P0AES9** | *hdeA* | RpoS, RpoD, RcsB, PhoP, GadE, TorR, FliZ, GadW, GadX, Lrp, H-NS, MarA | 5.2 | 10.9 | 282.3 | 387.2 | 15.4 | 4.8 | 6.9 | 9.3 | RpoS-dependent, stationary phase specific | (Loewen *et al.*, 1998) |
| **P63235** | *gadC* | RpoD, RpoS, AdiY, ppGpp, RcsB, GadX, GadE, FliZ, GadW, Fis, CRP-cAMP | 6.5 | 12.2 | 315.4 | 363.0 | 21.4 | 7.4 | 2.2 | 7.4 | RpoS-dependent | (Weber *et al.*, 2005) |
| **P69910** | *gadB* | RpoD, RpoS, ppGpp, AdiY, GadX, GadE, RcsB, Fis, GadW, CRP-Camp | 11.9 | 16.9 | 286.5 | 350.4 | 24.9 | 14.1 | 9.3 | 13.2 | RpoS-dependent, stationary phase specific | (Castanie-Cornet *et al.*, 1999; De Biase *et al.*, 1999) |
| **P37194** | *slp* | RpoD, YdeO, GadE-RcsB, GadX, H-NS, GadW, MarA | 7.2 | 18.6 | 284.8 | 321.0 | 18.2 | 9.6 | 8.8 | 13.5 | RpoS-dependent | (Patten *et al.*, 2004) |
| **P0AET2** | *hdeB* | RpoD, RpoS, ppGpp, RcsB, PhoP, GadE, TorR, FliZ, GadW, GadX, Lrp, H-NS, MarA | 14.4 | 19.4 | 270.4 | 329.3 | 30.4 | 12.3 | 7.3 | 11.4 | RpoS-dependent, stationary phase specific | (Loewen *et al.*, 1998) |
| **P0AAV6** | *ybgS* | ppGpp | 19.0 | 30.7 | 297.8 | 413.6 | 26.0 | 8.3 | 8.6 | 11.5 | RpoS-dependent | (Patten *et al.*, 2004) |
| **P76145** | *tam* | RpoS, ppGpp | 28.1 | 30.2 | 192.6 | 352.4 | 31.4 | 23.4 | N.D. | N.D. | RpoS-dependent, stationary phase specific | (Cai and Clarke, 1999) |
| **P37636** | *mdtE* | RpoS, RpoD, ppGpp, GadX, GadW, GadE, EvgA, YdeO, FliZ, H-NS, CRP-cAMP | 38.5 | 32.6 | 300.1 | 331.1 | 31.0 | 10.1 | N.D. | N.D. | RpoS-dependent, stationary phase specific | (Kobayashi *et al.*, 2006) |
| **P0A786** | *pyrB* | UTP, ppGpp | 27.2 | 31.4 | 243.5 | 295.8 | 41.5 | 38.1 | 42.8 | 80.4 | RpoS-dependent | (Patten *et al.*, 2004) |

| | | | Wildtype | | | | RpoS | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Accession ID** | **Gene** | **RNA regulation⁺** | **4 h** | **6 h** | **24 h** | **48 h** | **4 h** | **6 h** | **24 h** | **48 h** | **RpoS-dependent** | **Reference** |
| **P76402** | *yegP* | ppGpp | 15.2 | 34.3 | 284.7 | 302.2 | 74.6 | 14.2 | 7.6 | 15.4 | RpoS-dependent | (Patten *et al.*, 2004) |
| **C4ZXY4** | *dps* | RpoS, RpoD, ppGpp, OxyR, IHF, MntR, H-NS, Fis | 15.0 | 37.7 | 260.2 | 315.8 | 29.1 | 16.5 | 20.5 | 28.5 | RpoS-dependent, stationary phase specific | (Loewen and Henggearonis, 1994; Loewen *et al.*, 1998) |
| **P68206** | *yjbJ* | ppGpp, FliZ | 11.0 | 41.9 | 322.5 | 338.8 | 18.8 | 6.8 | 5.4 | 10.5 | RpoS-dependent, early stationary phase induction | (Lacour and Landini, 2004) |
| **P65292** | *ygdI* | | 13.6 | 47.4 | 251.1 | 383.8 | 16.4 | 5.2 | 18.2 | 27.5 | RpoS-dependent | (Dong and Schellhorn, 2009b) |
| **P31130** | *ydeI* | RpoD, RcdA | 26.3 | 41.5 | 271.5 | 327.9 | 23.7 | 14.6 | 19.1 | 25.7 | RpoS-dependent | (Patten *et al.*, 2004) |
| **P0A991** | *fbaB* | RpoS, ppGpp, Cra | 16.2 | 48.2 | 308.5 | 369.0 | 20.8 | 12.9 | 37.6 | 48.9 | RpoS-dependent, early stationary phase induction | (Lacour and Landini, 2004) |
| **C4ZRB7** | *pyrI* | UTP, ppGpp | 33.5 | 36.4 | 238.1 | 285.6 | 31.5 | 32.6 | 43.1 | 65.7 | | |
| **P0A867** | *talA* | RpoS, CreB, DksA, ppGpp | 13.6 | 45.0 | 282.4 | 340.2 | 22.4 | 10.5 | 9.2 | 18.3 | RpoS-dependent, early stationary phase | (Lacour and Landini, 2004) |
| **P0AGD1** | *sodC* | RpoS, ppGpp | 20.9 | 40.9 | 270.6 | 303.3 | 27.6 | 2.9 | N.D. | N.D. | RpoS-dependent, stationary phase specific | (Gort et al., 1999b) |
| **P0AG84** | *yghA* | ppGpp | 13.6 | 42.5 | 266.7 | 315.0 | 18.8 | 10.4 | 4.7 | 13.6 | RpoS-dependent | (Weber *et al.*, 2005) |
| **Q46857** | *dkgA* | RpoD, RpoS, YqhC, ppGpp | 19.7 | 43.0 | 272.9 | 313.1 | 20.5 | 17.3 | 36.8 | 41.7 | RpoS-dependent | (Lelong *et al.*, 2007) |
| **P77717** | *ybaY* | | 14.9 | 48.1 | 274.2 | 345.1 | 20.1 | 10.2 | 9.9 | 19.0 | RpoS-dependent | (Patten *et al.*, 2004) |
| **P0ADB7** | *ecnB* | RpoS, ppGpp, MicA, OmpR | 8.1 | 52.1 | 247.6 | 358.7 | 18.0 | 10.4 | 11.2 | 22.3 | RpoS-dependent | (Dong and Schellhorn, 2009b) |
| **Q2M7R5** | *yibT* | | 33.4 | 36.4 | 247.4 | 255.7 | 21.6 | 23.7 | 81.0 | 52.7 | RpoS-dependent* | (Lago *et al.*, 2017) |
| **P45470** | *yhbO* | ppGpp | 22.4 | 52.3 | 237.4 | 371.0 | 17.9 | 2.4 | N.D. | N.D. | RpoS-dependent | (Weber *et al.*, 2005) |
| **P75691** | *yahK* | | 14.1 | 46.0 | 273.0 | 315.1 | 30.2 | 17.5 | 18.8 | 46.5 | RpoS-dependent | (Traxler *et al.*, 2008) |
| **P33368** | *yohF* | | 23.8 | 47.7 | 264.6 | 309.5 | 13.9 | 4.2 | N.D. | N.D. | RpoS-dependent, stationary phase specific | (Loewen *et al.*, 1998) |
| **P06999** | *pfkB* | RpoS | 31.5 | 42.2 | 222.5 | 276.4 | 27.0 | 42.5 | 58.7 | 59.5 | RpoS-dependent | (Lacour and Landini, 2004) |
| **P21367** | *ycaC* | BaeR, Nac, FNR | 12.3 | 55.8 | 343.3 | 342.8 | 15.4 | 1.3 | N.D. | N.D. | RpoS-dependent | (Patten *et al.*, 2004) |

| | | | Wildtype | | | | RpoS | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Accession ID** | **Gene** | **RNA regulation⁺** | **4 h** | **6 h** | **24 h** | **48 h** | **4 h** | **6 h** | **24 h** | **48 h** | **RpoS-dependent** | **Reference** |
| **P77667** | *sufA* | RpoD, ppGpp, OxyR, IHF, IscR, NsrR, Fur | 34.1 | 57.7 | 239.6 | 365.1 | 33.1 | 5.5 | N.D. | N.D. | RpoS-dependent | (Patten *et al.*, 2004) |
| **P21179** | *katE* | RpoS, ppGpp, Fur, Fis | 14.2 | 74.1 | 246.7 | 449.6 | 24.7 | 4.5 | N.D. | N.D. | RpoS-dependent, stationary phase specific | (Loewen and Henggearonis, 1994; Loewen *et al.*, 1998) |
| **P31658** | *hchA* | RpoD, RpoS, ppGpp, H-NS | 17.4 | 44.9 | 232.5 | 268.4 | 23.8 | 20.8 | 54.0 | 57.0 | RpoS-dependent | (Patten *et al.*, 2004) |
| **P0AEH5** | *elaB* | RpoS, ppGpp | 11.9 | 60.0 | 269.6 | 356.4 | 19.2 | 11.5 | 8.3 | 21.2 | RpoS-dependent | (Patten *et al.*, 2004) |
| **P25738** | *msyB* | RpoS, ppGpp | 6.3 | 55.8 | 253.0 | 328.6 | 26.6 | 17.0 | 13.7 | 30.7 | RpoS-dependent | (Lacour and Landini, 2004) |
| **P09551** | *argT* | RpoD, DksA, Fis, DksA-ppGpp, ppGpp | 13.5 | 53.5 | 212.5 | 305.0 | 19.5 | 9.3 | 107.9 | 100.4 | | |
| **P76172** | *ynfD* | ppGpp | 12.2 | 57.8 | 264.5 | 324.5 | 25.6 | 18.1 | 61.1 | 25.9 | RpoS-dependent | (Traxler *et al.*, 2008) |
| **P0ADE6** | *kbp* | RpoS, CpxR, ppGpp | 16.2 | 42.8 | 119.0 | 306.2 | 35.9 | 14.2 | 22.5 | 77.9 | | |
| **P33570** | *tktB* | RpoS, PhoB | 20.3 | 50.1 | 252.7 | 272.1 | 25.5 | 22.5 | 14.8 | 32.2 | RpoS-dependent | (Patten *et al.*, 2004) |
| **P0AD59** | *ivy* | Nac | 18.2 | 50.5 | 226.0 | 270.4 | 21.0 | 11.2 | 122.2 | 89.1 | | |
| **P0C0L2** | *osmC* | RpoD, RpoS, ppGpp, RcsB, Lrp, H-NS, YaaB | 29.7 | 57.7 | 262.9 | 305.0 | 28.2 | 17.8 | 25.1 | 25.8 | RpoS-dependent | (Gordia and Gutierrez, 1996) |
| **P37685** | *aldB* | RpoS, CRP-cAMP, Fis | 19.7 | 60.8 | 309.6 | 306.0 | 18.4 | 9.8 | 23.2 | 32.0 | RpoS-dependent, stationary phase specific | (Loewen *et al.*, 1998) |
| **P76217** | *astD* | ArgR,NtrC,RpoS, RpoN, RpoD | 11.3 | 55.7 | 264.1 | 287.4 | 23.3 | 28.5 | 69.1 | 66.5 | RpoS-dependent | (Dong and Schellhorn, 2009b) |
| **P76113** | *curA* | ppGpp, Nac | 18.6 | 53.5 | 219.7 | 272.1 | 29.6 | 24.7 | 49.4 | 97.6 | | |
| **P0AFH8** | *osmY* | RpoS, RpoD, FliZ, Fis, IHF, Lrp, CRP-cAMP | 12.9 | 57.2 | 265.5 | 287.6 | 30.6 | 12.2 | 7.6 | 15.9 | RpoS-dependent, stationary phase specific | (Loewen and Henggearonis, 1994; Loewen *et al.*, 1998) |
| **B1X9C5** | *ECDH10B_1076* | | 12.3 | 59.6 | 262.1 | 291.3 | 19.0 | 24.4 | 37.7 | 26.1 | | |
| **P64581** | *yqjD* | | 15.5 | 59.9 | 224.1 | 291.7 | 19.8 | 18.3 | 16.9 | 28.6 | RpoS-dependent | (Patten *et al.*, 2004) |
| **P25906** | *pdxI* | | 12.3 | 33.1 | 77.9 | 170.1 | 56.5 | 19.6 | N.D. | N.D. | | |
| **P0AET8** | *hdhA* | | 16.2 | 44.8 | 147.8 | 218.5 | 19.4 | 41.5 | 127.6 | 188.0 | RpoS-dependent | (Weber *et al.*, 2005) |

| | | | Wildtype | | | | RpoS | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Accession ID** | **Gene** | **RNA regulation⁺** | **4 h** | **6 h** | **24 h** | **48 h** | **4 h** | **6 h** | **24 h** | **48 h** | **RpoS-dependent** | **Reference** |
| **B1X8V5** | *cbpA* | RpoD, RpoS, ppGpp, Fis | 27.3 | 59.2 | 214.8 | 285.5 | 24.4 | 32.2 | 61.1 | 85.8 | RpoS-dependent, stationary phase specific | (Loewen *et al.*, 1998) |
| **P0ABD3** | *bfr* | RyhB | 17.7 | 71.1 | 260.9 | 328.9 | 15.5 | 5.7 | 7.6 | 44.5 | RpoS-dependent, early stationary phase specific | (Lacour and Landini, 2004) |
| **P0ADB1** | *osmE* | RpoS, RpoD, IHF, Fis | 14.5 | 77.2 | 289.0 | 351.8 | 17.2 | 12.4 | 28.4 | 43.3 | RpoS-dependent, stationary phase specific | (Loewen and Henggearonis, 1994; Loewen *et al.*, 1998) |
| **P64451** | *ydcL* | ppGpp | 35.5 | 64.0 | 230.0 | 302.3 | 33.8 | 35.0 | 46.9 | 77.7 | RpoS-dependent | (Traxler *et al.*, 2008) |
| **P75818** | *ybjP* | RpoS, ppGpp | 32.0 | 49.8 | 201.5 | 227.9 | 61.5 | 28.3 | 43.0 | 53.2 | RpoS-dependent | (Traxler *et al.*, 2008) |
| **P16681** | *yjdN* | | 33.6 | 53.9 | 261.4 | 237.4 | 35.0 | 16.9 | N.D. | N.D. | | |
| **P0AB80** | *ilvE* | RpoD, ppGpp, IHF, Lrp, Lrp-Leu | 26.9 | 28.2 | 201.2 | 126.8 | 95.3 | 83.4 | 78.3 | 93.9 | | |
| **P0AAT9** | *ybeL* | ppGpp | 20.4 | 64.4 | 242.5 | 272.2 | 22.1 | 24.2 | 64.8 | 48.9 | RpoS-dependent | (Weber *et al.*, 2005) |
| **P27250** | *ahr* | | 34.0 | 84.8 | 258.8 | 338.6 | 44.1 | 8.7 | N.D. | N.D. | | |
| **B1XEA4** | *lsrB* | RpoS, CRP-cAMP, LsrR | 39.1 | 61.7 | 211.8 | 236.7 | 52.0 | 18.9 | 118.6 | 54.4 | RpoS-dependent | (Dong and Schellhorn, 2009b) |
| **P39451** | *adhP* | ppGpp | 25.9 | 69.5 | 262.8 | 290.5 | 41.2 | 12.6 | N.D. | N.D. | RpoS-dependent | (Weber *et al.*, 2005) |
| **P0AB14** | *yccJ* | RpoS, ppGpp, CsgD | 48.5 | 78.8 | 231.7 | 315.2 | 15.6 | 18.2 | 34.6 | 30.7 | RpoS-dependent | (Weber *et al.*, 2005) |
| **P33224** | *aidB* | RpoS, RpoD, ppGpp, Ada, Lrp | 22.7 | 46.7 | 292.0 | 186.5 | 109.9 | 23.6 | N.D. | N.D. | RpoS-dependent, stationary phase specific | (Loewen and Henggearonis, 1994; Loewen *et al.*, 1998) |
| **P42620** | *yqjG* | | 23.7 | 64.1 | 210.4 | 254.5 | 58.4 | 14.8 | N.D. | N.D. | RpoS-dependent | (Traxler *et al.*, 2008) |
| **P0ADQ7** | *ygaM* | | 28.9 | 64.8 | 207.1 | 256.6 | 64.7 | 31.0 | 22.2 | 33.8 | RpoS-dependent | (Weber *et al.*, 2005) |
| **P0A915** | *ompW* | MicA, RybB, NarL, CRP-cAMP, ArcA, FNR | 6.3 | 3.6 | 5.0 | 19.0 | 5.3 | 97.8 | 231.1 | 262.2 | RpoS-dependent | (Patten *et al.*) |
| **P0AC59** | *grxB* | RpoD, ppGpp | 23.9 | 52.3 | 209.2 | 202.2 | 21.9 | 34.6 | 141.2 | 103.8 | | |
| **P0A978** | *cspG* | | 87.6 | 45.0 | 8.5 | 218.0 | 155.4 | N.D. | N.D. | N.D. | | |
| **P77674** | *prr* | RpoS, Nac | 13.4 | 91.7 | 269.1 | 315.1 | 30.0 | 19.2 | 18.5 | 35.6 | | |
| **Q46845** | *yghU* | | 25.1 | 58.6 | 176.9 | 212.9 | 25.6 | 38.9 | 102.2 | 146.3 | | |

| | | | Wildtype | | | | RpoS | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Accession ID** | **Gene** | **RNA regulation⁺** | **4 h** | **6 h** | **24 h** | **48 h** | **4 h** | **6 h** | **24 h** | **48 h** | **RpoS-dependent** | **Reference** |
| **P0AAN5** | *yaiA* | RpoD, ppGpp, TyrR, TrpR | 17.5 | 84.9 | 293.3 | 293.1 | 17.9 | N.D. | N.D. | N.D. | RpoS-dependent | (Weber *et al.*, 2005) |
| **P77454** | *glsA1* | | 27.3 | 44.9 | 328.8 | 158.2 | 89.9 | 20.8 | N.D. | N.D. | RpoS-dependent, stationary phase specific | (Loewen and Henggearonis, 1994; Loewen *et al.*, 1998) |
| **P0AF50** | *yjbR* | GadE | 29.1 | 62.6 | 200.9 | 216.7 | 25.1 | 41.1 | 122.8 | 76.1 | | |
| **P0A912** | *pal* | MicA | 89.2 | 79.9 | 74.8 | 277.4 | 132.3 | 45.9 | 62.4 | 127.9 | | |
| **P76108** | *ydcS* | RpoS, Nac | 53.6 | 90.7 | 244.8 | 304.9 | 42.2 | 7.7 | N.D. | N.D. | RpoS-dependent | (Schellhorn *et al.*, 1998) |
| **P21363** | *yciE* | RpoD | 37.3 | 54.5 | 256.9 | 186.1 | 68.0 | 20.3 | N.D. | N.D. | RpoS-dependent | (Dong and Schellhorn, 2009b) |
| **P76143** | *lsrF* | RpoS, CRP-cAMP, LsrR | 28.1 | 58.6 | 162.4 | 188.8 | 26.7 | 44.4 | 141.6 | 167.6 | RpoS-dependent | (Dong and Schellhorn, 2009b) |
| **P25526** | *gabD* | RpoS, RpoD, Nac, ppGpp, CRP-cAMP, Lrp, H-NS, GlaR | 21.1 | 93.3 | 237.2 | 303.3 | 17.7 | 20.9 | 25.9 | 64.1 | RpoS-dependent | (Weber *et al.*, 2005) |
| **P22255** | *cysQ* | Nac, ppGpp | 43.9 | 69.8 | 205.5 | 222.7 | 51.1 | 42.6 | 71.6 | 49.6 | RpoS-dependent | (Traxler *et al.*, 2008) |
| **P02925** | *rbsB* | RpoD, CpxR, ppGpp | 102.3 | 91.2 | 160.5 | 293.5 | 104.6 | 24.2 | 216.9 | 123.1 | | |
| **P0A901** | *blc* | RpoS, ppGpp | 50.2 | 99.0 | 248.2 | 308.7 | 24.7 | 5.1 | N.D. | N.D. | RpoS-dependent | (Weber *et al.*, 2005) |
| **P0A6X7** | *ihfA* | RpoS, IHF | 47.2 | 64.1 | 189.5 | 197.0 | 45.6 | 61.7 | 101.1 | 65.8 | | |
| **P0A9D2** | *gstA* | | 24.2 | 55.8 | 151.1 | 169.2 | 28.5 | 46.5 | 177.5 | 146.0 | | |
| **P22256** | *gabT* | RpoS, RpoD, Nac, ppGpp, CRP-cAMP, Lrp, H-NS, GlaR | 15.9 | 100.5 | 246.3 | 292.9 | 18.5 | 10.8 | 21.0 | 30.0 | | |
| **P0C0S1** | *mscS* | | 96.6 | 67.7 | 180.8 | 204.7 | 66.2 | 49.2 | 62.0 | 77.6 | | |
| **P77754** | *spy* | RpoD, BaeR, CpxR | 46.5 | 66.7 | 210.2 | 198.7 | 34.6 | 26.5 | 130.5 | 92.2 | | |
| **P0ADV7** | *mlaC* | MarA | 51.4 | 81.2 | 163.5 | 242.3 | 88.4 | 15.2 | 102.1 | 65.6 | | |
| **P33362** | *yehZ* | ppGpp, RpoH, RpoS | 17.9 | 95.1 | 252.6 | 275.2 | 19.0 | 5.1 | N.D. | N.D. | RpoS-dependent | (Dong and Schellhorn, 2009b) |
| **P63284** | *clpB* | RpoD, RpoH | 56.7 | 57.0 | 136.1 | 167.4 | 45.2 | 72.0 | 115.5 | 169.0 | | |
| **P69776** | *lpp* | | 83.6 | 51.0 | 79.6 | 155.1 | 94.5 | 52.9 | 59.9 | 74.4 | | |
| **P64429** | *ypfJ* | | 57.3 | 73.1 | 127.2 | 208.9 | 87.3 | 82.1 | 60.4 | 94.0 | | |

| | | | Wildtype | | | | RpoS | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Accession ID** | **Gene** | **RNA regulation⁺** | **4 h** | **6 h** | **24 h** | **48 h** | **4 h** | **6 h** | **24 h** | **48 h** | **RpoS-dependent** | **Reference** |
| **C4ZZA4** | *astC* | ArgR,NtrC,RpoS, RpoN, RpoD | 13.8 | 90.7 | 274.1 | 251.4 | 34.3 | 29.1 | 56.0 | 73.1 | RpoS-dependent | (Dong and Schellhorn, 2009b) |
| **P0AGE6** | *chrR* | RpoE | 38.8 | 64.8 | 140.8 | 178.5 | 59.8 | 117.1 | 99.0 | 97.5 | | |
| **P30859** | *artI* | ArgR, RpoS, RpoD, Lrp | 38.0 | 100.4 | 195.1 | 272.7 | 55.3 | 11.4 | 43.5 | 23.4 | RpoS-dependent | (Lacour and Landini, 2004) |
| **P32157** | *yiiM* | | 47.1 | 69.6 | 162.5 | 189.7 | 49.7 | 57.7 | 99.7 | 116.3 | | |
| **P0ADG7** | *guaB* | RpoD, CRP-cAMP, Fis, PurR,DnaA | 115.5 | 62.6 | 59.0 | 171.3 | 130.6 | 74.3 | 55.4 | 248.1 | | |
| **P0A9S5** | *gldA* | | 13.9 | 24.8 | 52.6 | 67.8 | 18.9 | 105.5 | 219.5 | 298.9 | RpoS-dependent | (Dong and Schellhorn, 2009b) |
| **P19926** | *agp* | ppGpp, CRP-cAMP | 19.5 | 52.2 | 115.8 | 139.9 | 13.2 | 46.4 | 187.6 | 246.3 | | |
| **B1XD59** | *yaeP* | CRP-cAMP | 40.9 | 68.5 | 189.8 | 184.6 | 34.7 | 44.7 | 154.8 | 105.2 | | |
| **P37645** | *yhjG* | RpoS | 31.3 | 65.9 | 353.9 | 183.4 | 24.6 | N.D. | N.D. | N.D. | RpoS-dependent | (Weber *et al.*, 2005) |

*RpoS-dependent in *Salmonella enterica*. N.D. = Not Detected. ⁺Regulation at the transcriptome level as determined by RNA sequencing methodologies and Microarray analyses.

# Appendix 2

Bacterial cells were collected to assess the proteomic profile of *Escherichia coli* during growth phases. The main objective was to determine which proteins are present at a higher level in late stationary and prolonged stationary phase when compared to early stationary phase. Samples were collected at exponential phase (4 h), early stationary phase (6 h), late stationary phase (24 h) and prolonged stationary phase (48 h). To assess the presence of proteins, SDS-PAGE was conducted on the samples collected (Fig. 23). The SDS-PAGE gel shows reproducibility among triplicates samples.

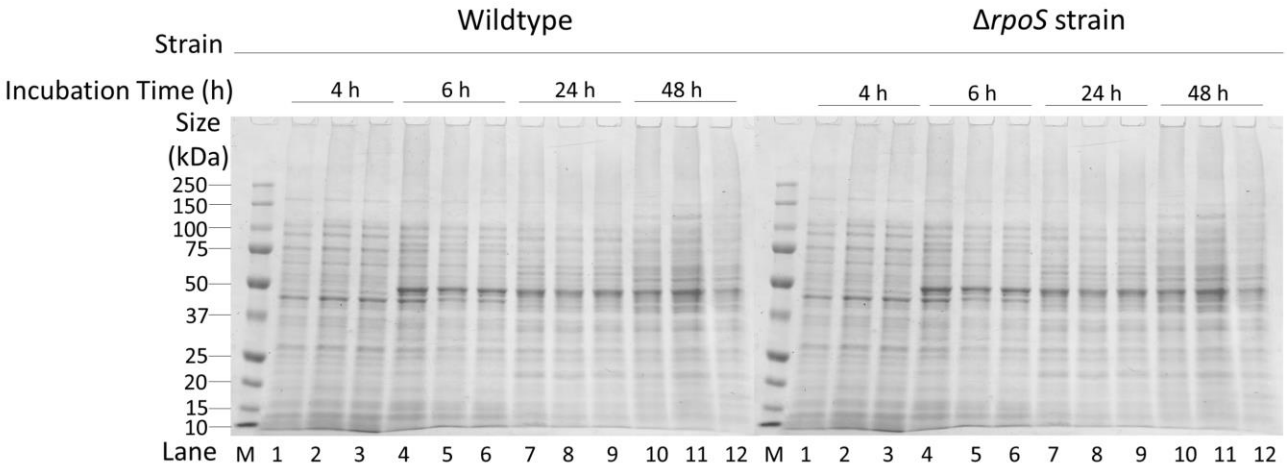


**Figure 23. Assessment of the *E. coli* proteome during long-term growth using SDS-PAGE**.
Gel image showing the extracted proteomes with RIPA buffer using a sonicator. Samples were collected in triplicate during exponential phase (4 h), early stationary phase (6 h), late stationary phase (24 h) and prolonged stationary phase (48 h). The gel, which contained 10 µg of each sample in each lane, shows good separation and reproducibility.

Samples were digested using trypsin and analysed by LC-MS/MS. Upon initial analysis of proteomic data, the abundance of RpoS in the wildtype was not as expected based on the known expression profile of RpoS. This prompted the confirmation of the expression of RpoS during growth using Immunoblot. In this work, RpoS expression peaks upon entry into early stationary phase, which is consistent with previous findings (Patten *et al.*, 2004). The Immunoblot was exposed for approximately 0.5 s (Fig. 23a). The expression of RpoS was indetectable by Immunoblot during exponential phase and was upregulated upon entry into early stationary phase. The level of RpoS slightly decreased during late stationary phase and was not detectable during prolonged stationary phase. To determine whether RpoS was present during exponential phase, the Immunoblot was exposed for 45 s. RpoS was barely detectable during exponential phase and prolonged stationary phase by Immunoblot (Fig. 23b). Furthermore, the expression of RpoS slightly decreased during late stationary phase and further decreased during prolonged stationary phase, almost to exponential phase levels. Additionally, immunoblot analysis of Δ*rpoS* samples indicated that RpoS was not present in these samples (data not shown).

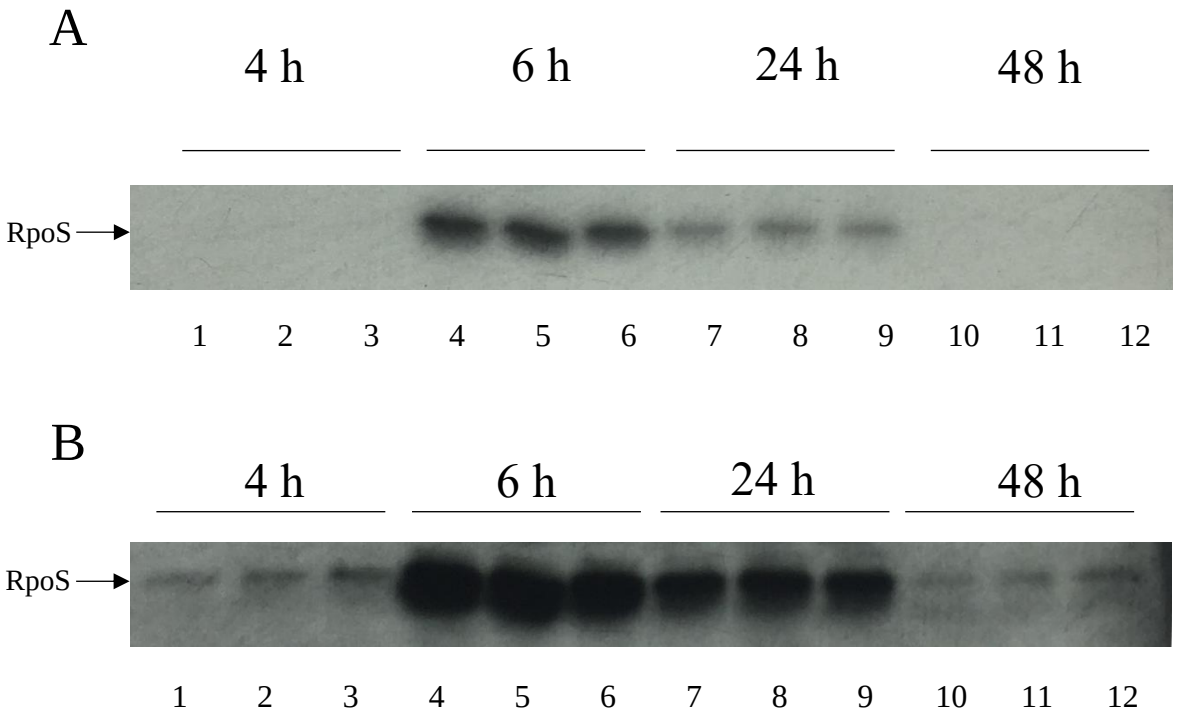


**Figure 24. RpoS expression in the wildtype during extended growth.**
Protein was extracted from cultures grown in triplicate to exponential phase (4 h, $OD_{600}$ 0.3), early stationary phase (6 h, $OD_{600}$ 1.5), late stationary phase (24 h) and prolonged stationary phase (48 h). (A) The level of RpoS was higher at 6 h than any other time point. (B) Extended exposure showed that the level of RpoS was upregulated at 6 h relative to 4 h, decreased at 24 h relative to 6 h and continued to decrease at 48 h.

# Appendix 3 – Standard Operating Procedures

## Bacterial growth

1. Streak strains from the -80 °C glycerol stock cultures without thawing onto LB plates to isolate single colonies.
2. Inoculate a single colony into 10 ml of LB in a 50 ml Erlenmeyer flask and incubate at 37 °C aerobically with shaking at 200 RPM (Innova 4000, New Brunswick Scientific) overnight (usually 12 h).
3. Subculture1:10000 into prewarmed 100-ml LB in 500-ml flasks and monitor $OD_{600}$ using Multiskan Spectrum (Thermo Labsystems).
4. Exponential phase is defined as $OD_{600}$ = 0.3 (typically 4 h post inoculation), early stationary phase as $OD_{600}$ = 1.5 (~ 6 h post inoculation). Furthermore, late stationary phase and prolonged stationary phase is defined as 24 h and 48 h post inoculation, respectively.

## Protein Extraction (Maintain samples at 4 °C)

1. Collect samples (in triplicate) at appropriate time points (exponential, early stationary, late stationary and prolonged stationary phase) and centrifuge at 3, 000 RPM for 15 min at 4 °C to collect the pellet.
2. Resuspend the pellet in phosphate buffered saline (PBS) and centrifuge at 3, 000 RPM for 15 min at 4 °C.
3. Remove the supernatant with a pipette and wash the cells two times with 1×PBS.
4. Resuspend the pellets in 0.5 ml 1× RIPA buffer (20 Mm Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM $Na_2EDTA$, 1 mM EGTA, 1% NP-40, 1% sodium deoxycholate, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM $Na_3VO_4$, 1 µg/ml leupeptin).
5. Cell lysis: Transfer the resuspended protein solution to a 15 ml polystyrene tube.
6. Turn on the Haake water cooling pump and ensure proper flow. Place the tubes in the tube holder. Remove (or loosen) the caps and ensure that there is a 2 mm gap between the bottom of the tubes and the bottom plate of the tube holder.

   *N.B. Turn on the Haake water cooling pump at least 30 min prior to use to ensure that the 4 °C is reached.*

7. Turn on the sonicator and switch the setting to # 9. Program the sonicator to pulse for 30 s and rest for 30 s with a total sonication time of 30 min.
   *N.B. Once the resuspended cell solution is clear, cell lysis is complete.*
8. Transfer the sonicated cells to a 1.5 ml microfuge tube. Centrifuge at 13, 000 RPM for 15 min.
9. Transfer the supernatant to a new 1.5 ml microfuge tube and store the cell extracts at 4 °C.

**Determining Protein Concentration Using the Bradford Assay** (Bradford, 1976)

1. Place 990 µl of dd$H_2O$ into borosilicate tubes. Add 0 to 10 µl of 1 mg/ml BSA protein standard and 10 µl 1× RIPA buffer to develop a range for protein concentrations.
2. Add 200 µl of the BioRad stock solution to the protein standards from step 1. Briefly vortex the tubes and then let them stand at room temperature for 5 min.
3. Zero the spectrophotometer with water at an of $OD_{595}$ and measure each of the protein standards.
4. Plot a standard curve relating the $OD_{595}$ to the concentration of the protein standards. Perform a regression analysis to fit the data and show the 'r' equation on the plot. Use the equation to calculate the concentration of the unknown samples.
5. Add an aliquot of unknown sample to 990 µl of dd$H_2O$ in borosilicate test tubes.
6. Add 200 µl of the BioRad stock solution to the diluted protein sample. Briefly vortex the tube and let stand for 5 min at room temperature.
7. Zero the spectrophotometer with water at an of $OD_{595}$ and measure each of the protein samples.
8. Use the equation derived from the standard curve (step 4), calculate the protein concentration of the unknown sample.

**SDS-Polyacrylamide Gel Electrophoresis**

1. The protein samples were diluted in 2× SDS-PAGE sample buffer. The protein sample is frozen at -20 °C for long-term storage.
2. Prepare the resolving gel acrylamide solution by combining resolver A and B solutions in equal volumes (6 ml each for 2 gels).

3. Add the associated volume of 10 % ammonium persulphate solution (APS) and Tetramethylethylenediamine (TEMED) (60 µl and 6 µl, respectively). Mix well and steadily and slowly pipet the solution into the cassette (avoid air bubbles below the tooth combs).
4. Immediately following, prepare the stacking gel acrylamide solution by combining equal volumes of stacker A and B (2 ml each for 2 gels).
5. Add the appropriate volume of 10 % APS and TEMED, mix well and dispense slowly into the cassette using a pipet. Immediately insert comb and allow gel to set (~ 30-45 min).
6. Assemble the gels in the gel box and fill with running buffer between the gels and to the appropriate mark in the gel box (10× Running Buffer: 30 g of tris base, 144 g of glycine, 10 g of SDS to 1 L total volume with dd$H_2O$).
7. Heat protein sample at 90 °C for 10 min prior to loading.
8. Load 10 µg of the protein samples in the wells and run the gel for 40 min at 200 V using PowerPack 1000/500 (BIORAD).

## Protein Gel Staining with Coomassie Brilliant Blue

1. Remove the gel from between the two glass plates and soak in the staining solution (0.5 g of Coomassie Blue dye, 200 ml of methanol, 50 ml of acetic acid, and 250 ml dd$H_2O$) with slow shaking at 55 RPM for 1 h.
2. Destain the gel in destaining solution (250 ml of sterile water, 200 ml of methanol, 50 ml of acetic acid) for 30 min, decanting and replacing the solution every 10 min.
3. Store the gel in 10 % acetic acid (this further de-stains the gel and hydrates the gel so that the protein bands become clearer).

## Immunoblot Analysis

1. Remove an Immuno-Blot® low fluorescence PVDF membrane (BioRad, 1620260) from the box using a pair of tweezers (take care not to touch the membrane with fingers).
2. Soak the membrane in methanol for 1-5 min, then transfer the membrane to 1× Transfer Buffer (200 ml 5× transfer buffer (BioRad, Catalogue #10026938), 600 ml dd$H_2O$, 200 ml ~90-98 % ethanol; stored at 4 °C).
3. Place two stacks (per gel) of transfer stacks (BioRad, 1620260) into 1× transfer buffer.

4. Remove the transfer stack on the bottom of the Trans-Blot® Turbo™ transfer system cassette tray. Remove the PVDF membrane from the 1× transfer buffer and place on top of the bottom transfer stack.
5. Remove the gel from between the glass plates and transfer the protein from the polyacrylamide gel to the PVDF membrane.
6. Place the second transfer stack on top of the gel, close the lid and re-insert the cassette into the Trans-Blot® Turbo™ transfer system.
7. Turn on the Trans-Blot® Turbo™ transfer system and select the appropriate program.
8. Prepare 5 % TBST-milk solution (50 ml 1× TBST, 2.5 g Carnation skim milk powder)
9. Once the transfer is complete, carefully remove the lid from the cassette. Slowly remove the top transfer stack. Cut along the sides of the PVDF membrane (so that it fits into the clear plastic box).
10. Remove the gel and transfer the PVDF membrane (using tweezers) to the clear plastic box. Submerge the membrane in 5 % TBST-milk and incubate at room temperature with shaking at ~55 RPM (this minimizes non-specific anti-body binding).
11. Decant the 5 % TBST-milk and incubate the membrane in a 1:10,000 dilution of the primary anti-body in 5 % TBST-milk overnight at 4 °C with slow shaking.
12. Pour the primary antibody back into the tube and wash the membrane with 1× TBST for 30 min, changing the solution every 10 min.
13. Incubate the membrane with 10 ml of the secondary antibody for 1 h with shaking at ~55 RPM.
14. Pour the secondary antibody back into its respective tube and wash the membrane with 1× TBST for 30 min, changing the solution at 10-min intervals.
15. Combine the substrate solution and enhancer solution in equal volume (3.5 ml) in a clean plastic box.
16. Transfer the membrane (blunt forceps) to the box containing the mixture of the substrate and enhancer solution and incubate with shaking at ~55 RPM for 1 min.
17. Remove the membrane from the box and securely wrap it in seran wrap (avoid any air bubbles).
18. In the dark room, expose the membrane for 5 s (increase as necessary) and image using the AlphaImager HP.

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