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By

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A thesis submitted to the school of graduate studies in partial fulfillment of the requirements of the degree of Master of Science

McMaster University

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MASTER OF SCIENCE
(Biology)

McMaster University
Hamilton, Ontario

TITLE: RNA extraction protocol development for the assay of temporal gene expression in batch-cultured *Escherichia coli* K-12

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NUMBER OF PAGES: vi, 78

Abstract

The sigma S subunit (RpoS) of RNA polymerase acts as the master regulator of stress in *Escherichia coli*, allowing adaptation and survival under unfavourable conditions such as nutrient deprivation. RpoS regulates and integrates the signals of hundreds of genes (about 10% of the genome) organized into complex networks and modules that govern the response to stress and entry of the cell into stationary phase. Although microarray studies have been performed on starvation models in *E. coli*, the expression of the RpoS regulon has not been studied in long-term cultures due to the difficulty of isolating RNA from starving cells. In this study, the development of a protocol for isolating RNA from stationary phase cells, employing hot acid phenol without the use of DNase, is described. In addition, the expression of several genes during different phases of growth is analyzed by RT-qPCR in order to validate preliminary microarray data obtained from 24 and 48 hour-old cultures. Although the results obtained by RT-qPCR agree well with the literature, they do not corroborate preliminary microarray data at the 24 and 48 hour timepoint.

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Acknowledgements

Thanks to Dr. Tao Dong for many helpful discussions and Dr. Richard Morton for his guidance. Special thanks to Amber Hamvai for maintaining her interest and support during my studies.

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1 Introduction

1.1 Long-term stationary phase in *E. coli*

It has been estimated that 60% of the world's biomass is composed of resting microorganisms (Gray *et al.*, 2004). Bacteria generally exist in nature under conditions of near-starvation or rapidly changing nutrient availability (Morita, 1997). When there is insufficient nutrients to sustain growth, *Escherichia coli* cells respond by activating biosynthetic pathways (Bremer and Dennis, 1996). If activation of these biosynthetic pathways fails to rescue growth, the cellular response shifts to preservation and long-term survival - a physiological state known as stationary phase (Hengge-Aronis, 1996). Stationary phase in bacteria is a complex state regulated by many exogenous and endogenous factors. These changes in the transcriptional network used by bacteria to orchestrate the balance of biosynthetic pathways with survival functions, known as the stringent response, is highly studied but remains poorly understood (Cashel *et al.*, 1996; Potrykus and Cashel, 2008). Studying bacteria under stationary phase is critical in understanding the processes that govern ageing, survival and mutation under typical conditions in microorganisms.

The stringent response is characterized by an accumulation of the alarmone guanosine 3',5' bispyrophosphate (ppGpp), leading to the downregulation of ribosome, nucleotide, phospholipid and cell envelope synthesis, as well as DNA replication and division (Cashel *et al.*, 1996; Magnusson *et al.*, 2005; Traxler *et al.*, 2008). Upregu-

lated processes include the stress response, core metabolism, nucleotide catabolism, oxidation of fatty acids, and amino acid turnover (Traxler *et al.*, 2008). The decrease in RNA, protein and DNA content is dramatic. For example, as the number of cell doublings per hour decreases from 2.0 to 0.6, stable RNA (ie. rRNA and tRNA) content decreases 10-fold, protein content decreases 4.5-fold, and DNA content decreases 2.2-fold (Bremer and Dennis, 1996). As mentioned, this global change in gene expression is controlled by ppGpp (Cashel *et al.*, 1996), which is itself regulated by proteins RelA and SpoT (Magnusson *et al.*, 2005; Srivatsan and Wang, 2008). RelA is the primary synthase of ppGpp, catalyzing its production in response to ribosome stalling as a result of amino acid starvation (Cashel *et al.*, 1996). SpoT recognizes depletion of carbon (Xiao *et al.*, 1991), phosphorus (Spira *et al.*, 1995), iron (Vinella *et al.*, 2005) and fatty acids (Seyfzadeh *et al.*, 1993) and is both a ppGpp synthase and hydrolase (Neidhardt *et al.*, 1996). During the stringent response, Dksa mediates binding of RNA polymerase and ppGpp, directly altering the expression of over 80 genes (Barker *et al.*, 2001; Chang *et al.*, 2002). The concentration of ppGpp varies inversely with growth rate (Lazzarini *et al.*, 1971), suggesting a gradient rather than binary response. Increased ppGpp has been shown to decrease open complex half-life at most promoters, resulting in down-regulation of short-half-life promoters such as those for stable RNA genes (Barker *et al.*, 2001). Recent work has suggested that the other main role of ppGpp lies in modulating the Lrp and RpoS regulons (Traxler *et al.*, 2011). These two regulons are briefly described below.

Lrp (leucine responsive protein) is a global transcriptional regulator, positively regulated by ppGpp (Landgraf *et al.*, 1996), known to affect more than 400 genes including almost three quarters of stationary phase genes (Tani *et al.*, 2002). The function of Lrp is to modulate cellular metabolism in response to available nutrients (Landgraf *et al.*, 1996), specifically by increasing amino acid anabolism, decreasing catabolism (Zinser and Kolter, 2000), and modifying transport (Cho *et al.*, 2008). Thus, Lrp plays a role complementary to that of ppGpp during the bacterial response to starvation.

The alternative sigma factor RpoS regulates the expression of up to 10% of the *E. coli* genome, acting as the key regulator of the general stress response (Dong *et al.*, 2008; Patten *et al.*, 2004; Weber *et al.*, 2005). The RpoS regulon is activated in response to nutrient deprivation such as low levels of carbon, nitrogen, phosphorus or amino acids (Hengge, 2008) as well as stress conditions as diverse as heat shock, cold shock, acid shock, osmotic stress, oxidative stress, and UV-light (reviewed in Storz and Hengge-Aronis, 2000). RpoS also serves to increase mutation rates by inducing the error-prone DNA polIV and repressing methyl-mismatch repair (MMR) (Saint-Ruf and Matic, 2006; Shee *et al.*, 2011) in an effort to adapt to environmental stress. Entrance into stationary phase is governed by a three- to fourfold increase in RpoS levels (Yuste *et al.*, 2006) resulting from increased transcription (Lange and Hengge-Aronis, 1994), efficient translation (Majdalani *et al.*, 2001) and increased protein stability (Bougdour *et al.*, 2008) (Fig.1). The alarmone ppGpp plays a central role in

RpoS activity as it is required for increased transcription of *rpoS* during stationary phase (Lange and Hengge-Aronis, 1994), facilitates competition of RpoS for core RNA polymerase (Jishage *et al.*, 2002), and increases transcription of *iraP* – an inhibitor of RpoS proteolysis (Bougdour *et al.*, 2008). RpoS is known to be maximally expressed at the onset of stationary phase and to decrease thereafter (Lange and Hengge-Aronis, 1994).

RpoS is subject to high regulation at all levels (Lange and Hengge-Aronis, 1994). Transcription is repressed by the phosphorylated response regulator ArcA (Mika and Hengge, 2005) and negatively regulated by cAMP-CRP (Lange *et al.*, 1995). The alarmone ppGpp has a positive effect on elongation of the transcript but not initiation (Lange *et al.*, 1995). The stability of the mRNA transcript is governed mostly by interactions with the 5' region (Cunning *et al.*, 1998). The two sRNAs DsrA and RprA stimulate translation of RpoS by unfolding the mRNA to make it more accessible to ribosomes (Majdalani *et al.*, 1998) under low temperatures (Sledjeski *et al.*, 1996) and in response to osmotic shock (Majdalani *et al.*, 2001), respectively. H-NS binds *rpoS* mRNA and enhances cleavage (Brescia *et al.*, 2004). Protein Hfq is required for translation of *rpoS* (Muffler *et al.*, 1996) but is inhibited by LrhA (Peterson *et al.*, 2006) and the sRNA OxyS (Zhang *et al.*, 1998). RpoS activity is mainly governed by increased binding to core RNA polymerase conferred by ppGpp (Jishage *et al.*, 2002) and Crl (Typas *et al.*, 2007). Post-translational control of RpoS is governed by the protease ClpXP (Zhou and Gottesman, 1998). The specific adaptor protein RssB

targets ClpXP to RpoS unless binding of RssB is inhibited by the antiadaptor proteins IraP, IraD, or IraM (Bougdour *et al.*, 2008; Merrikh *et al.*, 2009). RssB regulation is mediated by Crl (Typas *et al.*, 2007) and H-NS (Zhou and Gottesman, 2006).

The current model suggests that during nutritional stress, the increasing concentration of ppGpp is responsible for first upregulating the Lrp regulon and, if this reallocation of bacterial resources fails to rescue growth, then inducing stress survival genes under the control of RpoS (Traxler *et al.*, 2011). Thus, ppGpp drives a negative feedback loop controlled by Lrp in conjunction with a feed-forward loop that triggers entrance into stationary phase should the initial response fail (Traxler *et al.*, 2011). Transcriptional profiling of the stringent response has revealed a hierarchical response, where differentially expressed genes increase over time with far more up-regulated genes than down-regulated genes (Durfee *et al.*, 2008).

1.2 Cellular adaptations to stationary phase

The genes of the *rpoS* regulon are responsible for morphological changes, resistance to stress, metabolic processes, virulence and the Growth Advantage after Stationary Phase (GASP) phenotype (Hengge-Aronis, 1996; Raiger-Iustman and Ruiz, 2008). Reductive division increases the cell surface area and endogenous degradation results in dwarfing, thereby contributing to a smaller, spherical cell (Akerlund *et al.*, 1995). The production of a modified protective cell envelope is characteristic of stationary phase in gram-negative bacteria (Huisman *et al.*, 1996). The concentration of exter-

nal lipopolysaccharides increases in conjunction with a decrease of total protein in the outer membrane (Allen and Scott, 1979). Additional cross-links form between the peptidoglycan layer and outer membrane lipoproteins in addition to an increase in osmoprotectant, membrane-derived oligosaccharides in the periplasm (Huisman *et al.*, 1996). Changes occur in the inner membrane that reduce fluidity and allow more ordered structuring, such as replacement of monounsaturated fatty acids with polyunsaturated fatty acids (Huisman *et al.*, 1996). In the cytoplasm, the nucleoid condenses to protect the DNA, forming a structure known as biocrystal (Frenkiel-Krispin *et al.*, 2001; Wolf *et al.*, 1999). The DNA-binding protein Dps is required for this condensation and is abundant in the cell following induction in stationary phase (Almiron *et al.*, 1992). Dps exhibits a global protective role against various stresses through regulation of gene expression in combination with its functions: DNA-Dps cocrystallization, iron chelation, and ferroxidase activity (Ilari *et al.*, 2002; Nair and Finkel, 2004).

Metabolism of carbohydrates, amino acids, and phospholipids decreases dramatically in stationary cells (Brown *et al.*, 2002). Conversely, growth rate, protein synthesis, as well as rRNA and tRNA abundance are reduced (Reeve *et al.*, 1984). Cells entering stationary phase elevate synthesis of proteases and peptidases, increasing protein turnover 5-fold (Groat *et al.*, 1986). Ribosomes also undergo dimerization mediated by the ppGpp-induced ribosome modulation factor Rmf (Wada *et al.*, 2000). Expression of the *fad* operon, controlled by the FadR regulator, increases during sta-

tionary phase and is required for growth on long-chain fatty acids as a carbon source, presumably to provide energy during digestion of membrane constituents (Farewell *et al.*, 1996). Furthermore, the response regulator ArcB/ArcA/RssB induces synthesis of glycolysis enzymes, pyruvate formate lyase, phospho-transacetylase, and acetate kinase while decreasing synthesis of TCA enzymes (Nystrom, 1994; Nystrom *et al.*, 1996). The repression of aerobic metabolism is thought to protect against uncontrolled use of cellular reserves during autophagy and decrease production of reactive oxygen species. A large number of secondary metabolites, antibiotics and toxins are synthesized during stationary phase. The RpoS-dependent microcins McjA and MccA, small molecules with potent antibacterial activity against closely related bacteria, are also produced in stationary phase (Duquesne *et al.*, 2007).

1.3 Genetic diversity

Cultures of *E. coli* under stress demonstrate high mutation rates (Bjedov *et al.*, 2003), with one study inferring a mutation rate as high as 1 in 600 bp per genome (Finkel and Kolter, 1999). Upregulation of error-prone DNA polymerases, down-regulation of error-correcting enzymes, and an increase in transposition events is common in bacteria under stress (Foster, 2007). Stress-induced mutagenesis provides a method of generating genetic diversity upon which natural selection may act to adapt the bacterium to an environment (Loewe *et al.*, 2003). RpoS serves to increase mutation rates by inducing the error-prone DNA polIV and repressing methyl-mismatch repair

(MMR) (Saint-Ruf and Matic, 2006). The MMR system repairs post-replicative errors and inhibits recombination such that repression of MMR by RpoS and Hfq induces a mutator phenotype (Oliver *et al.*, 2002). PolIV lacks proofreading ability, can copy damaged DNA, and is thought to be responsible for some 85% of adaptive mutations described (Goodman, 2002). Furthermore, cells lacking any of the SOS DNA polymerases do not acquire the GASP phenotype (Yeiser *et al.*, 2002), described below. RpoS, in conjunction with IHF, has also been implicated in activating transposition events, such as the activation of a promoterless phenol-degrading operon in stationary phase (Kasak *et al.*, 1997; Kivistik *et al.*, 2007). Furthermore, most cells in stationary phase carry two, four or even eight chromosomes, presumably to protect themselves from loss of essential genes during stress-induced mutagenesis (Akerlund *et al.*, 1995).

1.4 The GASP phenotype

The GASP phenotype is characterized by the ability of aged cells (after 10 days or more) to outcompete younger cells when both are inoculated into fresh media, and has also been observed in gram-positive bacteria (Finkel *et al.*, 2000; Smeulders *et al.*, 1999) and Eukarya (Gray *et al.*, 2004). Stationary phase cultures have been shown to be highly dynamic due to the continuous propagation of mutants with greater fitness than the parental strain (Zambrano and Kolter, 1996). Indeed, 20-day-old cells will outcompete 10-day-old cells, and so on (Finkel and Kolter, 1999), until about 60 days due to the culture environment being sufficiently different as to prevent aged

cells from expressing a fitness advantage over unaged cells (Yeiser *et al.*, 2002). The most frequent mutation found in GASP mutants is one of attenuated RpoS activity (0.1-1%); *rpoS* null mutants do not exhibit GASP (Zambrano *et al.*, 1993). The presence of RpoS-down mutations is thought to be beneficial to cell-growth due to unbalanced competition with the RpoD and RpoN sigma factors (Dong *et al.*, 2011). The two main stresses in stationary phase cultures grown in Luria-Bertani broth is nutrient depletion and basification of the media (Farrell and Finkel, 2003). Therefore, cells with an elevated proportion of RNA polymerase-bound RpoD, which regulates glucose scavenging, and RpoN, which is responsible for ammonia assimilation, amino acid uptake, and protection from alkaline stress, will outcompete wildtype strains (Farrell and Finkel, 2003).

The best-characterized effect of GASP alleles in *rpoS* mutants is enhanced ability to catabolize certain amino acids (Zinser and Kolter, 1999). Another characterized GASP mutation, in *lrp*, confers enhanced amino acid utilization (Zinser and Kolter, 2000). Interestingly, GASP phenotypes conferred by mutations in *rpoS* are dependent on pH and the nutrient environment (Farrell and Finkel, 2003). While initial models described a system where a single GASP mutant would sweep the population until itself being displaced by a more fit mutation (Zambrano and Kolter, 1996; Zambrano *et al.*, 1993), it is now understood that stationary cultures in the laboratory have many subpopulations with distinct genotypes existing simultaneously (Farrell and Finkel, 2003; Finkel and Kolter, 1999). Many GASP mutations remain uncharac-

terized. However, it is clear that it is a phenomenon found across the microbial world (Finkel *et al.*, 2000). While the appearance of the GASP phenotype is not applicable to the timepoints described here, the co-existence of non-isogenic subpopulations is certainly relevant in long-term stationary phase which may be studied following further development of the methods described here.

1.5 Recent transcriptome studies on the stringent response in *E. coli*

Assays of long-term gene expression, beyond overnight batch culture, are absent in the literature. However, there have been several major studies of transcriptome reorganization as a response to starvation (Chang *et al.*, 2002; Durfee *et al.*, 2008; Traxler *et al.*, 2006, 2008, 2011). Each of these studies used a model to emulate famine, and therefore "artifically" achieve stationary phase, rather than cultures undergoing longer term starvation. Chang *et al.* (2002) and Traxler *et al.* (2006) used the classic glucose-lactose diauxie model to investigate the stringent response, wherein cells experience transient growth arrest during which metabolism is reoriented to the consumption of lactose following the depletion of glucose in the medium (Loomis and Magasanik, 1967). In contrast, Durfee *et al.* (2008) used serine hydroxamate to force the cellular response to amino acid starvation while Traxler *et al.* (2008, 2011) subjected cultures to isoleucine starvation. Each of these models induces a robust stringent response and has advantages and disadvantages. However, no model is as physiologically relevant as observing starvation in a stationary phase batch culture.

It is accepted that a core component of the stringent response is upregulation of amino acid biosynthesis. However, it was found that approximately half of the

90 amino acid synthesis genes are in fact downregulated during growth arrest under diauxie (Chang *et al.*, 2002). It is interesting to note that researchers have used histidine and arginine as induction models for the stringent response (Shand *et al.*, 1989; Williams and Rogers, 1987) yet both of these operons experience regulation that does not follow the general trend. Another confounding discovery is that the histidine and arginine pathways are actually downregulated under H_2O_2-induced growth arrest, in contrast to diauxie (Chang *et al.*, 2002). While it is possible that the ppGpp synthetases RelA and SpoT could sense carbon starvation independently of amino acid starvation, this is not likely due to the association of RelA with the ribosome wherein it monitors the amino acyl-tRNA pool through ribosomal stalling (Wendrich *et al.*, 2002). Thus, it is interesting that these pathways experience opposing regulation depending on whether growth arrest is induced by oxidative stress or diauxie.

In the most recent studies, serine hydroxamate (Durfee *et al.*, 2008) and isoleucine starvation (Traxler *et al.*, 2008, 2011) were employed to model amino acid starvation and induce the stringent response. Serine hydroxamate is a serine analogue that mimics serine starvation by inhibiting serine tRNA synthetase (Tosa and Pizer, 1971). While serine hydroxamate does cause a spike in ppGpp levels, it is not the most adequate model because no new protein can be synthesized after treatment such that there can be no reorganization of the proteome (Tosa and Pizer, 1971). Isoleucine starvation relies on the susceptibility of K-12 strains to valine toxicity (Leavitt and Umbarger, 1962). Briefly, when isoleucine is limiting and valine is in excess, K-12 strains are unable to synthesize isoleucine due to a framshift mutation in *ilvG* – the only one of three acetohydroxy acid synthases that would normally be active in *E. coli* under this condition. However, this system has metabolic consequences such as the accumulation of α-ketobutyrate (Herring *et al.*, 1995). Another issue is that accumulation of α-ketobutyrate in conjunction with upregulation of leucine biosynthesis

leads to production of norleucine (Bogosian *et al.*, 1989) – a toxic methionine analogue that can be incorporated into proteins (Barker and Bruton, 1979; Cohen and Munier, 1956).

There is also evidence that the models can lead to misleading general conclusions about the stringent response. Traxler *et al.* (2011) identified strong induction of the biosynthesis pathways for valine, isoleucine and leucine, as well as several genes involved in pyruvate metabolism, as a direct response to isoleucine starvation (particularly those directing flux of glycolytic intermediates and serine to pyruvate). Indeed, the authors point out that rather than a general upregulation of amino acid biosynthesis, the cells induce a range of synthetic and catabolic pathways to reroute metabolism towards production of the limiting amino acid (Traxler *et al.*, 2011). Presumably, there may also be an effect on the synthesis of isoleucine-rich proteins. Furthermore, isoleucine starvation induced over 130 members of the RpoS regulon compared to only about 20 regulon members in cells treated with serine hydroxamate (Durfee *et al.*, 2008; Traxler *et al.*, 2011). This difference suggests that isoleucine starvation is probably the best stringent response model available but that it may not provide a completely accurate picture of the general response in resting microorganisms. An analysis of gene expression in batch culture would provide a more physiologically relevant picture of the stringent response in addition to validating previous work on this topic.

1.6 Gene expression in long-term batch cultures

E. coli have been shown to be capable of surviving for over three months under near-starvation conditions in aquatic reservoirs (Lothigius *et al.*, 2010) and still retain viable counts after 5 years of continuous incubation under laboratory conditions

(Steinhaus and Birkeland, 1939). Natural aquatic populations are of considerable interest because they persist in water reservoirs and may be pathogenic (Lothigius *et al.*, 2010). Elucidation of the life processes that allow cells in stationary phase to persist in the environment under starvation is critical to understanding how cells respond to stress and adapt to maintain viability under typical environmental conditions. Genes belonging to the RpoS regulon confer resilience by affecting metabolism, resistance to stress, and genetic stability.

We hypothesize that RpoS-dependent transcripts play a role in long-term cultures. Microarray technology provides an ideal tool to study the transcriptional network that allows bacteria to survive stress and starvation, and has previously been used to analyze the RpoS regulon in our lab (Dong *et al.*, 2008; Dong and Schellhorn, 2009; Patten *et al.*, 2004). Studies have shown that under starvation conditions, cells reorganize their transcriptional network to balance biosynthetic and survival functions. Determining how these and other transcripts allow cells to survive for longer periods of time is a critical step in understanding microbial persistence in the environment. In order to carry out such a study, the first challenge is isolating RNA from cultures in the stationary phase of growth. An additional challenge is that growth becomes unbalanced at $OD_{600} > 0.3$ (Sezonov *et al.*, 2007), leading to an increase in experimental standard error at later timepoints.

1.7 Genes of interest

Six genes were chosen for this study, for which the reasoning is described in Section 1.8: *dps, emrK, rpoS, otsB, tomB*, and *tufA*. A brief overview of the function of their protein products is given below.

Protein Dps (reviewed in Calhoun and Kwon, 2011) is the fifth-most abundant

protein in stationary phase cells (Link *et al.*, 1997) and plays a key role in the stringent response (Almiron *et al.*, 1992) and long-term viability during stationary phase (Nair and Finkel, 2004). It has been shown to provide resistance to diverse stresses including oxidative stress (Dukan and Touati, 1996; Martinez and Kolter, 1997), fatty-acid starvation (Gong *et al.*, 2002), exposure to light in seawater (Gourmelon *et al.*, 1997), and exposure to the electrophile N-ethylmaleimide (Ferguson *et al.*, 1998). Dps binds DNA in a sequence-independent manner (Azam and Ishihama, 1999) and forms a complex known as biocrystal (Wolf *et al.*, 1999) that protects the nucleic acid from damage (Martinez and Kolter, 1997). The protein restructures chromatin upon entry into stationary phase (Frenkiel-Krispin *et al.*, 2004; Kim *et al.*, 2004) and binds iron, which acts as a reducing agent against oxidative damage (Zhao *et al.*, 2002) in conjunction with the ferroxidase activity of Dps (Nair and Finkel, 2004).

Less is known about EmrK – a component of the putative EmrKY-TolC multidrug efflux system (Tanabe *et al.*, 1997). EmrK confers resistance to the lethal effects of some stress (Han *et al.*, 2010). Indeed, *emrK* mutants are sensitive to mitomycin C and UV radiation (Han *et al.*, 2010).

OtsB, or trehalose-6-phosphate phosphatase, has long been implicated in the *E. coli* stress response (Giaever *et al.*, 1988). Expression is increased during osmotic stress (Rosenthal *et al.*, 2006; Weber *et al.*, 2006), low temperatures (Kandror *et al.*, 2002), and upon entry into stationary phase (Hengge-Aronis *et al.*, 1991). Interestingly, in *rpoS* mutants undergoing starvation, a mutation that allows RpoS-independent transcription of *otsBA* occurs leading to increased viability (Stoebel *et al.*, 2009).

TomB is encoded by the *tomB-hha* operon – a toxin-antitoxin module (Garcia-Contreras *et al.*, 2008). Thus, *tomB* expression decreases toxicity resulting from expression of *hha*. Both proteins are induced during biofilm formation (Ren *et al.*,

2004). Toxin-antitoxin systems are generally highly-expressed during stationary phase and have been implicated in stress responses and recovery from starvation (Blower *et al.*, 2011)

The product of *tufA*, elongation factor Tu (EF-Tu), is the most abundant protein in *E. coli* (Weijland *et al.*, 1992) and exhibits the highest expression during the stress response (Muela *et al.*, 2008). EF-Tu is encoded by two genes, *tufA* and *tufB*, which are identical in their coding sequence but differ in their regulatory regions. Essentially, EF-Tu binds aminoacyl-tRNAs and mediates entry into the free site of the ribosome (Weijland *et al.*, 1992). If there is a codon-anticodon match, guanosine triphosphate bound to EF-Tu is hydrolyzed to guanosine diphosphate, causing a conformational change in EF-Tu and dissocation from the tRNA molecule (Weijland *et al.*, 1992). The ribosome then catalyzes the transfer of the amino acid onto the polypeptide chain (Weijland *et al.*, 1992). It should be noted that any primers targeting the coding region of *tufA*, which is the case here, will also hybridize to *tufB*, which is identical in its coding sequence.

1.8 Rationale and project objective

Previous gene expression data for cultures in exponential growth and entering stationary phase was obtained from a previous study (Dong *et al.*, 2008). Microarray data for 24h and 48 hour-old cultures was also obtained from S. Chiang (unpublished, preliminary results). It is important to note that the latter data for 24h and 48 hour-old cultures cannot be directly compared to previous microarray data (Dong *et al.*, 2008) because the RNA was isolated using a different protocol which may have altered gene expression. Furthermore, this data was not replicated or confirmed by RT-qPCR. Therefore, the aim of this project was to validate the microarray data pertaining to

24h and 48 hour-old cultures, as well as provide a link between the two sets of data by performing the gene expression analysis on cultures across these timepoints. Previous attempts had failed due to the difficulty in isolating RNA from cultures in stationary phase. Therefore, a new protocol was to be developed that was capable of isolating RNA of sufficient yield and purity from cultures at later timepoints. This method was to be based on hot acid phenol extraction without the use of DNase to remove DNA contamination (as this was sufficient for previous studies on cultures in exponential phase growth and entering stationary phase (Dong *et al.*, 2008; Patten *et al.*, 2004)).

The genes chosen for validation of the 24h/48h microarray data were *dps, emrK, rpoS, otsB, tomB*, and *tufA*. These genes were chosen for their biological properties and their expression over time as shown in the microarray data (Figs.2 & 3). Gene *rpoS* was chosen because it is the key regulator of stationary phase and stress survival. The other genes were identified as being highly expressed at later timepoints, suggesting that they may be important for long-term survival. Genes *tufA* and *dps* have amongst the highest expression of all genes in *E. coli*. Genes *emrK* and *tomB* are not as highly expressed as the other chosen genes, but appear to exhibit a continued increase in expression over time. RpoS-dependent genes are represented by *otsB* and *dps*.

2 Materials and Methods

2.1 Bacterial strains, media, and growth conditions

E. coli MG1655 was the only strain used in this study. Stock cultures were initally streaked onto Luria-Bertani (LB)/agar plates as described by Miller (1992) and incubated overnight at 37°C. Independent colonies were inoculated (in triplicate) into 50ml flasks containing 10ml fresh LB and grown overnight at 37°C with shaking at 200rpm. The following day, the overnight cultures were subcultured into 200ml flasks containing 50ml fresh LB (10^{-4} dilution) and maintained in exponential phase for at least eight generations prior to experimentation. Growth was monitored by measuring optical density at 600 nm with a Shimadzu UV-1201 spectrophotometer. Cultures were harvested at $OD_{600} = 0.3$ and 1.5, and after 24 and 48 hours.

2.2 Previous microarray data

Two sets of previous microarray data were obtained for this study. Microarray data for four timepoints is presented here (3.8, 5.7, and 24/48 h representing exponential phase, entry into stationary phase, and stationary phase, respectively (Table 2)). Data for the first pair of timepoints (at 3.8 and 5.7 h) was obtained from Dong *et al.* (2008). The second pair of timepoints (at 24 and 48 h) was obtained from a single microarray experiment performed by S. Chiang. Although no microarray analysis was performed by the author, the methodology is briefly described here to aid in interpretation of the data.

Probe-level data was normalized using the dChip invariant set normalization method (Li and Wong, 2001). Replicate sample correlation was determined using the Pearson correlation coefficient (BRB ArrayTools v3.5.0, R. Simon and A.P. Lam, Biometric

Research Branch, National Cancer Institute). Gene expression intensities were quantified by dChip model-based expression analysis using the perfect match and mismatch probesets, then Log_2-transformed (**LI**) for subsequent analysis (Li and Wong, 2001).

2.3 RNA extraction and processing

RNA was isolated using a hot acid phenol extraction method, similar in principle to that described previously (Patten *et al.*, 2004). Cultures at the appropriate timepoint were mixed with ice-cold "stop solution" (Bernstein *et al.*, 2002), chilled on ice, and pelleted. Pellets were then washed with ice-cold PBS, flash-frozen in liquid nitrogen, and stored at -80°C. When appropriate, pellets were thawed on ice and mixed with 1 ml of boiling lysis solution (2% SDS, 16 mM EDTA, 100°C), then with 6 ml of ddH_20 (65°C), and then with acidic hot phenol (pH 4.3, 65°C) (Sigma-Aldrich) in a 15 ml glass tube (Corex). The mixture was centrifuged and the aqeuous phases transferred to a fresh tube. Two additional extractions from hot acid phenol were performed, followed by two extractions from phenol:chloroform:isoamyl alcohol (IAA) (25:24:1) and one extraction from chloroform:IAA (24:1). The nucleic acid in the aqueous phase was then precipitated with 2.5 volumes ethanol and 0.1 volumes sodium acetate (3 M, pH 5.2) at -20°C overnight. Precipitated RNA was pelleted, washed with 70% ethanol, and resuspended in nuclease-free ddH_2O. See Appendix A.4 for details.

2.4 Real-time reverse transcription PCR

Gene expression was assayed by real-time reverse transcription polymerase chain reaction (RT-qPCR) using the *Mx3000P QPCR System* (Stratagene) as previously described (Dong *et al.*, 2008). Primers amplifying 75-200 bp of target genes were designed (Table 1) using PerlPrimer (Marshall, 2004) (length between 19-24 nucleotides

and predicted annealing temperatures from 59°C to 61°C) and synthesized by the MOBIX laboratory at McMaster University. All primers were targeted to the mid-region of the coding sequence. Thus, the primers for gene *tufA* also hybridize to the identical-in-coding-sequence *tufB* transcript. 3 μg RNA per 20 μl reaction were reverse-transcribed into first-strand cDNA by using the *RevoScript cDNA synthesis kit* (Froggabio). Conditions for qPCR were 10 min at 95°C, followed by 40 cycles of heating at 95°C for 15 s and 60°C for 1 min, and final extension at 72°C for 5 min. *SYBR Advantage qPCR premix* (Clontech) was used to detect PCR amplification. The expression level of genes was determined by constructing a standard curve using 10^{-1} serial dilutions of MG1655 genomic DNA of known concentration. The 16S RNA gene *rrlA* was used as an endogenous control for normalization of differences in total RNA quantity among samples and efficiency of reverse transcription (Kobayashi *et al.*, 2006). Gene expression was represented as the Log_2-transformed mean expression ratio (**LMER**) (Patten *et al.*, 2004) of the gene of interest relative to *rrlA*.

2.5 Inference of absolute transcript copy number per cell

Determining the absolute number of transcripts per cell or unit mass by RT-qPCR using genomic DNA standards is not possible because there is no control for the efficiency of reverse transcription. These values were inferred using an approximation of the theoretical RNA yield and the proportion of RNA composed of the endogenous control *rrlA*.

The theoretical RNA yield at each timepoint was determined using literature data for the theoretical yield in exponential phase growth (200 fg/cell) (Neidhardt *et al.*, 1996), controlling for cell concentration, and assuming linearity between experimental

and theoretical yield.

$$\frac{m_{\mathrm{RNA}}}{\mathrm{ml}} = \frac{m_{\mathrm{RNA}}}{cell} \times \frac{cells}{\mathrm{ml}} \tag{1}$$

The proportion of RNA comprised of *rrlA* transcripts was calculated from previous literature data. The fraction of total RNA that is stable RNA (f_s) is 0.98 (Baracchini and Bremer, 1987; Kennel, 1968) and the fraction of stable RNA that is tRNA (f_t) is 0.14 (Dennis and Bremer, 1974; Rosset *et al.*, 1966). 5S, 16S and 23S rRNA are synthesized as a large precursor RNA transcript (Dunn and Studier, 1973; Ginsburg and Steitz, 1975; Nikolaev *et al.*, 1973; Pace, 1973), of which the individual lengths are 120 (Brownlee *et al.*, 1967), 1542 (Brosius *et al.*, 1978; Carbon *et al.*, 1979), and 2904 nucleotides (Brosius *et al.*, 1980), respectively. Given that there are seven rRNA operons in the *E. coli* genome (Kenerley *et al.*, 1977; Kiss *et al.*, 1977), it can be calculated that any given 16S gene, such as *rrlA*, comprises 4.1% of total RNA (2).

$$\frac{m_{rrlA}}{m_{\mathrm{RNA}}} = f_s - (f_t \times f_s) \times \frac{nt_{16\mathrm{S}}}{nt_{5\mathrm{S}} + nt_{16\mathrm{S}} + nt_{23\mathrm{S}}} \times \frac{1}{\#_{operons}} \tag{2}$$

From the mass of total *rrlA*, the number of *rrlA* transcripts was determined by calculating the sequence molecular weight (3), and finally the number of transcripts per cell (4).

$$M_{rrlA} = (n_{\mathrm{A}} \times 329.2) + (n_{\mathrm{U}} \times 306.2) + (n_{\mathrm{C}} \times 305.2) + (n_{\mathrm{G}} \times 345.2) + 159 \tag{3}$$

$$\frac{copies_{rrlA}}{\mathrm{cell}} = \frac{m_{rrlA}}{M_{rrlA}} \div \frac{cells}{\mathrm{ml}} \tag{4}$$

The absolute number of transcripts for each gene was then calculated by multiply-

ing the gene's mean expression ratio relative to *rrlA* by the inferred number of *rrlA* transcripts per cell.

2.6 DNA isolation

DNA was isolated from spent media by pelleting cultures at the appropriate timepoint and transferring 1 ml of supernatant to a 2ml microfuge tube followed by 1ml of basic hot phenol (pH 8.0, 65°C) (Sigma-Aldrich). The mixture was centrifuged and the aqueous phase transferred to a 2 ml "safe-lock" microfuge tube (Eppendorf). Extraction from phenol:chloroform:IAA was performed twice followed by one extraction from chloroform:IAA. The nucleic acid in the aqueous phase was then precipitated with 2.5 volumes ethanol and 0.1 volumes sodium acetate (3 M, pH 5.2) at -20°C overnight. Precipitated DNA was pelleted, washed with 70% ethanol, and suspended in nuclease-free ddH_2O.

2.7 Cell viability

Viability was determined using the standard plate-count method. Cultures were diluted in M9 salts at the appropriate timepoints and 100 μl of each dilution was plated onto LB plates and incubated overnight at 37°C. The number of colonies on each plate was then counted to determine the number of colony forming units per milliletre of culture.

There is a subset of cells that are viable but nonculturable (reviewed in Oliver, 2005), and would therefore elude quantification by standard plate count. In an attempt to quantify this subpopulation, fluorescence microscopy was used in conjunction with the *BacLight^{TM} RedoxSensor^{TM} Green Vitality Kit* (Invitrogen). Exponential and overnight cultures were stained according to the standard protocol.

3 Results

All timecourse data presented below corresponds to exponential phase, entry into stationary phase, and stationary phase at 3.8, 5.7, and 24/48 h, respectively (Table 2).

3.1 RNA extraction protocol development for stationary phase cultures

It was determined at the beginning of this project that a protocol was to be developed for the isolation of RNA from *E. coli* in stationary phase using hot acid phenol with the additional caveat of not using DNase to remove DNA contamination. A protocol based on hot acid phenol extraction was used successfully in this laboratory in the past (Dong *et al.*, 2008; Dong and Schellhorn, 2009) and it is important to keep methodologies consistent, particularly if they have already been accepted in the literature. A variation of hot acid phenol extraction has been shown to isolate RNA of increased integrity relative to *TRIzol* (Invitrogen) and *RNeasy* kits (QIAGEN) (Jahn *et al.*, 2008). Organic extraction is also well-known to give increased yields relative to kits – a key factor when attempting to isolate RNA from older cultures. Briefly, this method involves lysing the cells with boiling SDS/EDTA, followed by the addition of hot acid phenol, separation of the aqueous and organic phases by centrifugation and then extraction of the aqueous phase. RNA partitions to the aqueous phase, DNA partitions to the interphase in a pH-dependent manner, and proteins and lipids partition to the organic phase. This extraction from hot acid phenol is done three times, followed by extraction from phenol:chloroform:IAA and chloroform:IAA. Phenol:chloroform:IAA effectively removes remaining proteins that were not removed initially by the phenol as well as lipids. Extraction from chloroform removes remaining phenol from the

aqueous phase. Purified aqueous phase is then precipitated in ethanol under high salt concentration. The RNA can then be collected by centrifugation and resuspended in nuclease-free ddH_20.

It quickly became apparent that the original protocol for RNA extraction (Appendix A.2) was deemed to be inadequate for the extraction of high-quality RNA from stationary phase cultures for multiple reasons. Scale-up was required because the quantity of RNA drops precipitously in batch cultures over time. This drop in yield is due to the overall decrease in RNA synthesis that accompanies starvation (Neidhardt *et al.*, 1996) as well as a decrease in the number of viable cells (Fig.14) (cell viability is discussed in section 2.7). Indeed, extraction of RNA from cultures that were incubated for 7 days yields virtually no RNA. A comparison using the *RNeasy* kit illustrates the difference in yield over time (Figs.4-5). In addition, it should be noted that RNA isolated from week-old samples was always of inadequate purity ($OD_{260/280} \approx 1.5$). Indeed, J. Bernstein (of Bernstein *et al.*, 2002) has reported that the RNA yield from 1 ml of exponentially growing culture is roughly equivalent to 20 ml of stationary phase culture. Furthermore, when extracting the aqueous phase, a small volume must remain in order to avoid disturbing the interphase. Leaving a greater volume of aqueous phase results in RNA of greater purity but diminished yield. Therefore, there is always a trade-off between yield and purity. As such, using microfuge tubes, it was impossible to maintain adequate distance from the interphase during extraction to obtain final RNA preparations of sufficient quality for downstream processing while still obtaining sufficient yield. This problem was further increased by the fact that older cultures seem to produce more cellular material that associates with the interphase relative to younger cultures, such that even more care needs to be taken to avoid the interphase material. By scaling up and using 15 ml glass tubes, it was possible to obtain samples of much higher purity and

yield.

In the original protocol (Appendix A.2), liquid cultures at the appropriate time-point are centrifuged at room-temperature for 10 min before decanting of the media and snap-freezing in liquid nitrogen. This handling at room-temperature must have effects on the transcriptome, as the temperature steadily drops some 15°C from incubation conditions, with decreased aeration and increased compaction of cells. Cooling cells in this fashion may induce the cold shock response – a process intimately linked with the stress response (White-Ziegler *et al.*, 2008) which may therefore confound subsequent gene expression analysis. However, the major concern during handling is degradation of mRNA transcripts as they are the topic of study. Indeed, 80% of all mRNA species in *E. coli* have half-lives between 3 and 8 min (Bernstein *et al.*, 2002). Furthermore, half-lives under 2.5 min have been reported for operons involved in functions that are important under the nutrient limiting conditions of stationary phase such as amino acid biosynthesis, utilization of alternative carbon sources, and nucleotide biosynthesis (Selinger *et al.*, 2003). Fortunately, a "stop solution" was developed during previous studies of mRNA decay in *E. coli*, consisting of a solution of water-saturated phenol in ethanol (Bernstein *et al.*, 2002). This solution also has the added benefit of preventing transcription to some extent (Bernstein *et al.*, 2002), thereby reducing transcriptome reorganization due to handling. Thus, cultures are added directly to 0.1 volumes of ice-cold "stop solution" and incubated in an ice-water bath prior to centrifugation at 4°C. Pellets were washed quickly with ice-cold PBS for reasons described below prior to being flash-frozen.

By far the greatest obstacle in development of this protocol was that of DNA contamination. Normally, DNA is removed from preparations using DNaseI. Enzymatic digestion is useful because DNA and RNA are nearly chemically identical, differing only in the presence of a hydroxyl group on carbon C2' in the case of RNA (ie. ribose

instead of deoxyribose). However, because DNase could not be used, some additional steps and insight was required to solve the problem of DNA contamination. The differing functional group results in a pH difference between RNA and DNA, causing DNA to form a slightly closer, exploitable association with the acidic interphase. Instead of mixing the aqueous and organic phases by vortexing, samples were instead inverted 6-8 times. Vortexing shears genomic DNA, and smaller DNA molecules are believed to have a looser association with the interphase such that they are difficult to eliminate using only hot acid phenol without employing DNase. Further measures were taken to increase the pH-dependent association of DNA with the interphase. The original protocol lyses the cell pellet with 500 μl of boiling lysis solution (2% SDS/16 mM EDTA) and then adds 1ml of hot phenol, such that the lysis solution makes up the entirety of the aqueous phase and phenol is present in a 2:1 ratio. However, SDS and EDTA are both known to affect pH such that it is best to use only as much as required. In addition, phenol is known to be most effective at removing proteins and other contaminants whem mixed in a 1:1 ratio with the aqueous phase (Lin-Chao and Bremer, 1986). To this end, pellets were lysed with 1 ml of lysis solution before the addition of 7 ml of hot acid phenol and 6 ml of ddH_20 also heated to 65°C. Heating the ddH_20 had the added benefit of quickly bringing the relatively large sample volume to 65°C as required in the next stage of the protocol. Thus, the lysis solution was effectively diluted by 1/7th to decrease the effect on pH, and phenol was brought to a 1:1 ratio relative to the aqueous phase thereby providing more ideal conditions for the pH-dependent separation of DNA. Standard lithium chloride precipitation was also attempted to remove contaminating DNA (Barlow *et al.*, 1963), but unnacceptably decreased RNA yield in addition to increasing handling time.

Lysis and extraction of RNA from liquid cultures (Appendix A.3) rather than pellets has been used with great success during the exponential growth phase (Dong

et al., 2008) but was here found to be unsuitable for stationary phase cultures. At one point it was believed that the reason for the low yield of RNA was due to incomplete lysis of cells. Moreover, it was thought that the efficiency of lysis could be improved by decreasing the density of cells and lysing the cells in liquid culture (while still shaking on an air incubator). It was found that the problem of DNA contamination was greatly exacerbated when using this method and decreased cell density effectively reduced the number of cells lysed such that yield was actually decreased. One possible explanation for this increase in DNA contamination was that shaking of the culture following addition of the lysis solution contributed to shearing of genomic DNA. However, modifying the shaking speed from 300 rpm to 100 rpm did not appear to reduce DNA contamination, suggesting that shearing of genomic DNA during mixing was not responsible for the increase in DNA contamination. To posit an explanation for the increased DNA contamination, it was hypothesized that DNA from dead cells is released into the extracellular melieu and partially digested by nucleases to yield DNA molecules of low molecular weight. As described previously, these smaller DNA molecules have lower association with the interphase relative to full-length genomes and are not efficiently separated. To determine the concentration of DNA in spent media at the relevant timepoint, the laboratory DNA extraction protocol was modified (Appendix A.5) to be suitable and relevant for DNA isolation from media (discussed in secton 3.1.1). Indeed, stationary phase cultures were found to have greatly elevated concentrations of DNA in the media relative to exponential cultures (Fig.6). Furthermore, gel electrophoresis of these samples suggests that the DNA molecules are of low molecular weight as there is no detectable DNA in the wells (Fig.7). The tendency of stationary phase cultures to have increased DNA contamination, correlating with the age of the culture, suggests that the two are related. Thus, pellets were also quickly washed with ice-cold PBS, as previously mentioned,

to remove as much media as possible. Washing the cells could remove even more contaminating DNA but would require excessive handling.

Following extraction three times with hot acid phenol, an additional extraction from phenol:chloroform:IAA was included. Far more material is present on the interphase at this step when handling stationary phase cultures compared to exponential phase cultures, appearing to correlate with the age of the culture. Two extractions from phenol:chloroform was found to eliminate most visible material at the interface and improve the purity of the final sample.

The final extraction from chloroform was also modified in order to increase safety and reduce contamination by RNases. Inverting chloroform in 15 ml glass tubes or even 1.5 ml microfuge tubes results in spillage due to vapour pressure which results in sample loss and damage to the experimenter's gloves. Handling glass tubes is particularly difficult because parafilm is required for inversion, and this material is dissolved by chloroform. Furthermore, the tubes must be left open during centrifugation, virtually ensuring contamination by RNase during this step. This situation was remedied by aliquoting the aqueous phase obtained from the phenol:chloroform:IAA extraction into 2 ml Eppendorf "safe-lock" microfuge tubes that seal completely. These tubes effectively prevented sample spillage and RNase contamination.

Precipitation of RNA also required some changes. In the original protocol, tubes are left open to dry in a 37°C incubator which led to rapid degradation of the sample through contamination by RNases. Instead, tubes were double-aspirated to remove as much residual ethanol as possible, and then dried at a 45°downward angle next to a Bunsen Burner. Initially, samples were left to dry completely for some 30 min because it was believed that the low efficiencies observed for reverse transcription was a result of ethanol or phenol contamination. It was later found that purification of the sample on an *RNeasy* column (a commonly required step) led to greatly increased efficiency

of reverse transcription. Therefore, drying was only done for a few minutes because the column effectively removed all ethanol and organic solvents and an undried pellet allowed more rapid dissolution of RNA. RNA cleanup was initially attempted with Nucleospin columns but these gave yields too low for downstream applications.

Instead of precipitating RNA and then storing at -80°C, precipitation was done immediately prior to reverse transcription to minimize transcript degradation prior to this crucial step. RNA isolated by this method was of the highest integrity (Fig.8) and purity ($OD_{260/280} > 2.10$). Resulting samples provided a robust signal in qPCR and exhibited very low DNA contamination. Confirmation of reverse transcription by end-point PCR showed that the reaction was successful and DNA contamination was undetectable (Fig.9).

3.1.1 DNA isolation from spent media

In order to isolate DNA from spent media, a new protocol had to be developed for this purpose. The protocol used by Miller (1992) was inadequate for several reasons. Briefly, this protocol involved incubation of the sample with SDS and proteinase K to denature and hydrolyse proteins followed by one extraction from phenol:chloroform:IAA and one extraction from chloroform:IAA. The DNA is then ethanol precipitated by incubating for one hour on ice, then centrifuged and resuspended in ddH_20. When applied to spent media (with precipitation done overnight as one hour produced virtually no yield), this protocol yielded DNA contaminated with a brown pigment and gave erroneously high spectrophotometric readings.

The problems associated with the original protocol and resulting modifications are as follows. First, because the media could not be filtered due to loss and shearing of DNA on the filter, there was a probability that cells carried over in the supernatant after centrifugation would be lysed by SDS and therefore contribute to the assayed

concentration of DNA. Therefore, the media was simply extracted without a lysing agent from hot basic phenol, which is considered to be more efficient at removing proteins and other contaminants relative to SDS and proteinase K. Basic phenol is more suitable than neutral phenol because RNA is readily hydrolyzed under alkaline conditions. Another advantage of using hot phenol is that it more closely resembles the protocol used for RNA isolation and thus allows comparison with DNA contamination that may be present following RNA isolation. Second, an additional extraction from phenol:chloroform:IAA was required to remove visible white matter and yield pure DNA. Third, samples were precipitated overnight at -20°C because incubation on ice for one hour resulted in a reduced yield, likely due to inadequate time to precipitate the presumably low-weight DNA molecules in the media. Finally, centrifugation time for precipitation was increased to 30 min. All mixing was done by inversion rather than vortexing to avoid shearing of genomic DNA.

With the modifications above to precipitation time, temperature, and centrifugation time, the experimental yield is expected to be >90% (Zeugin and Hartley, 1985). Applying this protocol to spent media indicates that there is virtually no DNA present in the medium during exponential phase growth. However, at 24 h and later timepoints, it is clear that there is a greatly elevated concentration of DNA in the medium relative to exponentially growing cultures (Fig.6). Gel electrophoresis of these samples suggests that the DNA molecules are of low molecular weight as there is no detectable DNA in the wells, and sheared DNA >10 kb is visible starting at 5.7 h and diminishing afterwards (Fig.7).

3.2 Previous microarray data

Microarray data at the relevant timepoints was not obtained by the author (Fig.2). Data for the first pair of timepoints (at 3.8 and 5.7 h) was obtained from Dong *et al.* (2008). The second pair of timepoints (at 24 and 48 h) was obtained from a single microarray experiment performed by S. Chiang. This data is briefly summarized here as two different datasets.

Between exponential phase growth and entry into stationary phase (3.8-5.7 h), Dong *et al.* (2008) reported greatly increased expression for *dps*, moderately increased expression for emphotsB, and no change for *rpoS*, *tomb*, *emrK* or *tufA*. Between 24 and 48 h, S. Chiang reported a sharp decrease in *dps* and *otsB* transcripts and a moderate decline in *rpoS* and *tufA* transcripts. Conversely, *tomb* and *emrK* trascript levels increased. Combining the two datasets, it appears that all genes increased in expression from 5.7 to 24 h except for *tufA*, which declined slightly.

3.3 RT-qPCR assay of temporal gene expression

Gene expression data obtained by RT-qPCR for genes *dps, rpoS, tomB, emrK, otsB* and *tufA* was plotted against each other for each timepoint (Fig.10), as well as individually in order to visualize standard error (Fig.11). The 48 h timepoint was not included because the results did not appear meaningful – all values for gene expression from cultures incubated for 48 h exhibited standard error approximately twice as great as the mean (Table 3).

Several expression patterns were apparent from the qPCR data (Fig.10). Expression of *rpoS* and the RpoS-dependent genes *dps* and *otsB* was greatly elevated upon entry into stationary phase relative to exponential phase. However, *otsB* appeared to maintain elevated transcript levels while *rpoS* and *dps* steeply declined at 24 h, to a

level less than that of exponential phase in the case of *rpoS* . The gene *tomB* exhibited an expression profile similar to that of *rpoS*, although at overall decreased levels. Transcripts of *emrK* appeared slightly elevated upon entry into stationary phase but no meaningful change was observed at 24 h. While *tufA* showed high expression during exponential growth, a large and maintained decline was observed upon entry into stationary phase.

Analysis of the absolute number of transcripts per cell showed nearly identical expression patterns as that of the qPCR analysis (Fig.12). However, in this case there was no change in *emrk* expression (Fig.12D) in comparison to the qPCR data.

3.4 Quantifying viable but non-culturable cells

In an attempt to quantify viable but non-culturable cells, fluorescence microscopy was used in conjunction with the *BacLight*^{TM} *RedoxSensor*^{TM} *Green Vitality Kit* (Invitrogen). This kit contains two components: a proprietary stain that fluoresces green when it is reduced by cellular reductase (an indicator of cellular respiration), and propidium iodide, a stain that fluoresces red upon binding to nucleic acids. Therefore, viable cells are expected to fluoresce green and cells with compromised membrane integrity (non-viable) are expected to fluoresce red.

Unfortunately, it was not possible to score a ratio of viable to non-viable cells in overnight cultures (and therefore older cultures) because all cells appeared to fluoresce red at either high or low intensity, without any green cells visible (Fig.13a). However, the *RedoxSensor*^{TM} dye appeared to be functional and readily taken up by metabolically active cells given the relatively high-intensity green fluorescence that was observed in exponential cultures (Fig.13b). In the overnight culture, it appeared that a small population of cells fluoresced red with high intensity while the major-

ity of cells fluoresced red very dimly. It was tempting to conclude that the brightly fluorescing cells were non-viable, but motility was observed in some members of this population in addition to pairings with presumed sister-cells. Therefore, it was not possible to draw any conclusions about viability from this set of observations.

4 Discussion

4.1 Confirmation of microarray data

When comparing the qPCR data obtained in this study to the microarray data, one must reiterate the point that the 24h/48 h data is separate from the earlier data on cultures undergoing exponential growth and entering stationary phase (Dong *et al.*, 2008). The RNA used for the single 24h/48h microarray experiment was obtained using a different protocol and therefore the expression values may be inflated or deflated relative to the values obtained from the earlier microarray (Dong *et al.*, 2008). Furthermore, transcriptional changes may have resulted from differences in handling prior to lysis of the bacteria and RNA isolation (eg. cold-shock protein induction due to handling at room temperature). Indeed, one can not accurately compare values between the two microarray datasets. Furthermore, the protocol that was used for isolating RNA from the 24h and 48h cultures used for microarray analysis was unreproducible in terms of successfully isolating RNA, suggesting that it may not be entirely reliable. In contrast, the microarray data for earlier timepoints (Dong *et al.*, 2008) was replicated and validated by qPCR analysis, suggesting that it is robust and highly reliable. Thus, any qPCR data spanning these timepoints requires a multi-pronged analysis – comparison to the earlier microarray data (Dong *et al.*, 2008), comparison to the 24h/48 h microarray data, and comparison to the change in expression between the two micrarray datasets.

Regarding inference of absolute transcript numbers per cell, it is important to note that the onset of the stringent response results in downregulation of rRNA operons and upregulation of mRNA (Barker *et al.*, 2001), which is unnaccounted for in this model. This model also assumes that all isolated RNA is from viable (and culturable) cells. This method of inferring transcript numbers should be viewed as complementary

to the more reliable and previously published LMER presentation of RT-qPCR data (Patten *et al.*, 2004).

4.1.1 Comparison of qPCR data to Dong *et al.* (2008) (3.8 and 5.7h)

The gene expression data obtained by the author (Fig.10) appears to be consistent with the earlier published microarray data (Fig.2) (Dong *et al.*, 2008). The RpoS-dependent genes *dps* and *otsB* exhibited much higher expression upon entry into stationary phase in both datasets, as expected. According to the qPCR data, *rpoS* expression increased upon entry into stationary phase but this observation was not reflected in the microarray data (Fig.2), although one would expect *rpoS* expression to increase during stationary phase due to stabilization of the transcript by ppGpp. Indeed, Traxler *et al.* (2011) also reported a significant increase in *rpoS* expression upon entry into stationary phase (LMER = 2.752 relative to unstarved cells) as did Lange and Hengge-Aronis (1994). Gene *tomB* showed a drastically different expression profile when comparing the qPCR data and the earlier microarray data. The qPCR data shows a large increase in *tomB* expression upon entry into stationary phase whereas the microarray data indicates that expression does not change. A very small increase in *emrK* expression was detected in the qPCR experiments while no significant change was seen in the microarray data (Dong *et al.*, 2008). However, the inferred number of *emrK* transcripts/cell indicates no change in *emrk* expression (Fig.12D), which is in agreement with the earlier microarray data (Fig.3D). The expression profile obtained by qPCR for *tufA* shows maximal expression during exponential phase growth (Fig.11F) in contrast to high constant expression reported by the microarray data. This difference is difficult to explain and may be attributable to experimental or human error.

4.1.2 Validation of 24/48h microarray experiment

The difference between gene expression after 24 h incubation compared to cultures entering stationary phase was not validated by the qPCR data (Fig.10). The 24h/48 h microarray data indicates that the expression of all target genes increases at 24 h relative to entrance into stationary phase (except for *tufA*). In contrast, the qPCR data shows a decrease in expression for all target genes except *otsB*, as would be expected for starving cells experiencing a drastic decrease in growth rate following large-scale protein synthesis in preparation for stationary phase. As RpoS governs entrance into stationary phase and plays a smaller role during long-term stationary phase (Lange and Hengge-Aronis, 1994), one would expect that *dps* and *otsB* would decrease or maintain expression after 24 h, contrary to the 24h/48 h micrarray data. Unfortunately, meaningful expression data for the 48 h timepoint was not successfully obtained in this study so no comparison can be made on the microarray data at this timepoint. As mentioned, standard error for gene expression assayed at 48 h was approximately twice great as the mean (Table 3). This may be due to the fact that RNA from stationary phase cultures is degraded, growth is highly unbalanced at this time (Sezonov *et al.*, 2007), or other factors including high DNA contamination. However, it does appear that the 24/48 h microarray data sharply overestimates transcript levels, which may be due to residual DNA contamination. In particular, the data should reflect a decrease after the onset of stationary phase in *rpoS* (Lange and Hengge-Aronis, 1994) and *dps* transcripts. In contrast, the 24/48 h microarray data shows an increase, suggesting that the qPCR data is more reliable than the data obtained in the 24/48 h microarray experiment.

4.1.3 Comparison to Traxler *et al.* (2011)

The transcriptome studies performed by Traxler *et al.* (2008, 2011) (Table 4) agree with the qPCR data (Fig.10). In these studies, the RpoS-dependent transcripts *dps* and *otsB* are highly expressed upon starvation. Similarly, *rpoS* also exhibits increased expression in the Traxler *et al.* (2011) dataset as cells enter stationary phase, albeit slightly less than in the qPCR dataset. In agreement with the qPCR data but contrary to the published microarray data (Dong *et al.*, 2008), *tomB* expression was shown to increase upon starvation. Genes *tufA* and *emrK* were not included in the study by Traxler *et al.* (2008, 2011). However, *yciK*, a member of the emrKY-TolC system, was included and exhibited a minute increase in expression upon starvation in agreement with the qPCR data (Fig.11D). Thus, it appears that the qPCR data is in agreement with previous studies on the stringent response (Traxler *et al.*, 2008, 2011) and RpoS (Lange and Hengge-Aronis, 1994).

4.2 Cell viability

It is important to address the viability of cells in the cultures from which RNA is being isolated. Virtually all previous work on gene expression has been done on cultures that were 100% viable - the cells were either in exponential phase, entering stationary phase, or were subject to induced growth-arrest. However, after one day, cells virtually cease growth and many become dormant or non-viable. Cells that are no longer viable but have preserved membrane integrity still contain RNA molecules that contribute to any assay of gene expression that is not on the single-cell level. Although this contribution is probably minimal, it is important to keep in mind when analysing gene expression. For example, stable RNAs such as rRNA and tRNA may persist in non-viable cells and result in inflated measurements of these RNA species,

which affects normalization to these transcripts.

It is clear that viability drops quickly during one week of incubation (Fig.14). Given the co-occuring drop in cell RNA content, these results are suggestive of the extremely low observed yield when extracting RNA from cultures after 7 days. However, it should be noted that the concentration of viable cells observed here at 7 days (10^2 CFU/ml) is significantly less than 10^7 CFU/ml previously reported for *E. coli* MG1655 by Conter *et al.* (2001).

Regarding the *BacLight*^{TM} *RedoxSensor*^{TM} *Green Vitality Kit*, the stain used in this kit has been applied successfully in the past to varied environmental samples (Alvarez-Ordonez and Prieto, 2010; Franks *et al.*, 2010) so it is difficult to understand why results were so poor under these conditions. One possible explanation is that the contents of the kit has deteriorated - the kit is guaranteed stable for one year if unopened but was opened and used over three years ago. Indeed, the microscope settings required to obtain images (high gain and low offset) indicated a very low signal to noise ratio.

4.3 RNA isolation methodology

It is important for a laboratory to maintain congruent methodologies between publications for comparative purposes, particularly if the methods are widely accepted by the scientific community. However, there are occasions when a seemingly similar problem presents new variables that traditional methods are unable to address adequately. During earlier stages of growth such as during exponential phase and entry into stationary phase, rapid lysis by the addition of lysis solution and hot acid phenol directly to an incubating culture is ideal (Dong *et al.*, 2008). At later timepoints, the quantity of RNA/cell drops precipitously as the age of the culture increases in

conjunction with an increasing concentration of low-molecular-weight DNA in the extracellular milieu (Fig.6). Furthermore, the intracellular DNA content is greater as stationary phase cells contain as many as eight chromosomes (Akerlund *et al.*, 1995). This change in the concentration of nucleic acids in combination with decreasing viability (Fig.14) requires a more aggressive approach to obtain an adequate yield of RNA free of contaminating DNA, proteins, and cellular detritus. It may be possible to analyse gene expression in cultures as old or older than 48 h. However, this would likely require concentrating the cells, ensuring efficient lysis, scaling up volumes and additional organic extractions. The rapidly decreasing ratio of RNA to DNA, and the small size of this DNA (recalcitrant to association with the phenolic interphase), would likely necessitate one or more treatments with DNase.

5 Conclusions

Determining the key biological processes that allow bacteria to persist in the environment under starvation conditions is an important area of research that requires greater attention. Although the stringent response in bacteria has been actively studied using induction models, this data has not been validated using physiologically relevant conditions. Indeed, these induction models often do not agree with each other and introduce confounding factors such as the accumulation of metabolites or metabolic restructuring specific to the model. Unfortunately, these models have not yet been validated due to the technical and biological difficulties in performing assays on RNA from cultures in long-term stationary phase. A protocol was developed here which allows extraction of RNA of the highest integrity and purity from cultures as old as 24 h. At the time of writing, gene expression analysis was performed once on 48 hour-old cultures but failed, presumably due to failure of reverse transcription or degraded mRNA. However, obtaining the 24 h timepoint was sufficient for a preliminary validation of microarray data found in the literature (Durfee *et al.*, 2008; Traxler *et al.*, 2008, 2011). Unfortunately, the preliminary microarray data for 24 and 48 hour-old cultures was not validated by qPCR in terms of absolute expression relative to cultures in exponential phase and entering stationary phase. Indeed, it appears that the values are inflated given the increase in expression of every gene (except *tufA*) at 24 h relative to entrance into stationary phase. Following preotein synthesis of these transcripts upon entry into stationary phase, one would expect a decrease at this timepoint when *E. coli* is more at rest. However, the differences observed in the microarray data between 24 and 48 hour-old cultures may be valid as isolated RNA was obtained in parallel. Future work should validate the 48 h timepoint by qPCR. However, there may be a limit with current technologies in ob-

taining meaningful data at very late timepoints due to the decrease in RNA yield and RNA:DNA, in combination with increasingly unbalanced growth. Furthermore, there is some evidence that long-term batch cultures are composed of many dynamic populations, wherein successive GASP mutations occur that outcompete the previous population (Farrell and Finkel, 2003; Finkel and Kolter, 1999). In this case, multiple non-isogenic populations may make it very difficult to obtain meaningful gene expression data in long-term cultures. Determining protein abundance for the products of each target gene, and perhaps analyzing medium component concentrations, would provide a clearer picture of the biological processes occuring in bacteria at this time and aid in interpretation of the gene expression data. For example, large-scale protein synthesis upon entry into stationary phase may provide an explanation for the decrease in expression of stress-related genes at later timepoints as these vital proteins may already be in high abundance, negating the requirement for a large number of transcripts coding for these proteins. Analysis of batch cultures over time provides the best opportunity to study physiologically relevant stationary phase life processes in a laboratory environment.

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A Protocols

A.1 Media preparation and growth conditions

Adapted from Sathiasothy (2011)

All media and solutions used for bacterial growth testing were brought to pH 7.0

LB broth:

- LB is prepared by adding 25 g of powder to 1 L of distilled water to give a final concentration of 10 g of bacto-tryptone, 5 g of bacto-yeast extract and 10 g of sodium chloride per liter.
- Broth is sterilized by autoclaving at 121°C.
- All cultures grown in LB broth were inoculated from single colonies.
- Cultures were grown in Erlenmeyer flasks at a ratio of 1:5.
- All cultures were grown at 37°C with shaking at 200 rpm.

LB plates:

- To make LB plates, added 250 ml of 2X LB broth to 250 ml of 2X Agar.
- Plates were dried in biosafety cabinet for about 20 min and stored at 4°C.
- All cultures taken from -80°C storage freezer were first streaked onto LB plates for isolation of individual colonies.

A.2 RNA extraction from frozen pellet

Obtained from S. Chiang

1. Collect 1.5 mL culture at desired timepoint in microfuge tube
2. Centrifuge 30 s @ 10000 rpm
3. Decant supernatant
4. Flash-freeze in liquid nitrogen
5. Store at -80°C until required

6. Thaw pelleted cells on ice
7. Resuspend cells in residual supernatant
8. Add 0.5 mL boiling 2% SDS/16 mM EDTA, vortex
9. Add 1 mL acidic phenol, invert
10. Put in 65°C for 3 min, vortex occasionally
11. Put on ice for 10 min, invert occasionally
12. Centrifuge 10 min @ 12000 rpm
13. Transfer aqueous phase to new microtube
14. Add 1 mL acidic phenol (65°C), vortex
15. Centrifuge 10 min @ 12000 rpm
16. Repeat steps 8-10
17. Add 1 mL of 25:24:1 (phenol:chloroform:IAA) (SATP), vortex
18. centrifuge 5 min @ 12000 rpm
19. Transfer aqueous phase to new microtube
20. Add 1 mL of 24:1 (chloroform:IAA) (SATP), vortex
21. Centrifuge 5 min @ 12000 rpm
22. Transfer aqueous phase to new microtube
23. Add 0.1 volumes (40μL) 3 M Na acetate pH 5.2, vortex
24. Add 2.5 volumes ethanol (1 mL), vortex
25. Leave overnight at -20°C

26. Centrifuge 25 min @ 12000 rpm at 4°C
27. Discard supernatant
28. Add 0.5 mL 70% ethanol, invert
29. Centrifuge 10 min @ 12000 rpm
30. Discard supernatant
31. Dry tubes at 37°C for 15 min
32. Add 20 μL 0.1% DEPC ddH_20 to resuspend RNA
33. Take OD_{260} and OD_{280}
34. Store at -80°C

A.3 Large scale rapid lysis hot phenol RNA extraction

Obtained from T. Dong with slight modification

1. Add 10 ml of culture at set timepoint and allow to equilibrate at 37°C rotating shaker at 200 rpm.
2. Add 5 ml (0.5 volumes) of boiling 2% SDS, 16 mM EDTA directly to the liquid culture in the incubator and shake at 300 rpm for 30 s.
3. Add 15 ml (an equal volume) of acid phenol (pre-warmed to 65°C) and shake at 300 rpm for 60 s. Incubate at 65°C water bath for 3 min.
4. Cool the sample (still in the glass incubation flask) on ice for 10 min to permit complete dissociation of nucleoprotein complexes. Mix gently by swirling every 30 s.
5. The aqueous/organic phases are separated by centrifugation at 12000 g for 15 min (15 ml Glass COREX tubes with 8441 adapters in an SS-34 rotor, Sorvall RC5B centrifuge). Using a 10ml disposable pipette, transfer the upper aqueous phase to an RNase-free tube and avoid any interphase matter.
6. Repeat steps 6-8 two more times or until no white matter at the interphase is visible.
7. After transferring the aqueous phase to an RNase-free tube, add an equal volume of acidic phenol:chloroform:isoamyl alcohol (25:24:1) at room temperature. Invert to mix and centrifuge at 12000 g for 5 min to separate phases.
8. Transfer the aqueous phase to an RNase-free tube.
9. Add an equal volume of chloroform:isoamyl alcohol (24:1) at room temperature. Invert to mix and centrifuge at 12000 g for 5 min.
10. Transfer the aqueous phase to an RNase-free tube.
11. Add 0.1 volumes of 3 M sodium acetate pH 5.2, invert to mix
12. Add 2.5 volumes of 100% ethanol to precipitate the RNA. Gently invert the tube to mix.
13. Store the sample for 60 min at -20°C or overnight.
14. Pellet the RNA by centrifugation at 12000 g for 30 min at 4°C and carefully decant the supernatant.
15. Wash RNA pellet with 70% ethanol by gentle inversion and centrifuge at 12000 g for 15 min.
16. Decant supernatant, spin for several seconds and aspirate remaining supernatant.
17. Air-dry pellet in biosafety cabinet.
18. When the pellet becomes clear, thoroughly dissolve it in 200 μl of 0.1% DEPC water

A.4 RNA extraction from stationary phase cultures of *Escherichia coli*

Developed by J. Wasniewski

1. Add 13.5 ml stationary phase culture to 1.5 ml (0.1 volume) ice-cold stop solution (5% phenol in ethanol) in a 15 ml conical polypropylene tube and allow to chill in ice-water bath
2. Centrifuge for 7 min at 5000 g (4°C)
3. Wash pellet twice with ice-cold PBS
4. Snap freeze in liquid nitrogen
5. Thaw pellet on ice and resuspend in residual supernatant
6. Add 1 ml boiling lysis solution (2% SDS/16mM EDTA) and gently agitate until solution turns clear (20 s)
7. Add 7 ml 65°C acid phenol. Invert 6-8 times.
8. Add 6 ml 65°C ddH_20. Invert 6-8 times.
9. Incubate in 65°C waterbath for 3 min. Invert every 30 s.
10. Incubate on ice for 10 min. Invert every 30 s.
11. Transfer to a 15 ml glass tube (Corex 8445-15)
12. Centrifuge 10 min at 12000g
13. Transfer aqueous phase to a clean 15 ml glass tube avoiding any interphase matter (leave about 1 ml aqueous phase)
14. Add 1:1 volume 65°C phenol. Invert 6-8 times.
15. Centrifuge 10 min at 12000 g
16. Repeat steps 13-15 until no white matter is visible at the interphase (add phenol about 3 times)
17. Add 1:1 volume room-temperature phenol:chloroform:isoamyl alcohol (25:24:1). Invert 6-8 times.
18. Centrifuge 5 min at 12000 g
19. Repeat steps 17-18
20. Add 1 ml of aqueous phase to 1ml chloroform:isoamyl alcohol (24:1) in a 2ml safe-lock tube (Eppendorf). Invert 6-8 times.
21. Centrifuge 5 min at 12000 g
22. Transfer 400 μl to an RNase-free 1.5 ml microfuge tubes (two tubes for each tube used in step 20)
23. Add 40 μl (0.1 volume) 3 M sodium acetate pH 5.2
24. Add 1 ml (2.5 volumes) ethanol. Invert 6-8 times.
25. Incubate overnight at 20°C (RNA can be stored indefinitely in this form)

26. Centrifuge 25 min at 16000 g (4°C) then decant supernatant
27. Add 1 ml ice-cold 75% ethanol. Invert 6-8 times.
28. Centrifuge 15 min at 16000 g (4°C) then carefully double-aspirate the supernatant

29. Allow pellet to dry on benchtop for 15min
30. Resuspend pellet in 20 μl RNase-free ddH_20 and put on ice
31. Purify RNA using QIAGEN RNeasy kit (RNA cleanup protocol)
32. Synthesize first-strand cDNA using an adequate Reverse Transcription kit

A.5 DNA isolation protocol for spent media

Developed by J. Wasniewski

1. Collect culture and centrifuge to pellet cells
2. Transfer 1 ml supernatant to a 2 ml microfuge tube
3. Add 1ml 65°C basic phenol (pH 8.0) to 1ml spent media. Invert 6-8 times.
4. Incubate at 65°C for 3 min. Invert every 30 s.
5. Incubate on ice for 10 min. Invert every 30 s.
6. Centrifuge 10 min @ 12000 rpm
7. Transfer aqueous phase to a 2 ml safe-lock tube (Eppendorf). Invert 6-8 times.
8. Add 1:1 volume room-temperature phenol:chloroform:isoamyl alcohol (25:24:1). Invert 6-8 times.
9. Centrifuge 5 min at 12000 g
10. Repeat above two steps one more time
11. Add 1:1 volume room-temperature chloroform:isoamyl alcohol (24:1). Invert 6-8 times.
12. Centrifuge 5 min at 12000 g
13. Transfer 400 μl to an RNase-free 1.5 ml microfuge tubes
14. Add 40 μl (0.1 volume) 3 M sodium acetate pH 5.2
15. Add 1 ml (2.5 volume) ethanol. Invert 6-8 times.
16. Incubate overnight at 20°C

17. Centrifuge 30 min at 16000 g (4°C) then decant supernatant
18. Add 1 ml ice-cold 75% ethanol. Invert 6-8 times.
19. Centrifuge 15min at 16000 g (4°C) then carefully double-aspirate the supernatant
20. Allow pellet to dry on benchtop for 15 min
21. Resuspend pellet in 20 μl nuclease-free ddH_20 and store at -20°C

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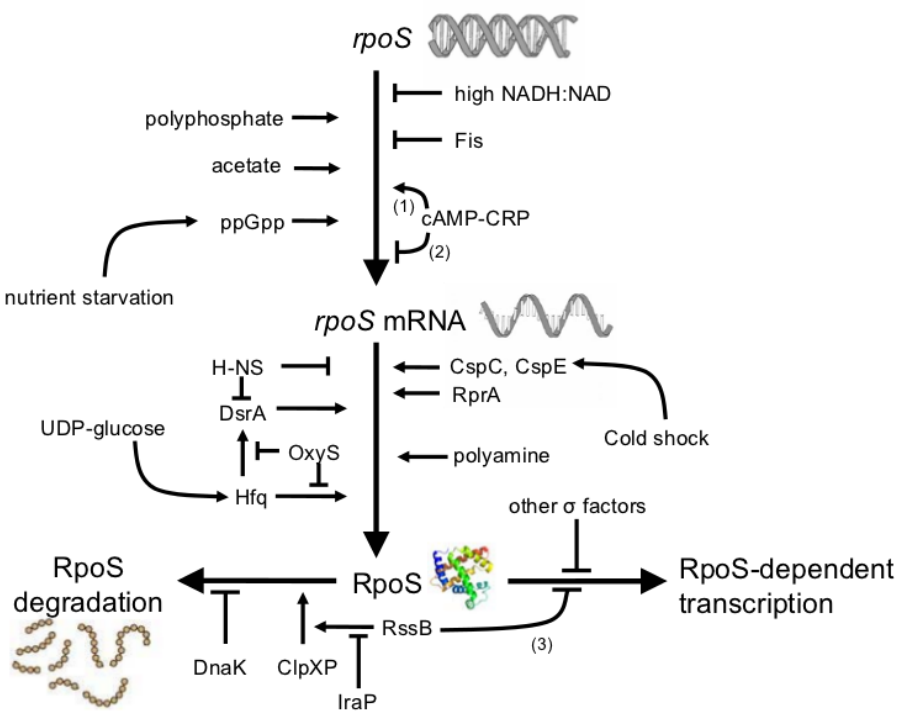

Figure 1: Schematic depicting the modulators of RpoS activity (Dong, 2010). Lines with arrowheads indicate an activating or upregulatory role while lines ending with bars indicate an inhibitory role. Effectors leading from the functional σ^S modulate transcription of other genes.

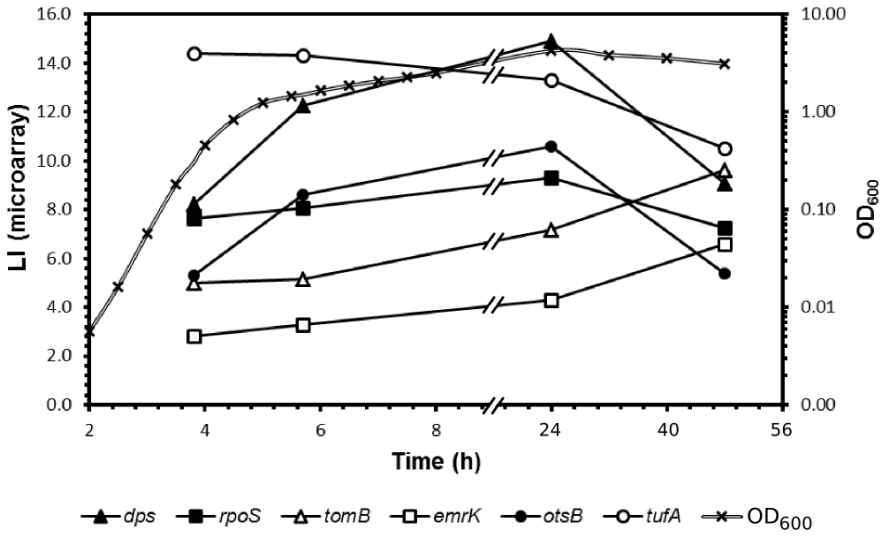

Figure 2: Gene expression data obtained by microarray analysis, expressed in LI units (dChip invariant set normalized Log_2-transformed fluorescence units (described in section 2.2)). Data for the first pair of timepoints (at 3.8 and 5.7h) was obtained from Dong *et al.* (2008). Standard error is not shown. The second pair of timepoints (at 24 and 48h) was obtained from a single microarray experiment performed by S. Chiang. Bacterial growth curve (x) is superimposed with secondary Y-axis.

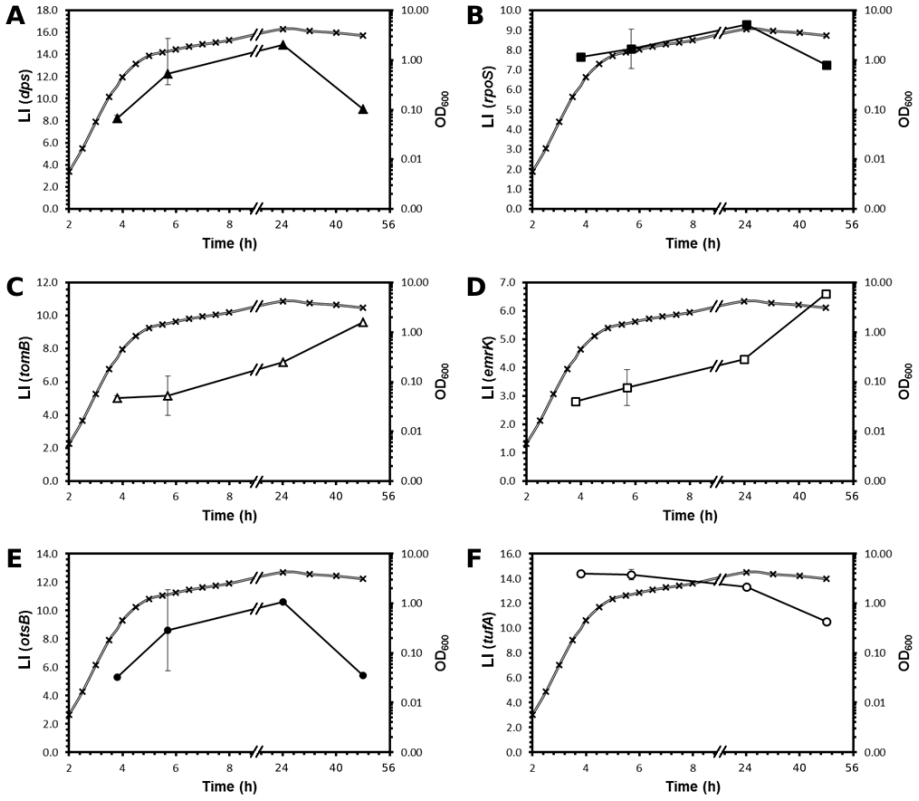

Figure 3: Gene expression data obtained by microarray analysis depicting standard error, expressed in LI units (dChip invariant set normalized Log_2-transformed fluorescence units (described in section 2.2)). Data for the first pair of timepoints (at 3.8 and 5.7h) was obtained from Dong *et al.* (2008). The second pair of timepoints (at 24 and 48h) was obtained from a single microarray experiment performed by S. Chiang. This data is identical to that shown in Figure 2. Bacterial growth curve (x) is superimposed with secondary Y-axis.

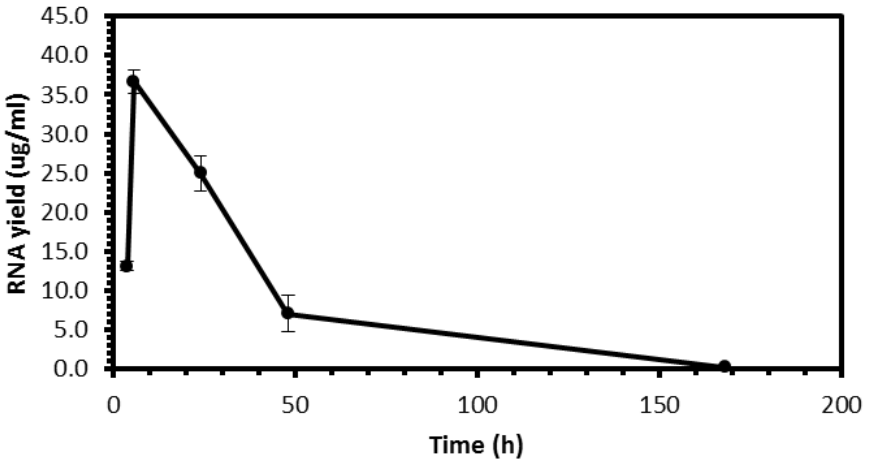

Figure 4: RNA yield from cultures over time using the *RNeasy* Kit standard protocol. At 3.8 and 5.7h, cultures are increasing in cell density (OD_{600}=0.3 and 1.5, respectively). At 24, 48 and 168h, cultures are saturated and exhibit rapidly diminishing RNA yield.

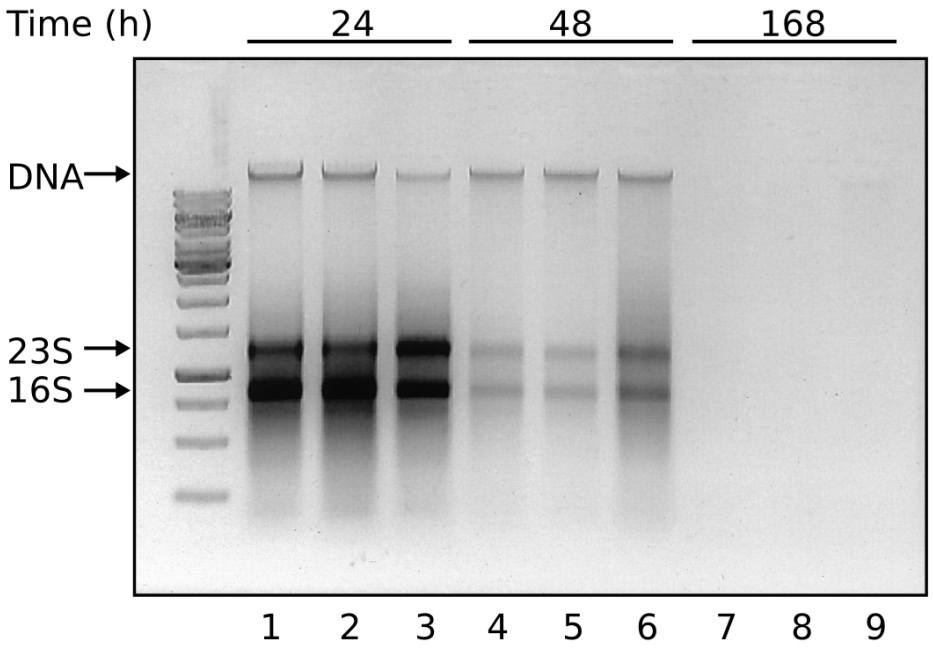

Figure 5: Gel image of RNA isolated using the *RNeasy* Kit standard protocol. Sheared DNA is visible as these samples were not treated with DNase. RNA yield is seen to diminish rapidly over time, evidenced by decreasing signal from bands corresponding to 23S and 16S RNA. No RNA is detectable at 168h.

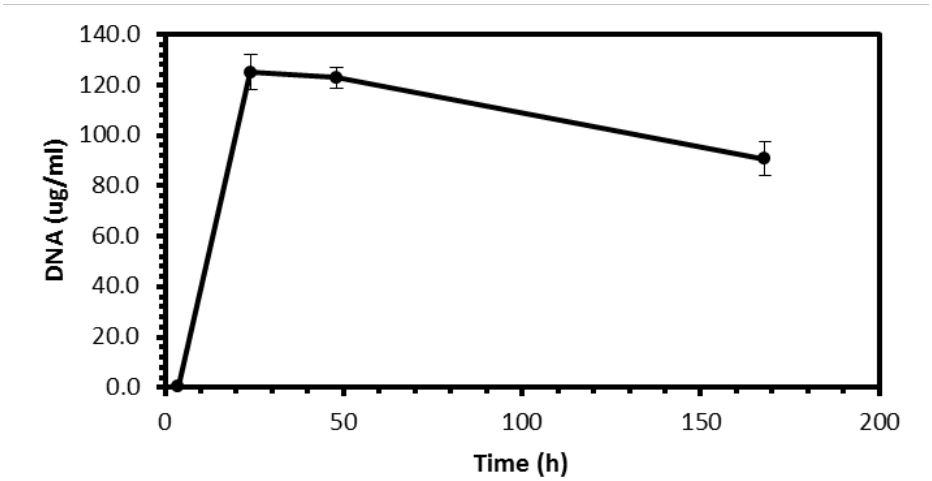

Figure 6: DNA concentration in spent media over time. The 5.7h timepoint was not included in this experiment. DNA concentration in media is greatly elevated after 24h relative to cultures in exponential phase growth (3.7h). See Figure 14 and Table 2 for corresponding viability and optical density at these timepoints, respectively.

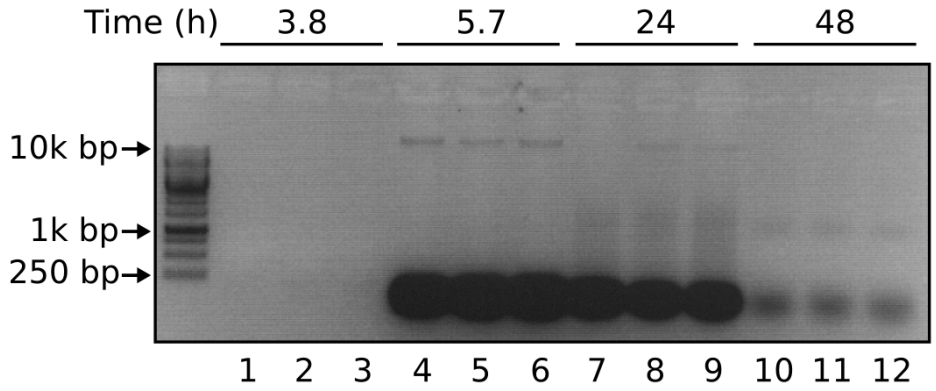

Figure 7: Gel electrophoresis image of DNA isolated from spent media. Lack of signal near the wells is indicative that the DNA is of low molecular weight. DNA is virtually undetectable at 3.8h. Higher-weight DNA can be seen in the media at 5.7h, with less visible at 24h (>10k bp) and none visible at 48h. Bands visible near 1k bp and below 250 bp are likely composed of degraded RNAs.

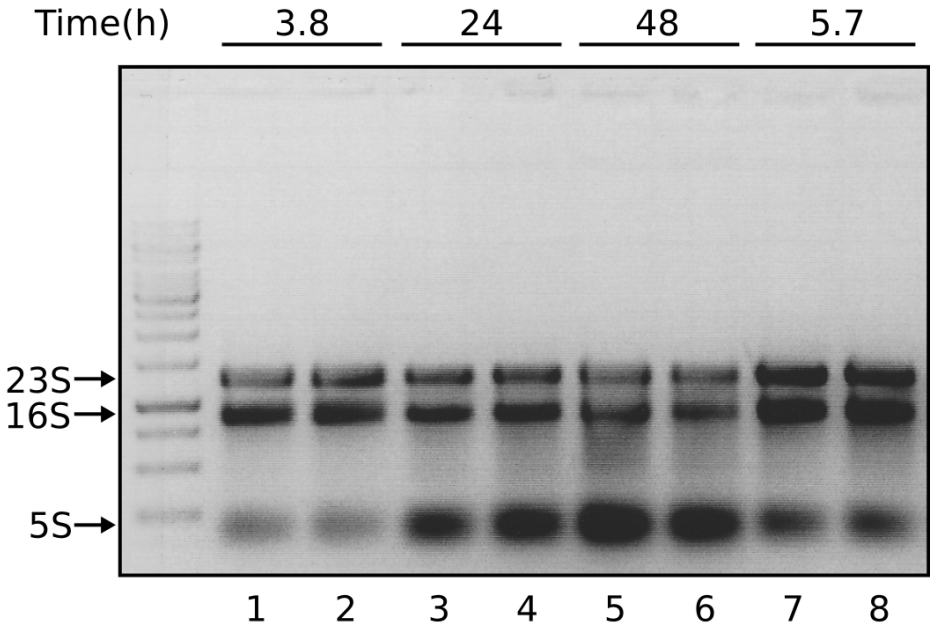

Figure 8: Gel image of isolated RNA for all timepoints using the developed protocol. Note that the 5.7h timepoint is included in lanes 7 and 8. Increased "streaking" below the 16S RNA band from 3.8 to 48h is indicative of increasing degradation over these timepoints.

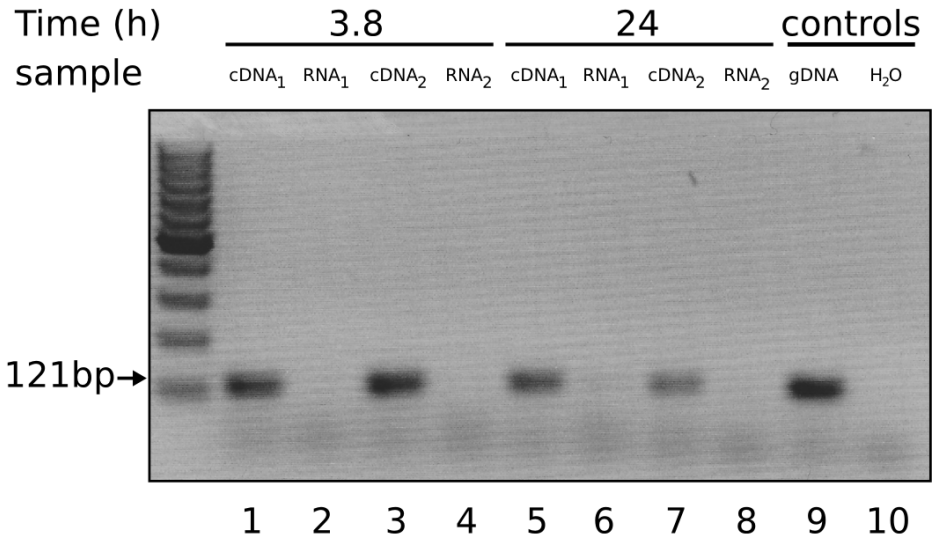

Figure 9: Gel image confirming cDNA synthesis from RNA isolated from exponential and 24 hour-old cultures (two of each) using primers targeting gene *rrlA* (amplicon = 108bp). Each cDNA sample is the reverse-transcribed RNA sample to it's right. A band in the cDNA sample indicates the reverse transcription reaction was successful. Absence of a band in the RNA sample indicates the absence of DNA contamination. Ribosomal genes, such as *rrlA*, have multiple copies in the genome thereby providing the best indicator of DNA contamination.

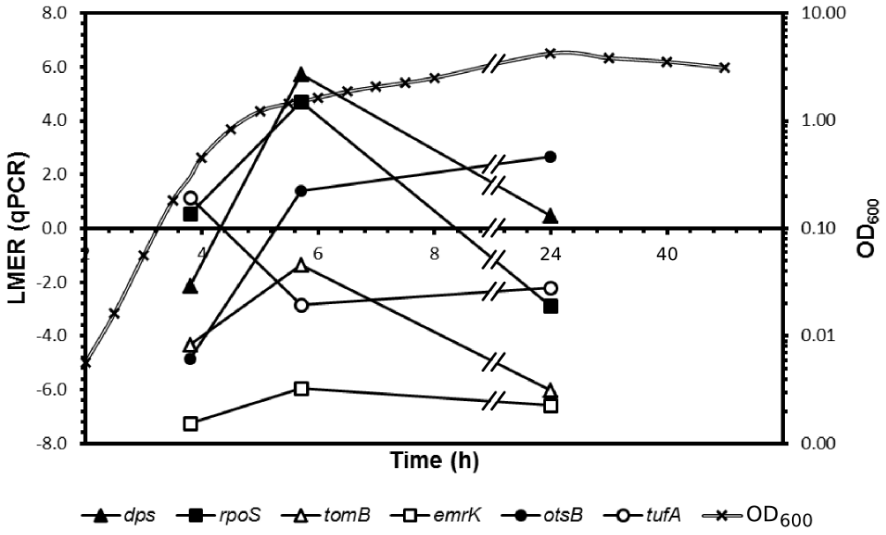

Figure 10: Gene expression data obtained by RT-qPCR, expressed as LMER (Log_2-transformed mean expression ratio of gene of interest to *rrlA*). Standard error not shown. Bacterial growth curve (x) is superimposed with secondary Y-axis.

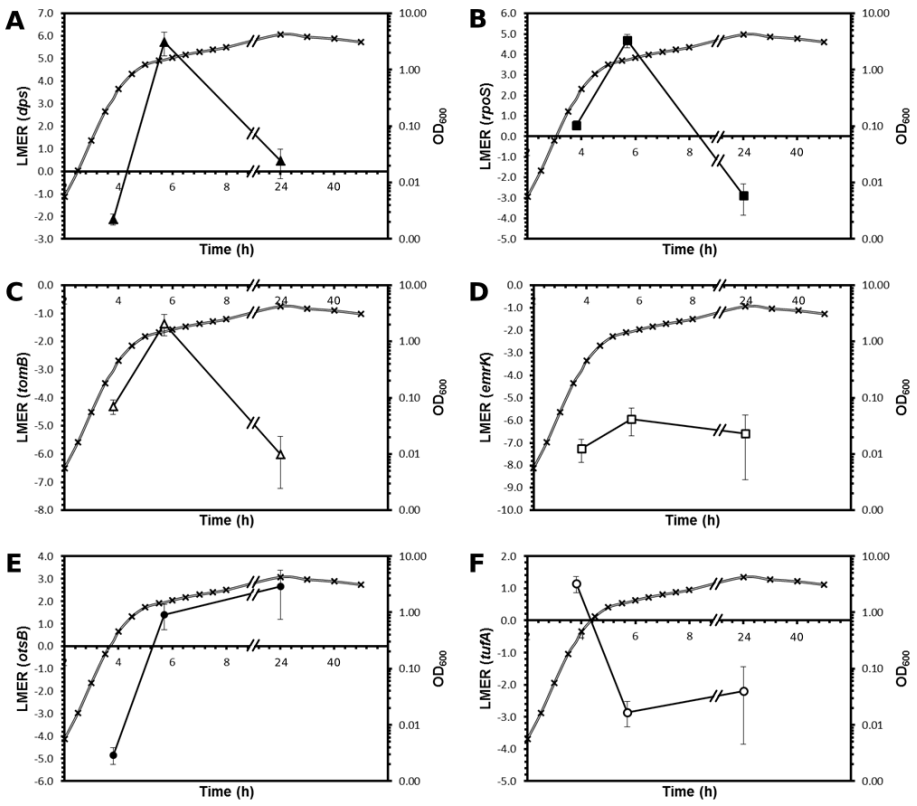

Figure 11: Individual gene expression data obtained by qPCR depicting standard error, expressed as LMER (Log_2-transformed mean expression ratio of gene of interest to *rrlA*). This data is identical to that shown in Figure 10. Bacterial growth curve (x) is superimposed with secondary Y-axis.

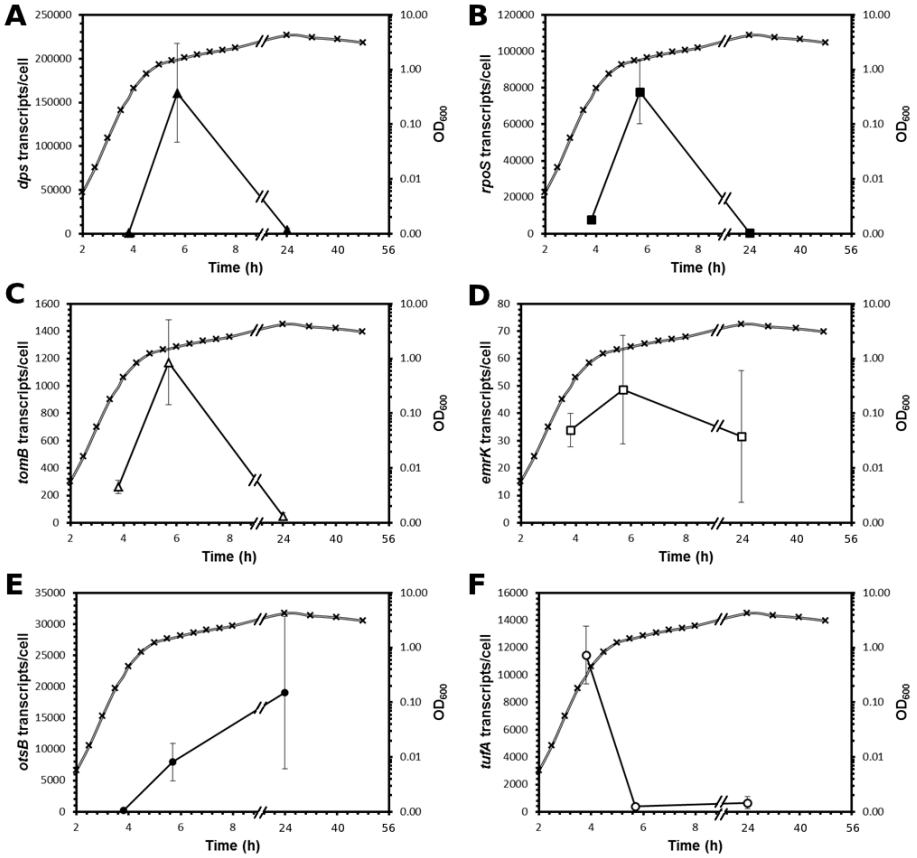

Figure 12: Inferred transcripts per cell for individual genes depicting standard error. The absolute number of transcripts per cell was calculated by determining the number of *rrlA* transcripts/cell and multiplying by the qPCR-determined mean expression ratio of the gene of interest relative to *rrlA*.

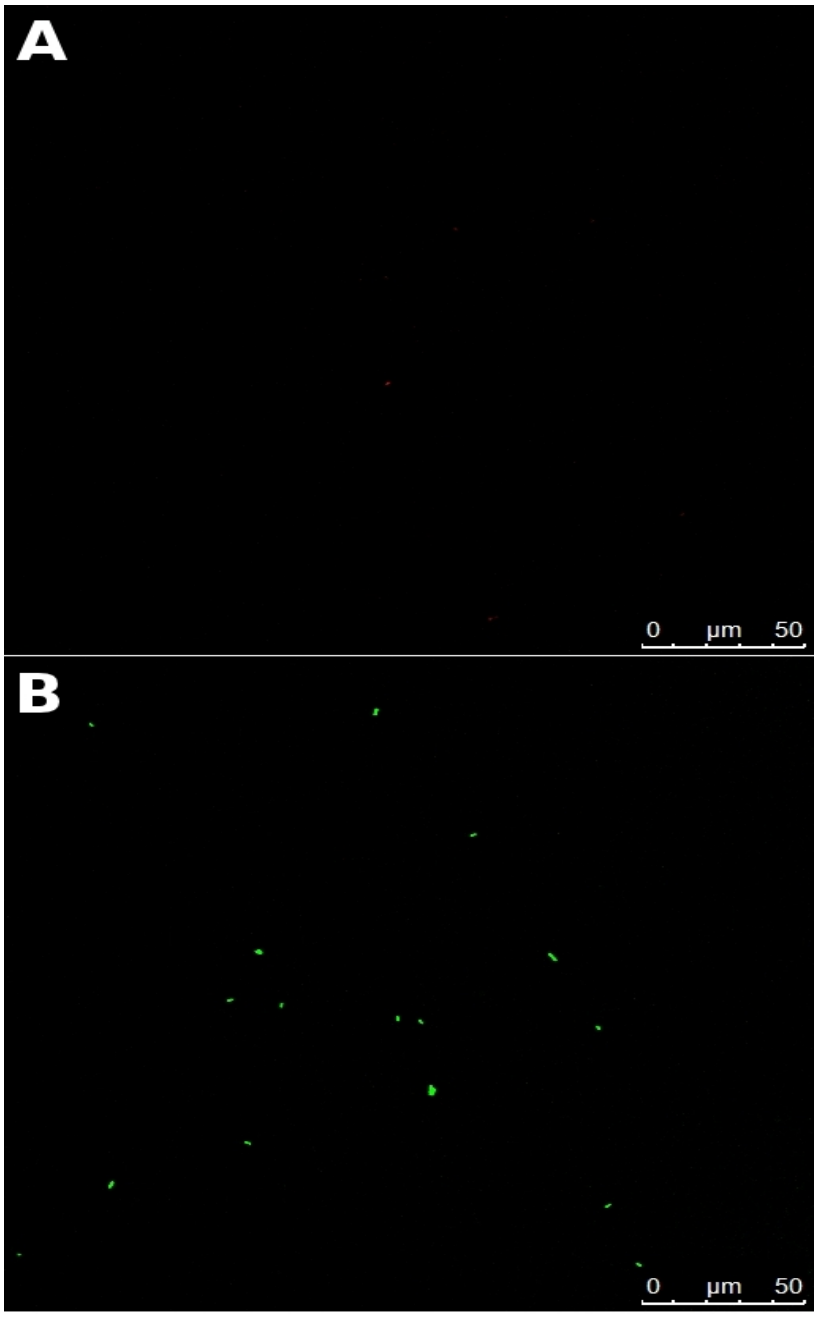

Figure 13: Confocal microscopy images of overnight and exponential culture subject to *BacLight*^{TM} *RedoxSensor*^{TM} *Green Vitality Kit*. Settings for both channels: Gain=1100V, Offset=-80%. A) Overnight culture depicting approximately 7 cells. Cells fluoresce red very faintly. B) Exponential culture depicting approximately 20 cells. Cells brightly fluoresce green.

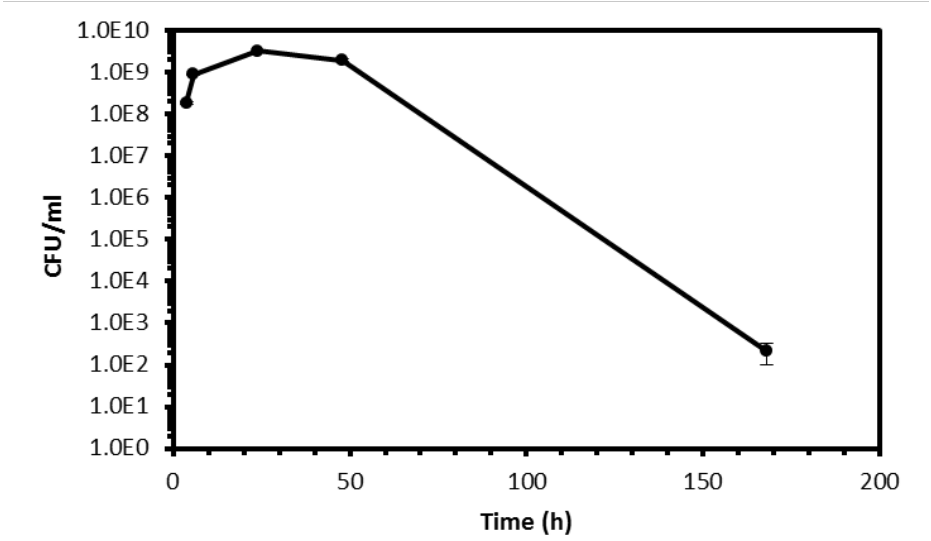

Figure 14: Cell viability of cultures at the relevant timepoints as determined by standard plate-count method. It should be noted that at 3.8 and 5.7h, cultures are increasing in cell density (OD_{600}=0.3 and 1.5, respectively). At 24, 48 and 168h, cultures are saturated. It should be noted that the concentration of viable cells observed here at 7 days (10^2 CFU/ml) is significantly less than 10^7 CFU/ml previously reported for *E. coli* MG1655 by Conter *et al.* (2001).

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Target	Forward Primer	Reverse Primer	Position
dps	ATTGCCGTACATGAAATGCT	GTTGATAACTTGAGTGGTCCC	178-297
emrK	AAAGAAACACAACTCACGGA	GGTTCCCATATTGATCCCTG	754-867
otsB	TTTGATCTTGATGGAACGCT	AGTCCTTGCAGAATATTGTCAG	55-134
rpoS	CTTCTCAACATACGCAACCT	AAACGAATAGTACGGGTTTGG	423-500
tomB	ACGAATATCTGGATGACACCT	CAACGGAATAGTCGATTACCT	230-329
tufA	CGTTGGTATCAAAGAGACTCAG	TACACTTCAGATTCGAACTTGG	735-929

Table 1: Sequences of primers (5' to 3') used for quantitation of gene expression by RT-qPCR. Primer melting temperatures are 59°C to 61°C. Amplicon length (75-200bp) is equal to the span of the position. Primers for gene *tufA* also hybridize to the identical-in-coding-sequence *tufB* transcript.

growth phase	**time(h)**	**OD_{600}**
exponential	3.8	0.3
intry into stationary	5.7	1.5
stationary (early)	24	4.2
stationary (late)	48	3.1
death	168	-

Table 2: Corresponding timepoints, growth phase, and optical density for experiments performed

gene	LMER
rrlA	1.0 ± 2.3
dps	3.7 ± 7.2
rpoS	22.3 ± 42.5

Table 3: LMER of assayed genes from 48 hour-old cultures as determined by RT-qPCR. Standard error is approximately twice that of the mean suggesting it is difficult to observe meaningful differences in gene expression at this time.

gene	LMER
dps	3.653
yciK(emrK)	0.701
otsB	4.085
rpoS	2.752
tomB	1.319

Table 4: Microarray expression data (Traxler *et al.*, 2008, 2011). Values are relative to unstarved cells. Gene *emrK* was not included in the microarray analysis but a member of the EmrKY-TolC system, *yciK* is presented here. Gene *tufA* was also not included in the microarray data, presumably because no change in expression was detected.