ABSTRACT

Title of Document: HARNESSING THE POTENTIAL OF THE *ESCHERICHIA COLI* RPOS PHENOTYPE VIA AN INDUCIBLE SMALL RNA REGULATORY PLATFORM

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Recent recognition of the pervasiveness of non-coding RNAs, in both prokaryotic and eukaryotic systems, has prompted metabolic engineers to reevaluate the role of RNAs in a traditionally protein dominated realm. More specifically, bacterial trans-encoded sRNAs have been implicated in the regulation of genes in several critical pathways from quorum sensing to stress responses. The task of responding to stressful conditions, as well as stationary phase, in a comprehensive manner falls to the *Escherichia coli* global stress regulator, RpoS. Genes transcribed by RpoS are involved in motility, biofilm formation and nutrient limitations. One of the challenges modulating RpoS control is its polymorphic nature. We think this can be addressed using an inducible sRNA regulatory platform.

Recent studies have confirmed RpoS to be post-transcriptionally regulated by at least four sRNAs: three activators, DsrA, RprA and ArcZ, and one repressor OxyS.

Each of these senses different stress conditions, allowing RpoS synthesis to increase or decrease in response to various stressors. This work investigates the potential of a genetically engineered interchangeable small RNA based gene regulation platform as a switch to affect the expression profiles and metabolic behavior of RpoS. RprA and OxyS were put under the control of an arabinose inducible promoter to test the ability to increase/decrease RpoS protein levels and subsequent changes in RpoS-dependent genes. We then assessed gene expression and phenotypic changes using RT-PCR, Western blotting, microarray and motility and biofilm assays. Positive modulation of RpoS using the pRprA platform resulted in a 2 fold decrease in motility in Top10 cells. This difference in motility improved biofilm formation levels up to 12 fold when compared to direct overexpression of RpoS protein. The positive effect of biofilm formation was further supported by the upregulation of other genes essential for biofilms. Conversely, negative modulation of RpoS using the pOxyS platform resulted in an increase in the transcription of the motility gene, flhD. Both systems were capable of positively and negatively regulating bacterial RpoS protective genes. The ability to deliberately and purposefully control RpoS protective genes, in conjunction with motility and biofilm formation, can potentially have broad impact on biotechnology applications.

HARNESSING THE POTENTIAL OF THE *ESCHERICHIA COLI* RPOS PHENOTYPE VIA AN INDUCIBLE SMALL RNA REGULATORY PLATFORM

By

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Dedication

To my parents, Edward I. Carter, Sr. and Beverly D. Carter, forever my heroes.

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It would be impossible to attempt to express my gratitude for all the people who have touched me during my time here. To my Bentley lab family, both past and present, know I appreciate all of you so much. It is not lost on me that I have had a chance to be a part of something special and rare, that I recognize cannot be duplicated anywhere else. I know without your support and encouragement I never would have lasted to see this thing through.

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Finally, I want to thank my family and closest friends, for their love and never ending support. I am unbelievably lucky to have such amazing people believe in me. You all wanted this so much for me, you practically willed me to succeed. My heart overflows with gratitude for all the strength you have given me. I am so fortunate to have you all in my life. This is for you.

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Chapter 1: Introduction

Research Motivation

Bacteria are one of the more investigated and understood microorganisms in the scientific community due to its single-celled structure. Consequently, the entire *Escherichia coli* (*E. coli*) genome was one of the first to be decoded and is readily accessible from a variety of sources. However, this seemingly simple organism actually hosts over 4,000 genes that contribute to an intricate network of coordinated processes. Even with all that has been elucidated about bacteria and *E. coli* in particular, in many aspects bacterial processes still remain somewhat enigmatic. Despite these challenges, researchers have successfully managed to make great strides not only in deciphering the nature of these vast networks but also employing them in a variety of technological advances, from industrial to medical. Concomitantly, we have also witnessed the emergence of new ways bacterial species have learned to adapt and improve their survival techniques in response to some of these advances.

This dilemma has probably received the most attention with regard to the medical community, as refractory microbes develop resistance to multi-spectrum antibiotics in the field of medicine. The current state of antibiotic resistance exists due to a multitude of reasons including over-prescription by doctors and wide spread use in the food industry for disease control and as growth enhancers for animals. Exposure to antibiotics creates a natural environment for variants, bacteria with traits that can withstand the attack, to flourish. Also, a bacterium that was once susceptible

to an antibiotic can gain resistance by mutations or by acquiring DNA with resistance properties from other bacteria. When these bacteria proliferate a new population of antimicrobial resistant bacteria emerges. Additionally, bacteria often live in protective sessile communities, or biofilms. The biofilm architecture is partially mediated by quorum sensing which allows bacteria to coordinate behaviors such as motility, virulence and stress responses, rendering bacteria increasingly resistant to antimicrobial agents.

Bacteria used in biotechnology resist genetic modifications to increase protein yield. Though the practice of overexpressing recombinant proteins in *E. coli* is widespread, the "metabolic burden" incurred by a host cell when producing a desired heterologous protein elicits a stress response that can result in reduced yields and degradation of the protein by cellular proteases. The RpoS protein has been at least partially implicated in all of these scenarios. RpoS leaves cells remarkably adaptive, yet resistant, to complete genetic manipulation.

The task of responding to varied and potentially stressful circumstances in a comprehensive manner falls to the *rpoS* encoded sigma subunit of the RNA polymerase, σ^S (RpoS). RpoS is responsible for protecting the cell against external stresses (e.g. nutrient limitations/starvation, temperature fluctuations, hyperosmolarity, heat, toxic chemical exposure, pH downshift, etc). Essentially σ^S plays a role in *E. coli* flexibility, working in concert with other systems, helping the cell to protect vital processes and most importantly survive. The ability to affect RpoS, by hindering or augmenting these characteristics, would allow a broader

strategy to affect metabolic pathways and approach the aforementioned problems from a more global perspective.

The persistent challenge present with working with RpoS, is also its most remarkable feature, it mutability. Studies have revealed numerous mutations in the *rpoS* gene resulting in different alleles not only among different strains but within the same strains, even strains that originated from the same parent. This is attributed to varying environmental conditions and the cell's constant trade-off between self-preservation and nutritional competence, or the SPANC balance, to maintain accurate levels of adaptation. RpoS polymorphism makes it an even more difficult target for genetic engineering. Any approach to modify RpoS behavior must take this factor into account.

Bacteria maintain an elaborate, but flexible, signaling network and adjust cellular physiology accordingly. Small noncoding RNAs are included in this complex regulatory circuitry. While initially many small RNAs (sRNAs) were discovered fortuitously, the last several years have revealed their pervasiveness in bacterial cellular regulation. Due to the nature of their mode of action many sRNAs fall into the category of riboregulators. Riboregulators provide a swift, adaptive response to diverse signals and modulate gene expression to accommodate versatile conditions. In this way, sRNAs may provide a simple way to impose global regulation of a group of genes.

The hallmark of sRNA riboregulation is rapid response to environmental cues and post-transcriptional regulation of mRNA and protein synthesis and/or stability, meaning most are synthesized under specific condition for a limited period of time.

These small RNAs essentially function as sensors responding to outside stressors and triggering a response, usually in the translation (repression or activation) of an mRNA target(s), though a few protein targets exist. In *trans*-encoded sRNAs particularly, the success of this regulation has a smaller dependence on base pairing, requiring generally ~10-25 nucleotide base pairing interactions. RpoS has been confirmed to be post-transcriptionally regulated by at least four small RNAs. The work here supports the use of small RNAs as a potential solution to genetically modulate RpoS metabolic gene expression profiles for directed downstream applications.

Literature Review

E. coli and sigma factor competition

As an enteric bacterium, *E. coli* experiences diverse conditions ranging from the mammalian gut to soil, from anaerobic to aerobic. The exposure to these ever changing surroundings requires bacterial populations to constantly switch between growth, survival, and death. To assist in the vacillation between these various states *E. coli* is equipped with a host of pleiotropic regulators of gene expression. The expression of these genes is dependent on the presence of seven sigma (σ) factors each selecting for the transcription of distinct promoters in the genome.

Transcription is the initial step in gene expression and is prompted by *E. coli* RNA polymerase core enzyme (denoted RNAP or E) binding a DNA promoter site, transferring genetic information into RNA and typically protein. The core RNA polymerase complex consists of five subunits ($\alpha_2\beta\beta'\omega$) [1]. The α subunit, encoded by RNA polymerase A or *rpoA*, is necessary for RNA polymerase core complex assembly and transcription termination or antitermination [2]. The β subunit,

encoded by *rpoB*, is involved in recruiting the sigma factors for RNAP holoenzyme assembly [3]. The β' subunit, encoded by *rpoC*, participates in promoter melting, plus stabilizes the open promoter complex [4]. The ω subunit, encoded by *rpoZ*, aids in β' folding, by preventing aggregation, and its subsequent assembly with rest of the catalytic core [5]. This subunit is key in the last stages of core enzyme assembly and has also been found to be necessary to restore denatured RNAP *in vitro* [6]. Only upon binding a sigma factor, the fifth subunit, is a complete holoenzyme formed which exhibits target promoter specificity (Figure 1) [7, 8]. A list of these seven sigma factors and their respective transcriptional functions is given in Table 1.

There are two primary sigma factors, the *rpoD* encoded σ^{70}, which is the main vegetative factor utilized for transcription during logarithmic or exponential phase [8], and σ^{S} (RpoS or σ^{38}) which is the prevalent factor during the transition from the exponential throughout the stationary phase, encoded by *rpoS* (also known as *katF*) [9, 10]. σ^{S} is also the master regulator of the general stress response [7]. The other five factors are usually only triggered by a particular stress signal(s) and hence are considered alternative, or minor, sigma factors. The alternative factors include the *rpoN* encoded σ^{54} (or σ^{N}), which is activated by nitrogen deficiency [11], the *rpoH* encoded σ^{38} (or σ^{H}), which is activated by heat shock [12, 13], the *fliA* encoded σ^{28} (or σ^{F}), which is involved in the synthesis of flagellar and chemotaxis genes [14, 15], the *rpoE* encoded σ^{24} (or σ^{E}), involved in the assembly, maintenance and repair of the cell envelope [16, 17] and *fecI* encoded σ^{19} (or σ^{FecI}), which regulates the ferric citrate transport system when iron is limiting [18]. The numbers denote the molecular mass in kilodaltons (kDa), though σ^{S} deviates from 38 kDa in some *E. coli* strains. The

seven sigma factors can be divided into two groups, the σ^{70} family, which consists of all the known sigma factors [19, 20] except σ^{54}, which constitutes the second family. σ^{54} has no homology to the other sigma factors and recognizes completely different promoter sequences [21].

Table 1 – ***E. coli*** **sigma factors and their corresponding functions**

	Function
σ^{70} (RpoD)	"housekeeping" sigma factor
σ^{S} (RpoS)	general stress/stationary phase sigma factor
σ^{54} (RpoN)	nitrogen limitation sigma factor
σ^{32} (RpoH)	heat shock sigma factor
σ^{28} (RpoF)	flagellar synthesis sigma factor
σ^{24} (RpoE)	extracytoplasmic (cell envelope) assembly and maintenance sigma factor
σ^{19} (FecI)	ferric citrate transport sigma factor

Calculations of core enzyme amounts suggest a limited amount of RNAP in the cell is actually available for transcription *in vivo* [22]. As a result, each of the seven sigma factors competes for affinity to the same amount of RNA polymerase [23, 24]. For steady-state cells growing in the logarithmic phase of growth, RNAP core enzyme amounts are estimated to be between 1,500 to 2,000 molecules per cell (in rich media) decreasing to approximately 65% of this quantity during stationary

phase [22, 25, 26]. It has been shown that a percentage of sigma factors are released from the core enzyme during elongation after every round of transcription to be exchanged with a new one [27]. Binding affinity is another determinant in sigma factor competition with σ^{70} possessing the strongest binding to the core enzyme followed by σ^{N}, σ^{F}, σ^{E}/σ^{FecI}, $\sigma^{H}$and σ^{S} with decreasing affinities [23]. This is particularly fascinating considering levels of σ^{S} increase to 30% of σ^{70} in stationary phase, while the latter and σ^{N} remain static [26, 28]. Clearly there are other dynamics contributing to the fluctuation of sigma factor binding to core polymerase depending on the environment. The emergence of unpredictable conditions is perceived by several intracellular signals that sense the nutritional quality of the environment, such as the alarmone guanosine 3'5'- bispyrophosphate (ppGpp) [29-32]. Such transduction signals elicit an enhanced affinity of a particular sigma factor for the available RNA polymerase by providing a selective advantage for that factor or allowing several sigma factors to work in concert to shift their relative competitiveness [27, 33, 34].

Being the primary factors, much of the shift between sigma factors occurs between σ^{S} and σ^{70}, occasionally working in tandem with one of the other five alternative factors. This is due to the fact that during a cell's life cycle it must constantly trade-off between growth/reproduction and maintenance/survival [35] which are predominantly regulated by the complementary roles of these two sigma factors. *In vitro*, both factors are able to transcribe many of the same genes, indicating the presence of other signals occurring *in vivo* to account for variable gene expression pattern, in addition to the alarmone ppGpp [36]. The formation of a σ^{S}-

holoenzyme is also supported by the protein Crl [37, 38]. Aside from the simple abundance of RNAP core enzyme complex there are other features that contribute to the switch between σ^S and σ^{70} selectivity [39, 40]. Another feature implicated is the promoter consensus sequences surrounding the -35 and -10 upstream regions. σ^S and σ^{70} have different preferences for the nucleotide at position -13. Cytosine in this position is highly conserved for σ^S promoters and directly interacts with K173 residue found in σ^S. σ^{70} possess glutamate in this position creating a preference for guanine at -13 [41]. The superhelicity of the DNA template is significant as σ^{70} shows high efficiency transcription of supercoiled DNA while σ^S transcribes more relaxed DNA [42] . Rsd is a protein that interacts with σ^{70} inhibiting transcription of its dependent genes and is therefore referred to as an anti-sigma factor [43, 44]. The 6S RNA operates similarly to Rsd by interacting with σ^{70}-holoenzyme reducing its activity and promoting the utilization of σ^S [45] and increased stress resistance and survival [46]. Other aspects include the fact that σ^S tolerates degenerate promoter sequences and spacer lengths, as well as, additional regulatory RNAs and proteins.

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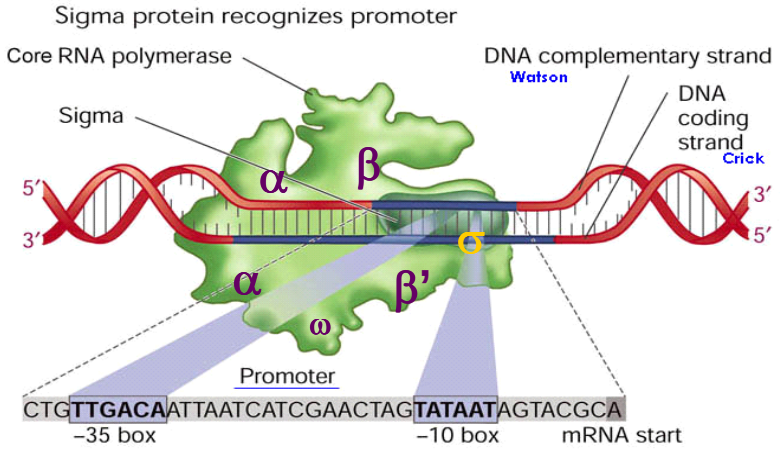

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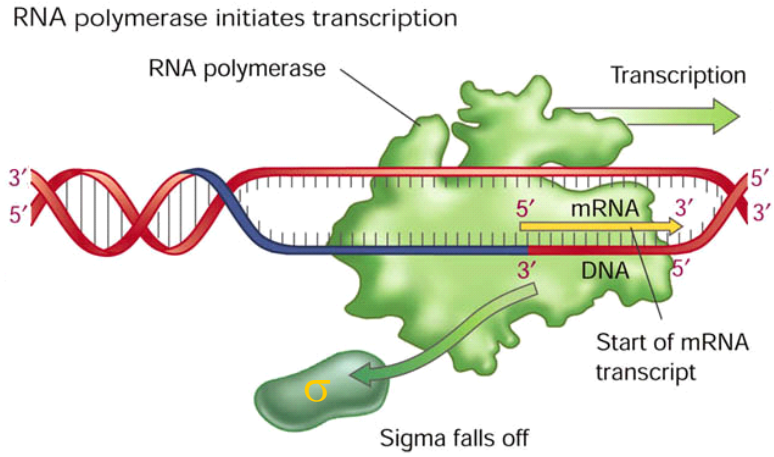

Figure 1 – The RNA polymerase holoenzyme – A) The sigma factor directs the RNAP holoenzyme to specific promoters. B) The holoenzyme then binds to the promoter and mRNA transcription is initiated. After elongation begins, the sigma factor falls off to bind to new core enzyme. (http://www.bio.miami.edu/~cmallery/150/gene/mol_gen.htm)

RpoS- stationary phase and the general stress response

Cells experience proliferation and reproduction during the exponential phase in a nutrient rich, stress free environment that supports growth. When nutrients become exhausted or when concentrations of toxic waste products becomes too high, cells cease reproducing and growing and enter into the stationary phase. In stationary phase, cell resources divide and some shift from proliferation and production to survival and maintenance, also the characteristic of a stress response. This phase does not specifically describe a fixed physiological state and is reversible if the stress can be combated or nutrient levels replenished [47].

In *E. coli* the general stress response is under the control of the master regulator, σ^S (RpoS) which renders cells broadly resistant [9, 48]. RpoS induction is a response cells transiently resort to by inducing a more economical system during times of perceived stress or in anticipation of stress. This may explain why during exponential phase undetectable amounts of σ^S are present in the cell, though there an abundant amount of *rpoS* mRNA is present, whose transcriptional induction starts in mid-exponential phase. When stationary phase is reached, the cells are experiencing some of the same characteristics that designate a stress response (e.g. nutrient limitation) and increased *rpoS* mRNA translation ensues [49]. This presence of σ^S can be further augmented by the simultaneous stabilization of σ^S already available in the cell. Upon entry into the stationary phase the amount of σ^S is about 30% the amount of σ^{70} [26]. As many as 500 genes have been shown to be controlled by RpoS, in exponential phase, stationary phase or both, include genes required for DNA repair, shifts in nutrient composition, osmotic shock, chemotaxis and flagellum

biosynthesis, cell structure, energy metabolism, amino acid biosynthesis and metabolism, enzymes involved in the TCA cycle, transport proteins and virulence factors in pathogenicity [7, 47, 50-55].

RpoS is intricately regulated on all levels, mRNA synthesis, stability and translation and proteolysis, though most occurs post-transcriptionally [48, 56]. At the post-transcriptional level, RpoS mRNA is controlled by at least four small noncoding RNAs and several proteins (e.g. LeuO, HF-I, and HU) which target its secondary structure [57, 58]. Protein stability is controlled by the ClpXP protease, which is recruited by the response regulator, RssB and the chaperonin, DnaK [59, 60]. Phosphorylated RssB acts as a proteolytic recognition factor directly interacting with RpoS, delivering it to the ClpXP protease degradation complex which recognizes 20 amino acids between residues 170 and 190. No other protein factor is required for RpoS degradation [61]. During exponential growth at 37^{o}C, RpoS is extremely unstable with a half life of less than two minutes. Entry into the stationary phase increases the half life to 30 minutes. RpoS mutants show decreased survival during stasis and stress [62, 63]. Though there is a divergence in genomes across species, the RpoS regulon still has similar functions in many proteobacteria [64].

Small non-coding RNAs

Environmental cues are sensed and responded to by untranslated specific small RNAs (sRNA) resulting in changes in both mRNA and protein synthesis and stability [65] . These sRNAs do not encode for proteins and function solely as regulatory RNAs [66]. Sizes for small RNAs vary dramatically, generally between 50-250 nucleotides (nt) though some as long as 500 nt have been identified. These

RNAs differ in the number of secondary structures that can be achieved, as well. Many of the earliest known sRNAs were discovered unexpectedly by analysis of RNA transcripts observed during studies of *E. coli* promoter regions and mutations of known neighboring genes, identified by their association with proteins of interest or resulting phenotypes of multicopy plasmids. Until the beginning of this decade only 13 sRNAs had actually been characterized [67]. Since then, systematic computational, microarray and cloning based screens have been performed based on criteria deduced from the previously characterized sRNAs including conserved intergenic regions, rho-independent terminators and co-immunoprecipitation with Hfq [68, 69], an RNA chaperone protein. Noncoding RNA regulation has been found to extend to several species including eukaryotic microRNAs (miRNAs) and small interfering RNAs (siRNAs), however, the term small RNA is usually reserved for small bacterial noncoding RNA.

While a couple noncoding RNAs are essential to *E. coli* serving in structural or quality control capacities, most are regulators synthesized under specific conditions to execute a specific action for a limited time period. Such sRNAs either bind a protein, altering its activity, or a target mRNA, functioning as an antisense regulator and affecting the translation or stability of that target. *Cis*-acting sRNAs are encoded on the opposite strand of their targets and exhibit complete complementarity to this one target transcript. Most of these sRNAs are expressed from a plasmid and control cell copy number [70]. Conversely, *trans*-acting sRNAs, which constitute the majority of sRNAs, are encoded at other loci in the genome, are only partially complementary to their targets and often have multiple targets. A few are actually

encoded adjacent to the gene that regulates their transcription. Most of this regulation is negative, resulting in repression by inhibiting mRNA translation, degradation by RNase E/RNase III, or both, requiring binding to the Hfq protein to facilitate the interaction with the mRNA target(s) [71]. This is accomplished through binding the 5'-untranslated region (UTR) region, sequestering the ribosomal binding site (RBS) or base pairing within the first five codons of the coding region, preventing ribosomal binding or increasing stability of its target [72, 73]. There is only one sRNA known to bind the 3'-UTR of its target, GadY [74]. Even more interesting is the fact that though base pairing only needs to occur between a small group of nucleotides (about 10-25), an even smaller number of these pairings are critical for effective regulation [75, 76].

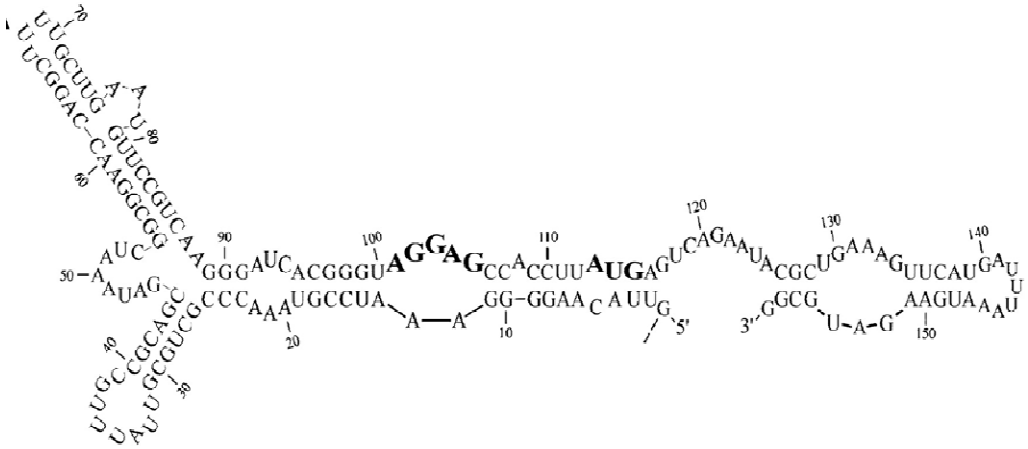

Figure 2 – The predicted secondary mRNA structure of RpoS. The extended 5'-UTR region of the *rpoS* mRNA forms a self inhibitory hairpin loop that obstructs the ribosomal binding site (bold AGGAG) and start codon (bold AUG) preventing translation.

Hfq

Approximately 30 sRNAs in *E. coli* operate with Hfq at the interface between them and their specific target(s) [77]. Initially Hfq protein was identified as a bacterial host factor for RNA phage Qβ replication but was later found to be a requirement for the function of several sRNAs [78-80]. This protein is also denoted host factor I (HF-I). Hfq is a conserved bacterial homologue protein much like Sm and Sm-like proteins in eukaryotic systems [81]. It tightly binds RNA but does not have a precise target sequence, though it appears to bind unstructured AU- rich sequences. This AU-rich element was recently confirmed to be necessary [82-85]. Estimations of the quantity of Hfq proteins have been calculated to be between 30,000-60,000 molecules per cell [86]. Many cellular processes are affected by Hfq including stimulation of RNA-RNA association and RNA binding and stabilization of sRNAs [85]. Recent studies have suggested that Hfq may stimulate the binding of both RNAs simultaneously in RNA-RNA interactions [87]. Hfq interacts with many small RNA species exhibiting chaperone activity, controlling regulation and stabilizing sRNA transcripts by protection from RNase E cleavage [77, 88]. Binding to Hfq by sRNAs occurs 1:1 stoichiometrically [89]. Hfq is only required to facilitate the formation of the sRNA-mRNA complex and has been predicted to cycle by transiently associating with several competitors at the same time, where one sRNA eventually displaces the other causing it to dissociate, effectively changing sites [90]. This interaction may also be influenced by salt concentrations [91]. Cycling allows Hfq to interact with all of its potential targets, however, other studies suggest that Hfq is limiting, resulting in its sequestration by some RNA transcripts and unavailability

for activity by others [92]. Dynamic competition supports better alignment of Hfq and its target RNA and consequent annealing efficiency [93]. X-ray crystallography has confirmed the structures and two binding surfaces of Hfq in *E. coli* [79, 94], *Staphylococcus aureus* and *Pseudomonas aeroginosa*.

RpoS and small RNAs

The secondary structure of *rpoS* mRNA, together with small regulatory RNAs, control translation under different stress conditions. The inhibitory secondary structure of *rpoS* mRNA is comprised of a hairpin created by an extended 5'-untranslated region which occludes the ribosomal binding site and prevents translation. The predicted Mfold secondary structure as reported by Worhunsky and colleagues is shown in Figure 2 [95]. To date there are at least four small RNAs that have been shown to interfere with this inhibitory leader region disrupting its regulatory structure: three activators, DsrA, RprA, and ArcZ and one repressor OxyS [85, 96]. Low temperature and cell surface stress stimulate RpoS translation respectively mediated by DsrA and RprA. Very recently, ArcZ was shown to play a role sensing aerobic and anaerobic conditions. While these three sRNAs sense different environmental conditions, they still trigger RpoS translation in the same way (Figure 3). In response to oxidative shock, OxyS represses RpoS translation. There is mounting evidence that there exist several more sRNAs that interact with RpoS [68, 85]. These sRNAs are all members of the trans-acting group of sRNAs with an Hfq requirement, though DsrA establishes comparable tight binding to the RpoS header region even in an *hfq* mutant [85]. Currently, RpoS is the only known case with four identified sRNAs for post-transcriptional regulation, though analogous

cases may exist, reinforcing the idea of its role as an intracellular adaptor for many microorganisms. The RpoS paradigm is thus far the best illustration of the intricacies of sRNA translational control.

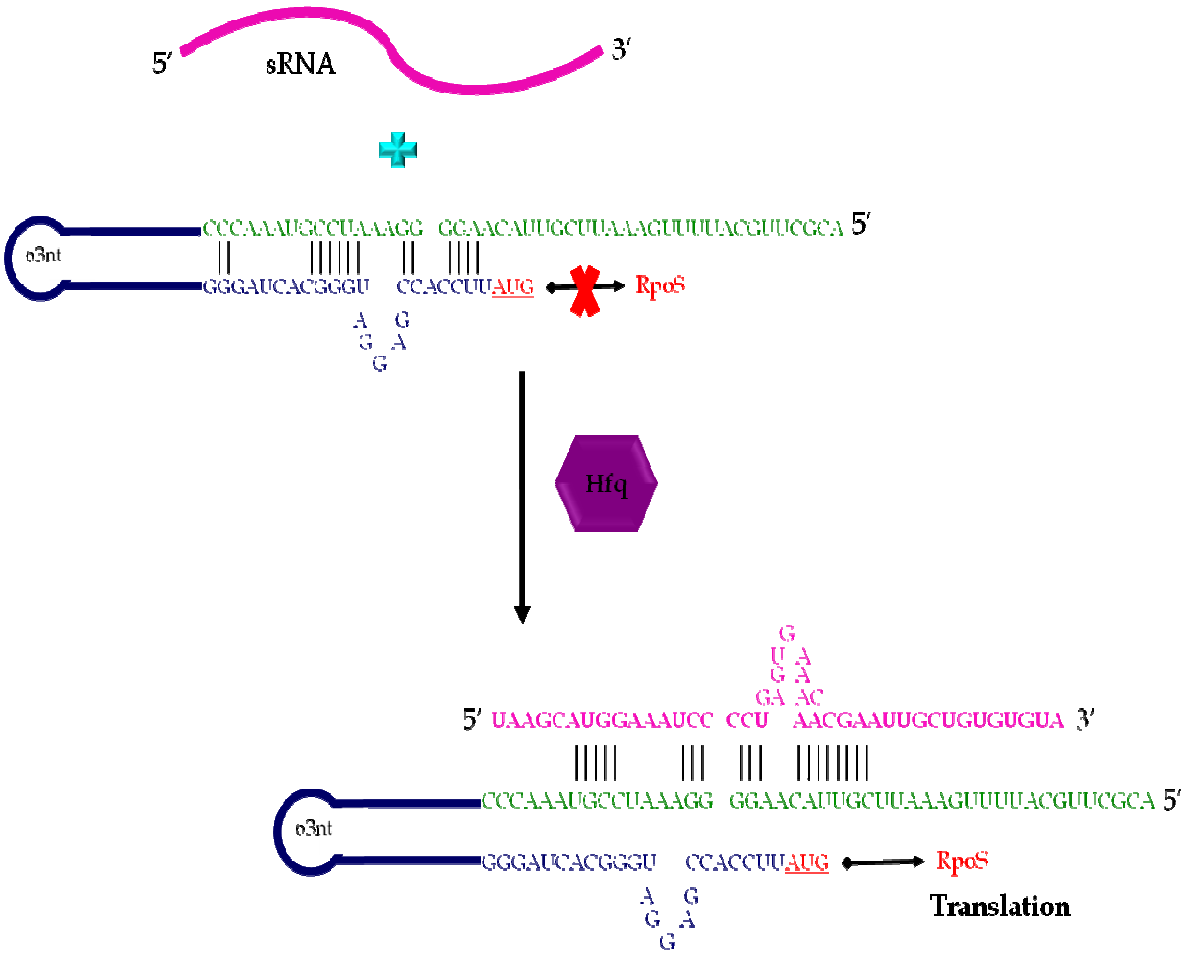

Figure 3– RpoS mode of activation by sRNAs. The inhibitory structure of RpoS is relieved by the interaction of the 5'-UTR leader region with an activating sRNA and Hfq resulting in translation. The region complementary to activating sRNAs is shown in green. The *rpoS* start codon is shown in red.

DsrA

One of the first small untranslated RNAs, DsrA (downstream region A), an 87 nucleotide sRNA, was discovered downstream of the capsular polysaccharide controlling gene, *rcsA* [97]. DsrA was later found to be essential for stimulating *rpoS* mRNA translation at low temperature (30^{o}C and below) during the exponential phase of growth [98]. Synthesis of DsrA is under temperature control. Temperature affects both the synthesis and stability of DsrA, leading to thermoregulation of *rpoS* translation [99]. Transcription profiling using DNA arrays revealed multiple acid resistance genes when DsrA is overexpressed [100]. DsrA also has a second target, overcoming H-NS mediated transcriptional silencing of genes, including *rcsA* which positively regulates capsule synthesis by negatively affecting transcription. These activities are independent with the *rpoS* complementary region residing in the first stem loop of the secondary structure and the *hns* complementary section is located in the second stem loop. The third predicted stem loop is the transcription terminator of *dsrA* (Figure 4A) [101-103]. The predicted points of base pairing with *rpoS,* as depicted by Mandin and colleagues, are shown in Figure 5B [104].

As an early detected sRNA, DsrA is also one of the most studied and has largely been the sRNA prototype for studying the interplay between small RNAs and their molecular mechanism. At least two functions for Hfq in the process of DsrA-mediated regulation of RpoS mRNA have been proposed. One role appears to be to induce the conformational changes needed by DsrA to interact with the *rpoS* mRNA header region, while the second suggests it accelerates DsrA annealing to the same region [85, 105]. DsrA is also reported to stabilize *rpoS* transcripts, which are

rapidly degraded in its absence, by redirecting RNase III cleavage [106]. There are conflicting viewpoints on whether DsrA interacts with ribosomal protein S1, which binds to poly-U stretches and may enhance translation initiation [107].

RprA

RprA, RpoS regulating, RNA is a 105 nucleotide untranslated RNA uncovered in a screening for potential suppressors of *dsrA* mutants. Like DsrA, RprA alters the secondary structure in the *rpoS* mRNA leader sequence activating RpoS translation in response to osmolarity and cell envelope stress [108]. RprA does not possess all the same points of complementarity as those involved in DsrA couplings, however, both predicted structures have pairing requirements within the same area of the *rpoS* mRNA regulatory region (Figure 5C) [109]. Expression of RprA is activated via the RcsC/YojN/RcsB phosphorelay system and specifically by RcsB [110]. The phosphorelay is activated by solid surfaces and regulates genes associated with the cell membrane or cell surface, many which are speculated to participate in biofilm formation [111]. RprA levels increase in stationary phase but this increase is RpoS-independent [109]. Other functions for RprA are still being investigated.

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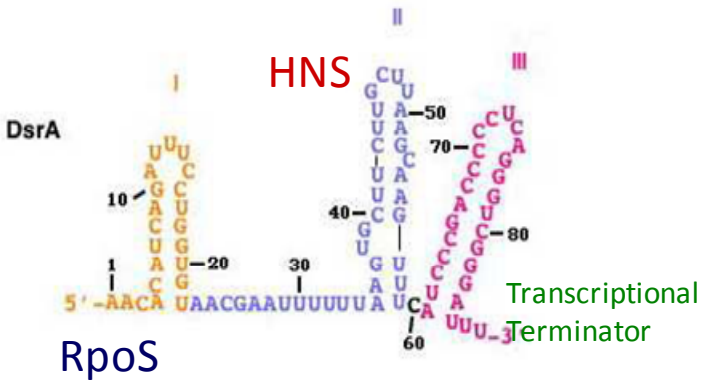

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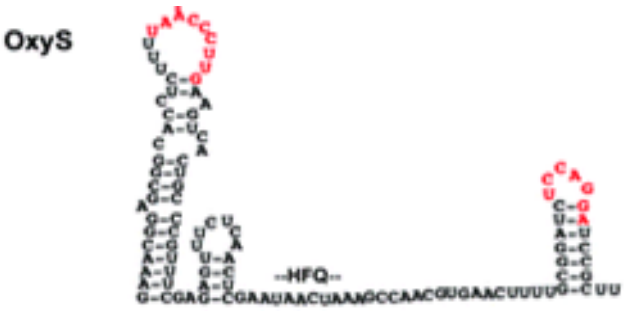

Figure 4 – The predicted secondary mRNA structures of DsrA and OxyS. A) DsrA has three stem loops. The first loop interacts with RpoS, the second loop interacts with the global transcription regulator HNS, and the third loop is the transcription terminator. B) OxyS has three stem loops binding Hfq between loops two and three. The exact mechanism of target interaction is still unknown.

ArcZ

ArcZ, formerly identified as RyhA and SraH, was identified in two separate genomic searches for small RNAs in *E. coli* [68]. The name was changed to ArcZ because it was found to be encoded concurrently with *arcB* which is involved in the growth transition from aerobic to anaerobic in *E. coli* [104] . It is well conserved and can be processed to a truncated form [112]. The interaction of its secondary mRNA structure with the RpoS is shown in Figure 5A. In *Salmonella enterica*, ArcZ was

shown to affect many genes, including a methyl accepting chemotaxis protein STM3216 and a serine uptake gene, *sdaCB [113]*.

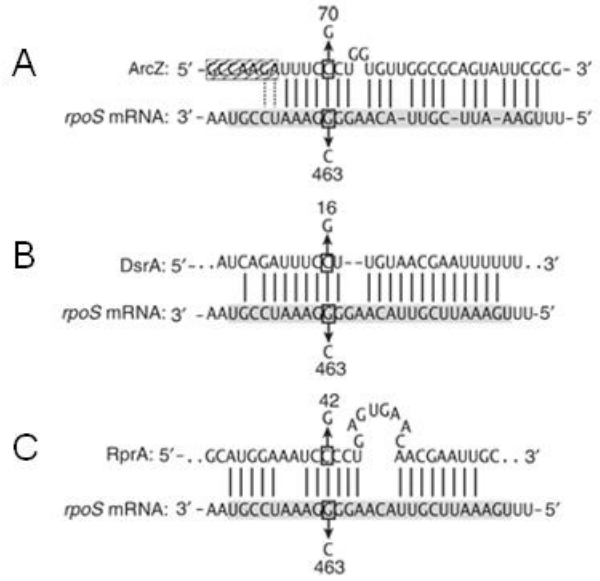

Figure 5– Pairing of activating sRNAs to RpoS regulatory hairpin structure. A) ArcZ B) DsrA and C) RprA.

Oxys

OxyS, or oxidative stress, RNA is a109 nucleotide untranslated RNA transcriptionally regulated by OxyR in response to oxidative stress. OxyS behaves as a global regulator both repressing and activating the expression of multiple genes, as well as, an antimutator protecting cells against DNA damage [116]. Translation of two target genes is repressed via OxyS regulation: *fhlA* and *rpoS*. FhlA activates formate hydrogenase synthesis in the presence of formate which can lead to H_2O_2 - induced damage in the company of metal cofactors. OxyS also forms a predicted three loop secondary structure (Figure 4B). While OxyS RNA directly binds to *fhlA* mRNA

inhibiting translation by interfering with ribosomal binding in two places [114], the mechanism of OxyS in the case of RpoS inhibition is still remains unclear [115]. However, the role of OxyS has been proposed to be indirect by heavily binding Hfq, occupying the protein so that is unavailable to act on *rpoS* mRNA [116]. This notion is further supported by suggesting this mode of action reduces the redundancy of RpoS- induced oxidative genes when the OxyR regulon is induced and able to handle the stress.

Riboregulators

When a gene transcript binds to target RNA in such a way as to promote the blocking or activation of ribosomes affecting translation to acclimatize to environmental fluctuations, this gene is considered a riboregulator. Small RNAs rapidly respond to response to diverse signals and modulate gene expression to accommodate versatile conditions qualifying them as riboregulators [102, 117, 118]. This newly realized critical role for sRNAs in mediating gene expression has spurred a large effort to engineer this activity *in vivo*.

Small RNAs make attractive engineering targets due to the simplicity of base pairing interactions and the wide variety of secondary structures possible [117, 119, 120]. The concept of a modular engineered riboregulator system was successfully demonstrated by Isaacs *et al*. by applying knowledge of natural small RNA regulators and their target mRNAs [121]. The engineered regulators used an independently transcribed trans-activating small RNA that was able to bind a *cis*-repressing sequence in the 5' untranslated region of the regulated gene. The design of this system also considered the YUNR [Y denotes a pyrimidine base (U or C), U denotes

a uracil base, N denotes any base, and R denotes a purine base (A or G)] motif of the loop sequence that has been shown to be important in the interactions of RNA in natural antisense systems [122]. This example and many others like it often engineer sequences inserted into the 5'UTR that promote the formation of aptamers for ligand binding or hammerhead ribozymes to create riboswitches. While the use of antisense RNA has also become widespread [123], the use of engineering *trans*-acting switches is still relatively new idea. This is largely due to the uncertainty of the actual signal transduction cascade of these RNA biosensors and their mode of action. Use of such systems is also limited by the fact that while hundreds of trans-encoded small RNAs have been revealed still only a handful has identified targets.

Advantages of using sRNA riboregulation

While there have been several successful investigations on studying the effects of various controls on every level of RpoS regulation, sRNA offers some unique advantages to these other methods including but not limited to the following:

- sRNAs expend less cellular energy to produce than proteins because they do not require translational resources
- the small size of most sRNAs allows a rapid response to environmental stimuli
- sRNAs are surprisingly stable and persist long enough to interact with their target transcripts
- sRNAs that target translation provide a simple way to impose global regulation on an operon

- multiple sRNAs made under different conditions can regulate a single target
- sRNA regulation removes the uncertainty of using base pairing techniques in conditions that spur gene mutations or when targeting genes which tend to be polymorphic in nature (e.g. RpoS)

Project Objectives

Traditionally, metabolic engineering targets proteins or enzymes for modification to affect cellular regulation. Due to its polymorphic nature this approach would be limiting for controlling RpoS gene expression profiles due to the uncertainty of the nature of the particular RpoS gene that may be present in the system. The obvious advantage to using small RNA riboregulation is that the RpoS genetic profile can potentially always be influenced regardless of genetic makeup.

RpoS regulation embodies a prime example of the potential magnitude of the extent of sRNA modulation. In this instance at least four such sRNA riboregulators are involved. DsrA, RprA, ArcZ and OxyS are able to sense stressors in the environment resulting in an increase/decrease in RpoS translation and RpoS-dependent genes to enhance cellular fitness and survival. This study attempted to gain a better understanding of the role of small RNAs serving as riboregulators in the control of σ^S function on *E. coli* cellular behavior. Overexpression of small RNAs is typically used to investigate its function. We attempted to create an inducible small RNA expression system that can be optimized to affect specific phenotypic

alterations in a desired *E. coli* strain by varying the amounts of inducer. We then examined the feasibility of exploiting the properties of these small RNAs in an interchangeable inducible riboregulatory platform, where different sRNAs can be used to influence diverse gene expression profiles in several strains of *E. coli*. This concept was explored through the analyses of both immediate gene transcription modulation and downstream phenotypic effects of the riboregulation of *Escherichia coli* σ^S, or RpoS.

Globally, we hypothesize that the modulation of activation or repression of *rpoS* mRNA translation on a molecular level via small RNA riboregulators will permit the effective exploitation of the desired characteristics of the cellular phenotypes conferred by σ^S. More specifically our research sought to acquire a better understanding of the prospective of engineered small RNA riboregulation through the following objectives:

1) Create an arabinose inducible promoter system for the RpoS activator, RprA, and repressor, OxyS, which effectively overexpresses the specific sRNA and increases/attenuates RpoS levels in various *Escherichia coli* strains.
2) Analyze whether the effect of positive modulation of RpoS translation in exponential phase results in a subsequent transcriptional up- or downregulation of confirmed RpoS-dependent genes, potentially increasing the overall stress resistance of various *Escherichia coli* cell strains.

3) Examine if the positive/negative modulation of RpoS provides a selective advantage to σ^{70}-dependent genes which support growth by means of either cross-protection or loss of some RpoS function.
4) Examine if the positive/negative modulation of RpoS results in an ensuing decrease in σ^{S} -dependent genes and a diminished capacity for stress protection, leaving cells less equipped to adapt and survive, hampering or promoting the cells ability to adopt protective states.
5) Investigate the downstream phenotypic results of the aforementioned effects on differences in motility, biofilm formation and heterologous recombinant protein yields.

Chapter 2: Characterization of RpoS Genetic Profiles and Regulatory Effects as a Consequence of Overexpression from RprA Activation and OxyS Repression Platforms

Introduction

The ability to isolate microbes has undoubtedly allowed scientists to effectively characterize microbial behavior for decades. The additional challenge has often been to interpret these findings in relation to the larger environmental architecture. This simply translates to the fact that microbes exist in heterogeneous populations and surroundings that obviously influence their function. The ability to respond to dynamic surroundings renders bacteria remarkably adaptive and resilient. *Escherichia coli* are able to thrive and subsist in a multitude of environments by altering gene expression. This shifting environment is monitored by the cell which requires constant competition among the seven sigma factors for limited amounts of core RNA polymerase for transcription [22, 23].

RpoS is the master regulator of the general stress response [9, 48] and involved in the regulation of hundreds of genes throughout the cell cycle required for DNA damage, shifts in nutrient composition, osmotic shock, chemotaxis and flagellum biosynthesis, cell structure, energy metabolism, amino acid biosynthesis and metabolism, enzymes involved in the tricarboxylic acid cycle (TCA) cycle, transport proteins and virulence factors in pathogenicity [7, 47, 50-55]. Considering the vital role of RpoS in cell survival, the ability to modulate it, by hindering or enhancing its characteristics would allow a broader strategy to affect metabolic

pathways. This could have extensive implications for global coordination of gene expression profiles and cellular behavior.

Although RpoS plays an important role in regulating global gene expression for survival and adaptation to a wide range of stresses, many widely used E. coli laboratory strains carry point mutations and deletions within the *rpoS* gene resulting in various levels of RpoS activity [124]. This can occur among stocks of different strains in different laboratories or between various strains of the same lineage because stored bacterial can be heterogeneous, contributing to strain variation particularly in RpoS function. There are a number of sequence differences even in the three "wild-type" K-12 strains. RpoS also regulates many of the genes responsible for maintaining the fidelity of gene transcription, for example *mutS* and *dinB* (DNA Polymerase IV). *mutS* is responsible for repairing DNA mismatches and *dinB* both generates spontaneous mutations at a higher rate than other replicative polymerases and plays a role in adaptive mutations [125-130]. Such genes make it possible for the cell to produce variants that survive. One of the most common mutations is in codon 33, which results in an amber stop codon (CAG goes to TAG) and premature termination of RpoS synthesis [131, 132]. It has been demonstrated that a secondary translational initiation region (STIR) exists in the *rpoS* gene that still confers a survival advantage by allowing translation of truncated forms [133]. This phenomenon has been explained by the fact that bacteria consistently try to balance between self-protection and nutritional capability, the SPANC balance, for an advantage in a given environmental condition, including selective loss of RpoS function [134]. Still this polymorphic nature of *rpoS*, resulting in possibly unknown

allelic variations, provides some challenges if scientists are to be able to exhibit an element of control over its regulation. However, it is possible that targeting this gene for modulation by sRNAs may be a solution.

Traditionally, genetically engineering factors to influence the control of metabolic pathways has involved overexpression or alteration of proteins that confer the desired phenotype [135]. A majority of cellular engineering strategies using RNA have been in eukaryotic systems, but more bacterial systems have surfaced. RNA participates in the modulation of almost every aspect of cell metabolism through a wide range of regulatory functions, as well as, serves as a biosensor to facilitate the direction of bacterial gene expression. This fact, coupled with its modularity, simple mode of action and diversity of structure and function, makes RNA a particularly attractive tool for genetic engineering design [117, 136]. Thus far one of the most popular employments of genetically engineered RNA in bacteria has come in the form of antisense technology. Antisense RNA has actually been effective in downregulating RpoS itself [123, 137, 138]. Antisense agents can both downregulate targets without disrupting the genome and transiently inhibit genes that are lethal as knockouts. Other systems use riboswitches comprised of ribozymes and ligand-aptamer couplings which have been used successfully for many types of regulation from transcription termination and translation initiation to splicing in eukaryotes [139] with great specificity. These systems are usually confined to controlling gene expression via a single mechanism. *Trans*-encode sRNAs can not only target global regulators, such as RpoS, but are themselves potentially considered

global regulators. We envision the use of these biosensors as a type of switch to be used to control RpoS regulation.

This research attempts to circumvent the uncertainty associated with the requirement of a precursor stimulus by directly exciting RpoS translation with sRNAs overexpressed from an inducible platform. We have engineered both an activator, RprA, and a repressor, OxyS, to be expressed from a variable arabinose induced promoter (Figure 6). This engineered platform will not only serve to examine the changed gene expression profiles but to attempt to observe the direct alteration of desired phenotypes that can be conferred by RpoS.

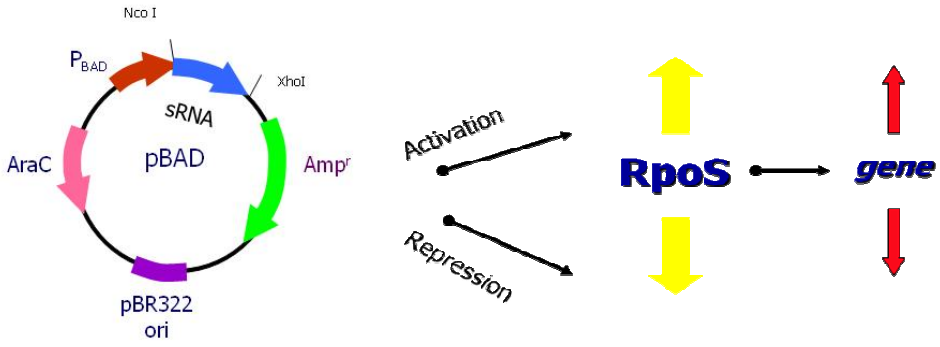

Figure 6 – sRNA riboregulatory platform. Arabinose induced overexpression of different sRNAs from a plasmid will interact with RpoS, either activating or repressing RpoS protein levels, and affecting downstream genes that are regulated by RpoS.

Materials and Methods

Bacterial Strains and growth conditions

Bacterial strains used are included in Table 2. Overnight cell cultures were inoculated in 100 mL of Luria-Bertani broth (LB) to an OD_{600} 0.01 in a 250 mL flask. All 100 mL experimental cultures were prepared from one larger inoculated OD_{600} 0.01 culture volume. Cultures were grown at 37^{o}C with 250 rpm shaking to an OD_{600}

0.3 at which point culture volumes equivalent to1 mL at an OD_{600} 2 were collected for protein samples and two 2 mL samples were collected for total RNA. Cultures were then induced for RprA sRNA transcription with the appropriate concentration of L-arabinose (0-2%). RNA samples were collected 15 minutes post-induction and both RNA and protein samples were collected at 30 minutes, one and three hours post-induction. Cultures were supplemented with ampicillin (50 μg/μL) when needed.

Table 2 – Bacterial strains used in this study

Strain	Genotype	Source
Top10	F–, *mcr*A, Δ(*mrr-hsd*RMS-*mcr*BC), φ80*lac*ZΔM15, Δ*lac*X74, *deo*R, *rec*A1, *ara*D139, Δ(*ara*, *leu*)7697, *gal*U, *gal*K, *rps*L(strr), *end*A1, *nup*G	Invitrogen
MG1655	F^- λ^- ilvG- rfb-50 rph-1	ATCC
W3110	F^- λ^- rph-1 INV(rrnD, rrnE)	Laboratory stock
MC4100	F^- *araD139*Δ(*argF-lac*)*U169 rspL150 relA1 flbB5301 fruA25 deoC1 ptsF25*	UMCP Molecular Biology Department laboratory stock

Small RNA and RpoS plasmid construction

The construction of pRprA was as follows. The *rprA* gene was cloned from K-12 genomic DNA with NcoI and HindIII restriction enzyme sites using the following PCR primers: RprA_For (5'- TCG CCC ATG GAC GGT TAT AAA TCA AC- 3') and RprA_Rev (5'- ACT TAA GCT TAA AAA AAG CCC ATC GT- 3'). This product was then purified, digested with the appropriate enzymes and ligated into the pBADHisA vector (Invitrogen) in the corresponding sites, removing the N-terminal polyhistidine (6xHis) tag, immediately downstream of the *araBAD* promoter. The pOxyS vector was created the same was as pRprA with the following

primers: OxyS_For (5'- ATA CCA TGG AAA CGG AGC GGC AC- 3') and OxyS_Rev (5'- TAA TAA GCT TAG CGG ATC CTG GAG A - 3'). pRpoS was constructed similarly except the *rpoS* gene was placed immediately after the polyhistidine tag by replacing the NcoI restriction enzyme site with NdeI using the following primer set: RpoS_For (5'- ACC GCT AGC ATG AGT CAG AAT ACG - 3') and RpoS_Rev (5'- ATT AAG CTT TTC ACG GGT GAG GCC - 3') (Figure 7). All primers were from Integrated DNA Technologies (Coralville, IA). DNA sequencing of all constructs was performed to verify integrity at the DNA core facility of the Institute for Bioscience and Biotechnology Research (University of Maryland, College Park).

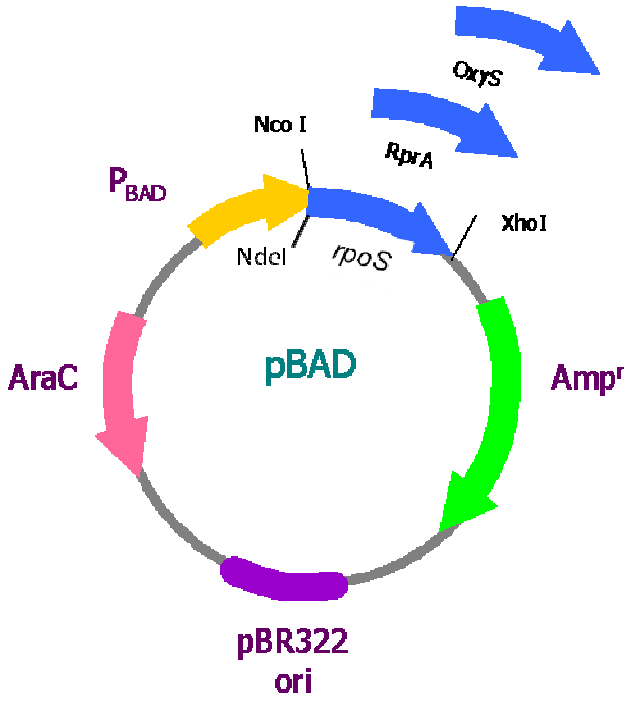

Figure 7 – pRprA, pRpoS and pOxyS expression vectors. The small RNA genes, *rprA* and *oxyS* were cloned between the NcoI and HindII restriction enzyme sites, directly after the arabinose induced pBAD promoter. The *rpoS* gene was cloned after the pBAD promoter and a polyhistidine tag between the NdeI and HindIII restriction enzyme sites.

RNA isolation, cDNA cloning and semi-quantitative RT-PCR

A 2 mL sample was collected at the indicated times and spun down at 10 x g for five minutes. The pellets were resuspended in Trizol Reagent (Invitrogen) and frozen at -20^{o}C until needed. Total RNA was isolated per the manufacturer's instructions and the RNA pellet was resuspended in 50 μL of RNase/DNase free water (VWR). RNA concentration was quantified using a NanoDrop Spectrophotometer (ThermoScientific). Samples were treated with DNase I, Amplification Grade (Invitrogen). First strand templates of each target gene were synthesized from 500 ng of total mRNA using random hexamer primers and the Superscript RT III kit (Invitrogen). PCR for all genes was performed for 27 cycles using Accuprime *Taq* Polymerase (Invitrogen). 16S was used as a control for all samples. PCR products were visualized on 1% agarose gels and analyzed using the AlphaImager®HP System and accompanying software from AlphaInnotech (Santa Clara, CA). Primers for all of the genes tested are listed in Table 3.

SDS-PAGE and Western blotting

Whole cell pellet samples equivalent to 1mL at OD_{600} 2 were collected for all cultures to be compared at the indicated time points, spun down at 10,000 x g for five minutes and stored at -20^{o}C until needed. The pellets were thawed on ice and resuspended in SDS-PAGE sample loading buffer (0.5 M Tris-HCL (pH 6.8), 10% glycerol, 5% sodium dodecyl sulfate (SDS), 5% β-mercaptoethanol, and 0.25% bromophenol blue). The samples were heated to 100^{o}C for five minutes and vortexed. Ten microliter samples were loaded into a 12.5% polyacrylamide gel and run by electrophoresis at 130V for one and a half hours using BioRad Mini Protean 3

system. Gels were then incubated for 30 minutes in Bjerrum and Schafer-Nielsen (BSN) buffer and transferred to nitrocellulose membranes using a Trans-Blot SD Semi-Dry transfer cell (BioRad) for one hour at 100 mA and then at 20 V for 15 minutes. RpoS protein level was detected by 1:1000 dilution of a monoclonal σ^S primary antibody (NeoClone Biotechnology, Madison, WI). Goat-anti-mouse was diluted 1:5000 for the secondary antibody (Sigma-Aldrich, St. Louis, MO). The blots were developed colorometrically for ~30 minutes and the band intensity levels were analyzed by Image J Software (NIH).

Table 3 – Oligonucleotides used for semi-quantitative RT-PCR

Name	Sequence (5' to 3')
16S_For	AGC GCA ACC CTT ATC CTT TGT TGC
16S_Rev	TCG CGA GGT CGC TTC TCT TTG TAT
bolA_For	AAG CTA TCG TCA CAA TGT CCC AGC
bolA_Rev	TAA GTA TGC AGA GCC AGC GCA TGA
flhD_For	CAT TCA GCA AGC GTG TTG AGA GCA
flhD_Rev	CAT TCA GCA AGC GTG TTG AGA GCA
rssB_For	TAA TTC GCG CGT TGA GGA AGA GGA
rssB_Rev	CCG AAA GTG CGG CAA TAT CAA GCA
ibpA_For	CAT TGC TAT CGC TGT GGC TGG TTT
ibpA_Rev	ACC AGG TTA GCA CCA CGA ACA TGA
pgaB_For	TCG TGA AGC ACA TCG AGG AGG AAA
pgaB_Rev	TGA AGA ATT GGG AAG ACG CGG GTA
rpoD_For	AAA TAC ACC AAC CGT GGC TTG CAG
rpoD_Rev	AGG TTG CGT AGG TGG AGA ACT TGT
ycdT_For	GAA CAT TGC ACA TCG CGA TCC CTT
ycdT_Rev	TTT ATC ACC TGA TCG CCA ACC GGA
Lon_For	CCA AAC TGT GTC GCA AAG CGG TTA
Lon_Rev	TTA TCC GCG CGA CCA TAG TCG AAA

DNA microarray

Samples equivalent to 1mL at OD_{600} 1 were collected for all cultures. Total RNA was isolated from the cultures using an RNeasy Mini kit (QIAGEN, Inc., Valencia, CA) according to the manufacturer's instructions. RNAprotect bacteria reagent (QIAGEN, Inc., Valencia, CA) was immediately added to the cultures to stabilize RNA before isolation. The RNase-free DNase set (QIAGEN, Inc., Valencia, CA) was used for on-column DNase digestion to remove residual DNA. Gene expression was analyzed using a 4x72K (4 x 72,000 probes) NimbleGen array (NimbleGen, MadisonWI). cDNA was created from 10 μg total RNA using the SuperScript Double-Stranded cDNA synthesis Kit (Invitrogen). The cDNA was labeled using NimbleGen One-Color DNA Labeling Kit and hybridized to an array using the NimbleGen hybridization system. The array was then washed, dried and scanned. Data was extracted from the scanned image and analyzed for gene expression levels.

Results and Discussion

pRprA overexpression of rprA transcript levels and corresponding RpoS protein levels

The *rprA* gene was put under the arabinose inducible *araBAD* promoter. This vector is capable of inducing the gene of interest over a wide range of inducer concentrations [140]. We were interested in adding another layer of control by potentially varying the amount of transcript produced. Most chemical inducer systems have an all or nothing level of induction due to initial limitations of transporters. We wanted to determine if the levels of RprA RNA could at least be

slightly varied. Cells were grown at 37^{o}C in LB broth with ampicillin when necessary. At OD_{600}0.3 cultures were induced with the indicated final arabinose concentrations. Samples were taken and total RNA was isolated. Cultures were tested and their corresponding RprA RNA levels by semi-quantitative RT-PCR were visualized on a 1% agarose gel. Representative gels are shown in Figure 8. Each experiment included a control with no plasmid (Top10), two controls containing an empty pBADHisA vector (one induced with 0.2% arabinose) to test for plasmid effects, and five containing the pRprA vector. Experiments were evaluated for the effect of arabinose percentage (0%, 0.002%, 0.02%, 0.2% and 2%) on overexpression of RprA RNA. At 15 minutes after induction, the levels of RprA RNA are present and do slightly vary across the range of concentrations tested, even in the uninduced pRprA culture (Figure 8A). This difference is no longer seen by 30 minutes and all levels are similar except for the uninduced culture, which has a hardly discernable amount (Figure 8B). There are still levels of RprA detectable even as late as 1 hour post-induction indicating the transcripts appear to be quite stable over a long period of time (Figure 8C). This is unexpected as induction is normally a transient event, initiated quickly and subsiding within the hour. This result can possibly be explained by the fact that the Top10 strain contains an *araBAD* deletion, meaning arabinose will not be metabolized by this strain, therefore remaining available as inducer for the vector. It has also been shown that RprA levels can increase in stationary phase in an RpoS-independent manner. This may also explain the persistence of RprA RNA. As expected, none of the control cultures expressed any RprA RNA.

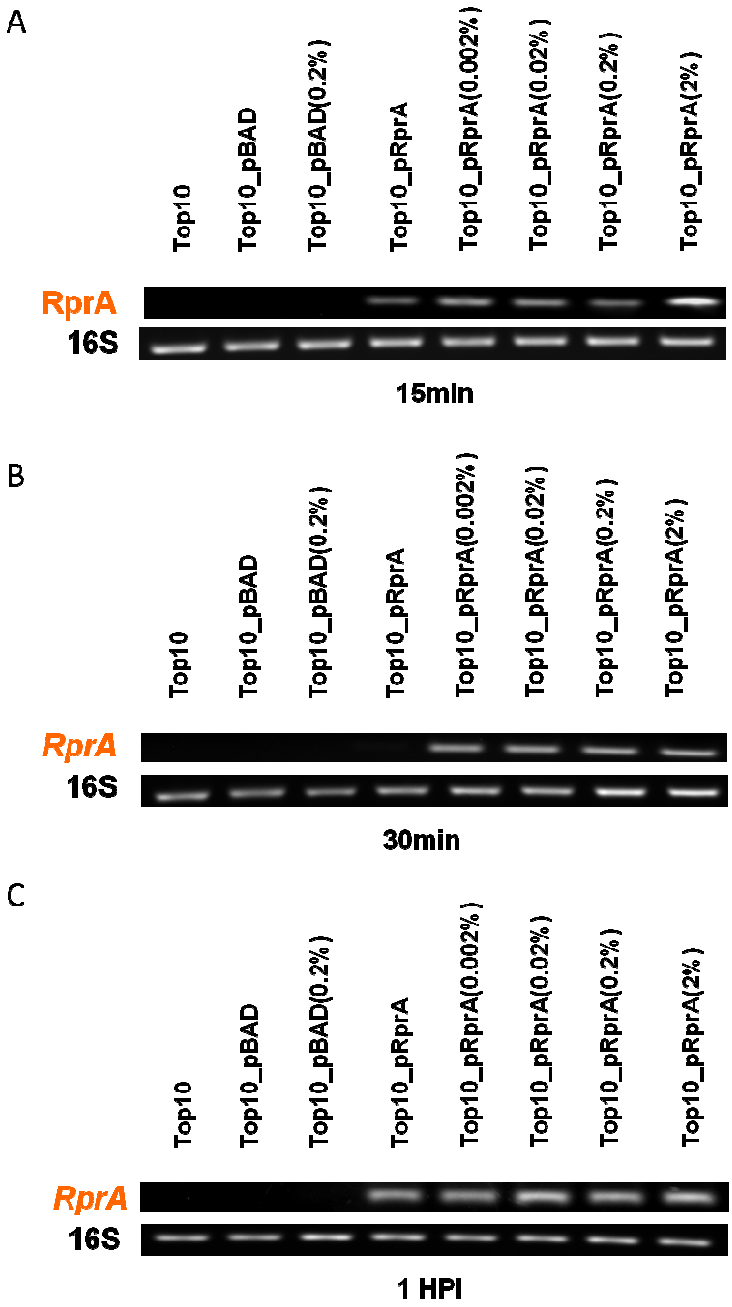

Figure 8 –RT-PCR results for RprA RNA overexpression in Top10pRprA cells in response to arabinose. Top10 was transformed with pRprA (pBAD-*rprA*) or a vector control (pBADHisA). Cells were grown at 37°C in LB with ampicillin (50 μg/μL) when required. A control with no vector was also grown. At OD_{600}0.3 cultures were induced with the indicated final arabinose concentrations. Samples were taken, total RNA was isolated and semi-quantitative RT-PCR was performed. RprA RNA levels were analyzed at A) 15 minutes, B) 30 minutes and C) 1 hour post-induction (HPI). 16S transcript levels were used as a control. Bands were visualized on a 1% agarose gel using ethidium bromide and UV light.

Cultures were also evaluated for a corresponding subsequent level of RpoS protein. Whole cell protein samples were collected and separated by SDS-PAGE and probed with σ^S antibody for Western blot analysis. Representative Western blots for each time point are shown in Figure 9. All band intensities were normalized to the value of the Top10 control at the respective time point. As expected in early exponential phase, the pre-induced cultures contain similar amounts of RpoS protein. By 30 minutes, the pRprA cultures induced with 0.002%, 0.02%, and 0.2% arabinose expressed RpoS protein levels at least twice as high as the control cultures. The uninduced and 2% cultures are comparable to the controls and the induced (0.2%) pBAD vector is actually half the amount of the control. This plasmid would be producing a very small nonsense transcript upon arabinose addition. It is possible this caused a different stress response and the coincident upregulation of another sigma factor in this culture, such as σ^{32}. σ^{32} is responsible for the heat shock response and transcribes chaperone proteins and proteases that help address an acute stress. By one hour post- induction, the protein levels in all of the pRprA induced cultures increase to ~3 fold the amount in the Top10 control. The protein levels in all the control cultures, as well as, the uninduced pRprA culture remain relatively consistent for all time points. All cultures expressed similar levels of RpoS by three hours post-induction. This result suggests the pRprA overexpression system can both effectively overexpress RprA and that this RNA expression is enough to positively modulate RpoS protein levels for at least an hour post-induction when induced by arabinose.

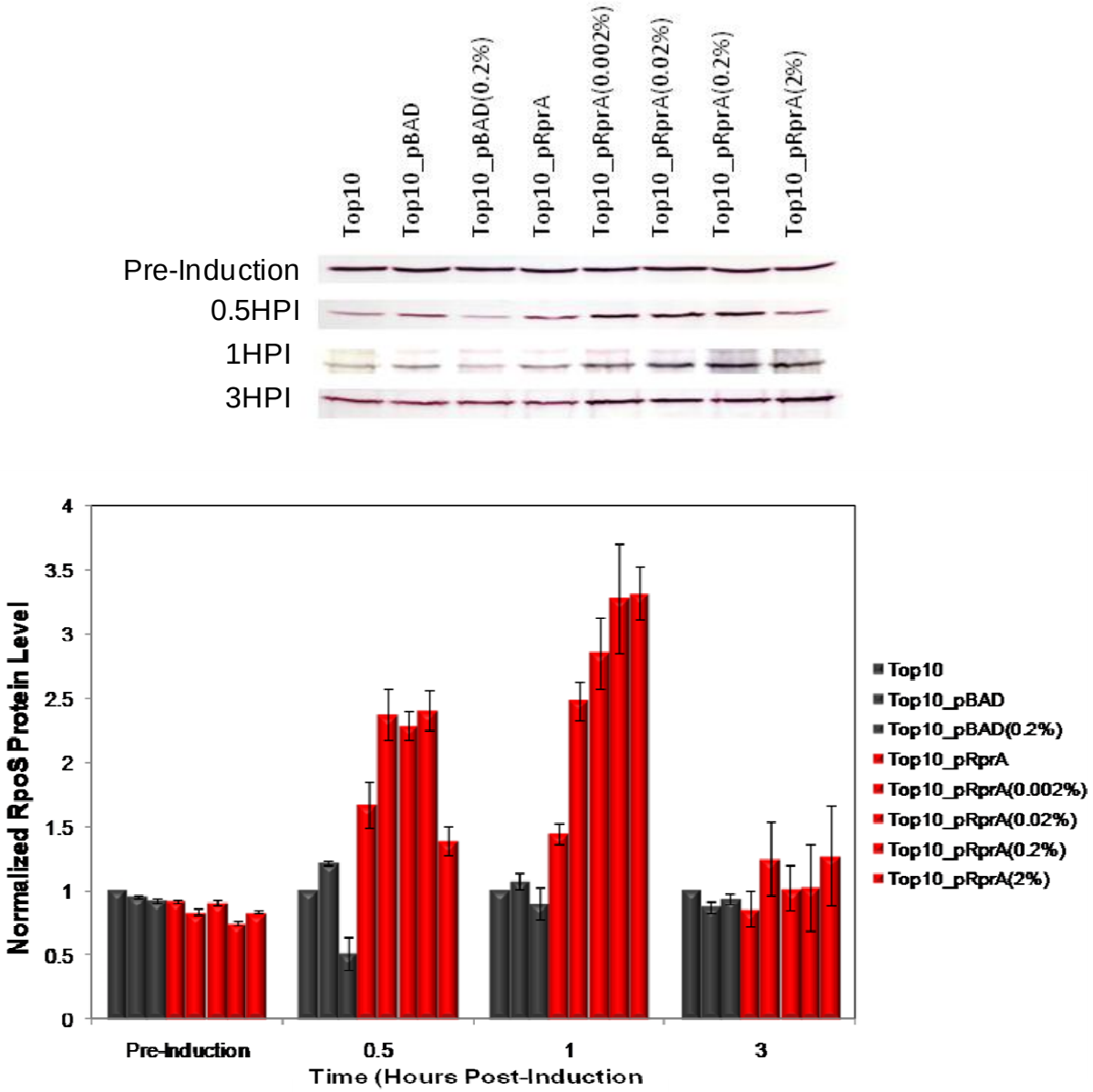

Figure 9 – RpoS Western blot analysis of Top10pRprA. Cells were grown at 37°C in LB. At OD_{600}0.3 cultures were induced with the indicated final arabinose concentrations. Whole cell protein samples were taken at the indicated time points. Samples were resuspended in protein loading buffer. Ten microliter samples were used for SDS-PAGE and Western blotting. Representative Western blots for the samples are shown (top). Bands from the Western blot were quantified using Image J software and normalized against the Top10 control sample for each respective time point (bottom). Error bars represent the standard of deviation for two replicates each for at least two independent experiments.

Downstream effects on RpoS-dependent genes

The pRprA system was shown to effectively overexpress RprA RNA which led to increased RpoS protein levels. This, however, does not automatically mean this system translates into active RpoS control of downstream genes. To test this, we performed RT-PCR for two genes known to be RpoS-dependent. The first is *bolA*, a cell morphology gene that protects the cell in stationary phase and during cellular stress, resulting in a smaller ovoid shape [141]. The second is *flhD*, which is part of the *flhDC* regulon that controls flagellar synthesis and ultimately cell motility and chemotaxis [142]. These genes are positively and negatively regulated by RpoS respectively. Figure 10A reveals no obvious RpoS-dependent *bolA* regulation with respect to the RprA overexpression system at 15 minutes and at 30 minutes. The level of *bolA* transcripts seems fairly similar across all the cultures, except for a few fluctuations in the 0.002% and 0.2% pRprA cultures. Conversely, the system did seem to have an effect on *flhD* transcript levels at 15 and 30 minutes in all of the pRprA induced cultures (Figure 10B). *flhD* transcript levels are markedly lower in these cultures and at 15 minutes this downregulation seems to increase modestly as arabinose concentration increases. At 30 minutes, there is no pronounced difference across the pRprA induced cultures. At both time points, the uninduced pRprA culture was similar to the controls, suggesting arabinose induction is necessary to affect this gene. This also implies that the increase in RpoS protein levels does correspond to the regulation of at least one identified RpoS-dependent genes, *flhD*. The complicated level of control of RpoS means that the gene expression profile for RpoS control will logically be different depending on the factors presented, one being

growth phase. Considering the fact that we are expressing RpoS in the exponential phase, previously identified RpoS-dependent stationary phase genes need not be affected. This is evident from the various DNA microarray reports on the gene expression profiles of *rpoS* mutants in different strains, phases and conditions [7, 50, 52, 55]. This also reveals the potential of using various sRNA overexpression systems to achieve a diverse range of genetic profiles and altered cellular phenotypes and behaviors.

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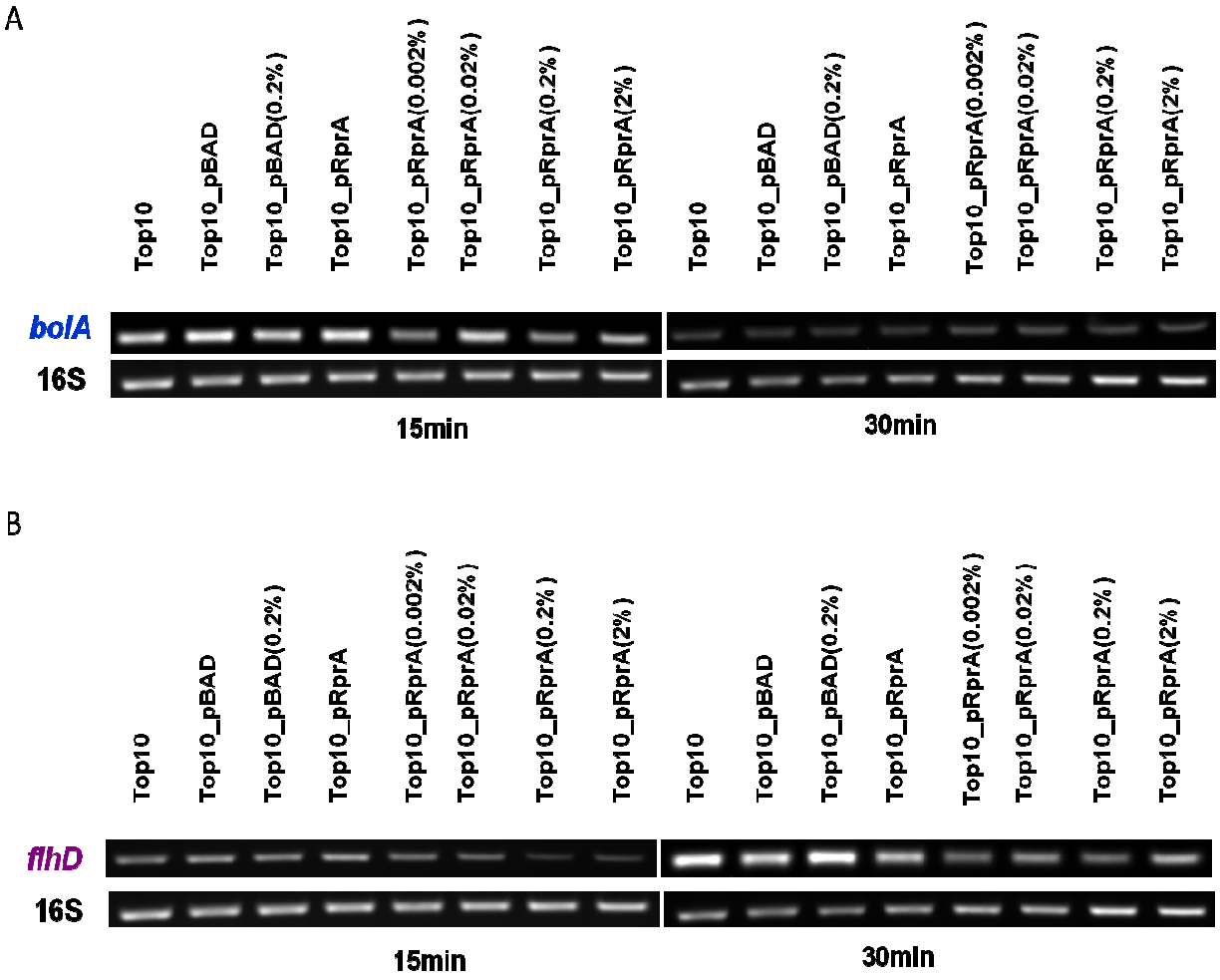

Figure 10 – *bolA and flhD* RT-PCR results for RprA RNA overexpression in Top10pRprA. A) *bolA* and B) *flhD*. Top10 was transformed with pRprA (pBAD-*rprA*) or a vector control (pBADHisA). Cells were grown at 37°C in LB with ampicillin (50 μg/μL) when required. A control with no vector was also grown. At OD_{600}0.3 cultures were induced with the indicated final arabinose concentrations. Samples were taken, total RNA was isolated and semi-quantitative RT-PCR was performed. RNA transcript levels were analyzed at 15 minutes and 30 minutes. 16S transcript levels were used as a control. Bands were visualized on a 1% agarose gel using ethidium bromide and UV light.

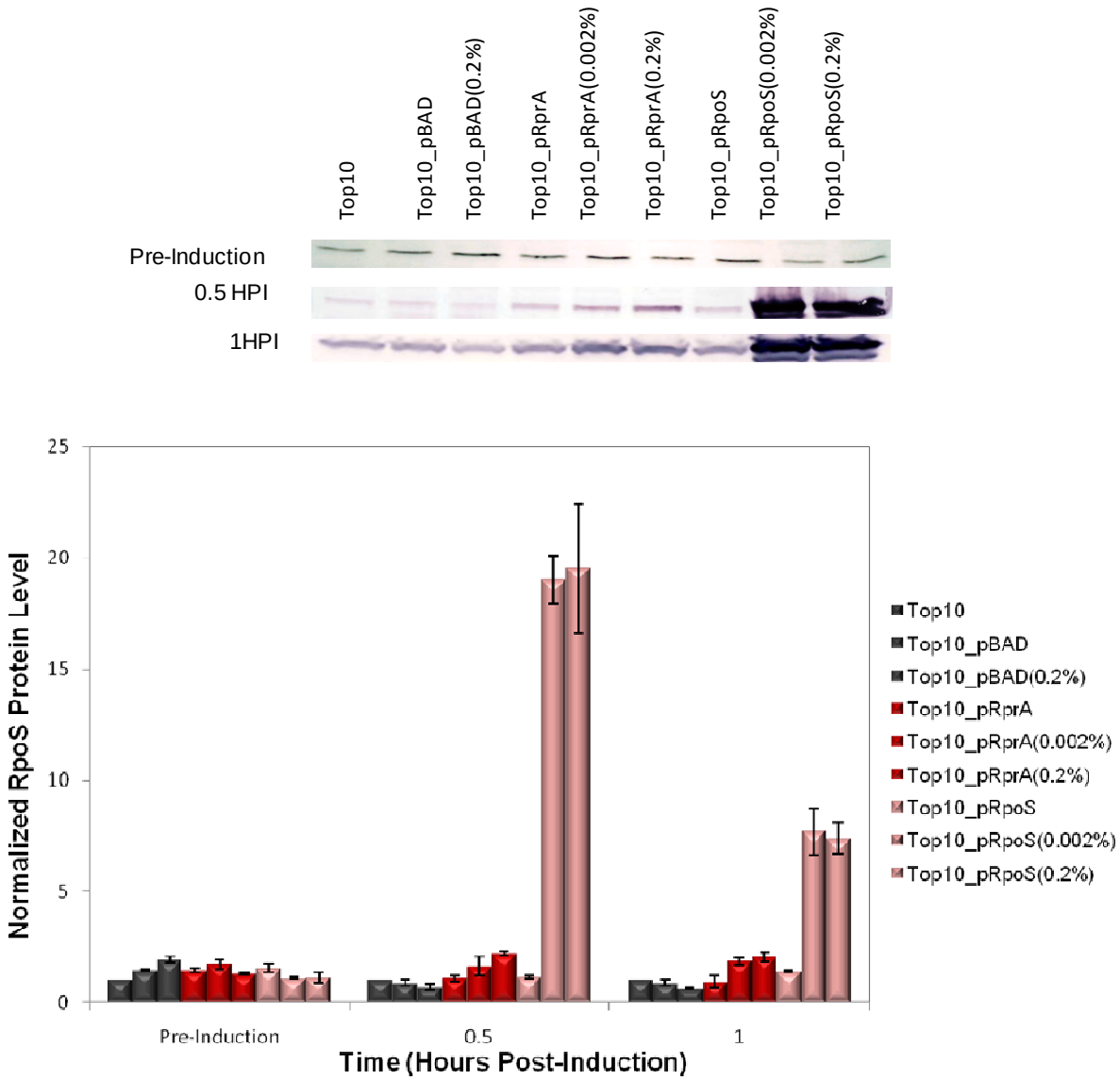

Figure 11 – RpoS Western blot analysis of Top10pRprA and Top10pRpoS. Cells were grown at 37°C in LB. At OD_{600}0.3 cultures were induced with the indicated final arabinose concentrations. Whole cell protein samples were taken at the indicated time points. Samples were resuspended in protein loading buffer. Ten microliter samples were used for SDS-PAGE and Western blotting. Representative Western blots for the samples are shown (top). Bands from the Western blot were quantified using Image J software and normalized against the Top10 control sample for each time point (bottom). At 0.5 and 1 HPI, induced pRprA cultures have a twofold increase in RpoS protein levels over controls. Induced pRpoS cultures have a ten- and fourfold increase in RpoS protein levels at the same time points, respectively. Error bars represent the standard of deviation for two replicates each for at least two independent experiments.

pRprA mediated RpoS protein regulation compared to direct RpoS overexpression

In an effort to further validate the pRprA overexpression system, we wanted to compare this indirect RpoS regulation with regulation from directly overexpressed RpoS protein from the *araBAD* promoter. Representative Western blots are shown in Figure 11. It was not surprising to see that RpoS protein levels from the RpoS plasmid were almost 20 fold higher than the control cultures and 10 fold higher than pRprA cultures at 30 minutes, when induced by both 0.002% and 0.2% arabinose. This difference is decreased to about 7 fold and 3.5 fold higher at one hour post-induction. Employing a low concentration of arabinose (0.002%) and a higher concentration (0.2%) for induction in the pRprA system seemed to produce varying amounts of RpoS protein, slightly increasing. This is not observed in the pRpoS system. It is feasible that either this does not occur when RpoS is directly overexpressed, or the direct overexpression results in such a rapid accumulation of RpoS protein that the system becomes saturated and the difference in levels could not be visualized by 30 minutes after induction. Uninduced pRprA and pRpoS cultures stayed at levels similar to the controls. Induced pRprA cultures maintained the 2-fold increase over controls previously seen in earlier experiments.

We also wanted to observe if there was a difference in regulation of the *bolA* and *flhD* genes between the two systems. We also chose to look at the *rssB* gene, which is required to direct RpoS to ClpX degradation. It was possible that by increasing the levels of RpoS protein, the cell could respond by trying to promote higher rates of degradation. The RT-PCR results are depicted in Figures 12A, 12B

and 12C, corresponding to *bolA*, *flhD*, and *rssB*, in that order. For *bolA* transcription, again there is no real discernable difference in expression across all cultures at 15 minutes. However, both systems had a modest increase over the controls when induced by 30 minutes. The case of *flhD* was very interesting in that pRpoS cultures had a slight increase in *flhD* levels when induced at 15 minutes post-induction. This is in direct contrast to what was expected as this would indicate that direct overexpression of RpoS increased flagellar synthesis. Even more, is the fact that by 30 minutes induced pRprA cultures are significantly lower than controls and pRpoS cultures, which corresponds to the previous study. Clearly in the area of motility, the two systems vary in regulation. This could suggest that the direct overexpression of RpoS finds a selective advantage in keeping flagellar synthesis normal to modestly higher in the cell. At 15 minutes, *rssB* did not reveal a particular trend across all the cultures. Thirty minutes did again reveal very modest increases in the cultures containing pRprA and pRpoS over the controls. This indicates that the overexpression of RpoS protein levels from both plasmids does not seem to result in such more *rssB* transcription than the control cultures. Still, this does not mean that RssB protein levels are not increased by higher levels of *rssB* mRNA translation or RssB protein stabilization.

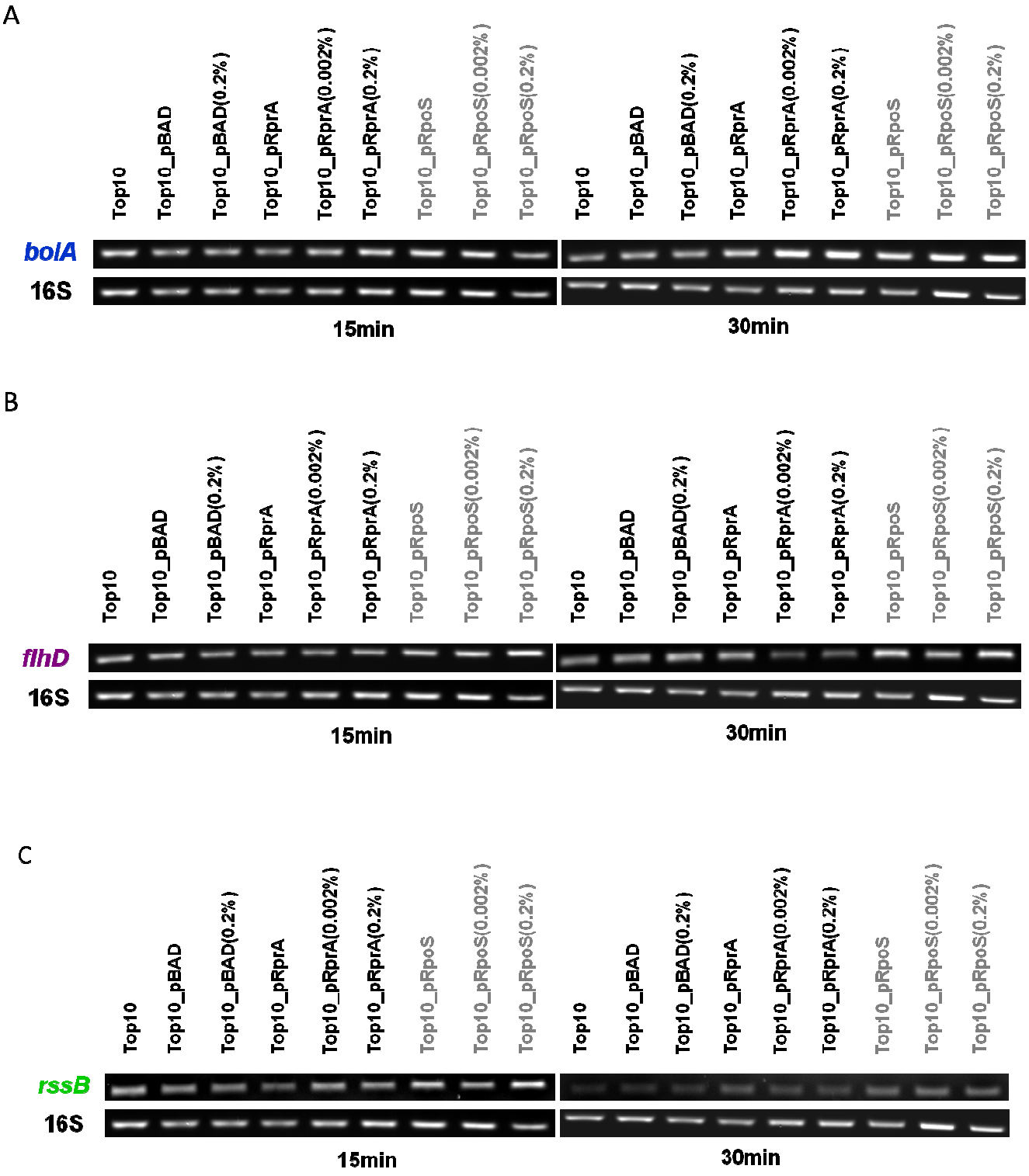
A
Top10
Top10_pBAD
Top10_pBAD(0.2%)
Top10_pRprA
Top10_pRprA(0.002%)
Top10_pRprA(0.2%)
Top10_pRpoS
Top10_pRpoS(0.002%)
Top10_pRpoS(0.2%)
bolA
16S
15min
30min
B
flhD
16S
15min
30min
C
rssB
16S
15min
30min

Figure 12 – *bolA, flhD* and *rssB* RT-PCR results for RprA RNA overexpression in Top10pRprA and Top10pRpoS. A) *bolA*, B) *flhD* and C) *rssB*. Top10 was transformed with pRprA (pBAD-*rprA*) or a vector control (pBADHisA). Cells were grown at 37^{o}C in LB with ampicillin (50 μg/μL) when required. A control with no vector was also grown. At OD_{600}0.3 cultures were induced with the indicated final arabinose concentrations. Samples were taken, total RNA was isolated and semi-quantitative RT-PCR was performed. RNA transcript levels were analyzed at 15 minutes and 30 minutes. 16S transcript levels were used as a control. Bands were visualized on a 1% agarose gel using ethidium bromide and UV light.

The pRprA overexpression platform across strains

A key characteristic for this system was for it to be able to be efficient across various laboratory strains to study the effects. To this end, pRprA was transformed into the strain, MG1655, which is widely considered the wild-type strain for K-12 *E. coli*. As was done for Top10, the pRprA system was evaluated for its ability to overexpress the RprA RNA and affect subsequent RpoS protein levels. MG1655 can metabolize arabinose as a carbon source, meaning all inducer did not solely go to the transcription of RprA RNA as in Top10. From Figures 13A and B, again we see that there are detectable amounts of RprA RNA with the addition of different amounts of inducer at both 15 minutes and 30 minutes. There are very small levels in the uninduced pRprA cultures but these amounts are barely detectable when compared to the induced cultures. Figure 14 displays representative Western blots of the RpoS protein levels expressed from the system. It should be noted that there were no detectable pre-induction levels of RpoS protein in the wild type strain. This supports the fact that *rpoS* transcription in wild-type strains does not begin until mid-log phase and up to that point *rpoS* is only present at basal levels. The levels seem to correspond to an increase in inducer with the 0.2% and 2% induced cultures being 2fold higher in the first half hour. By 1 HPI, these two cultures are at least three times as high as the control cultures. One notable difference in this system is that the induced pBAD culture does show a response to arabinose addition with a modest increase in RpoS protein levels. As noted previously, this system is producing amounts of nonsense transcript which could result in mild stress and therefore increased RpoS protein levels. By 3 HPI all the cultures have similar levels of RpoS

protein, except both cultures induced with 0.2% arabinose which have slightly less amounts.

As done previously, the effect on downstream RpoS-dependent genes was also examined, for *bolA* (Figure 15A) and *flhD* (Figure 15B). In this strain, *bolA* results were fairly similar across cultures at 15 and 30 minutes. The trend for *flhD* at 15 minutes is the same across all cultures except for the 2% induced pRprA culture which begins to show some decreasing transcript levels. At 30 minutes, the 0.02%, 0.2% and 2% pRprA cultures all showed appreciable downregulation of *flhD* transcript levels when compared to control cultures. By 30 minutes post-induction, as in Top10, the pRprA system in MG1655 does show that the overexpression of RprA RNA results in downregulation of an RpoS-dependent gene, *flhD*. Downregulation in Top10 was visible earlier, by 15 minutes post-induction, for induced pRprA cultures. Overall, this demonstrates that the pRprA expression system is not only viable in more than one strain but that the same system can result in slightly variable regulation.

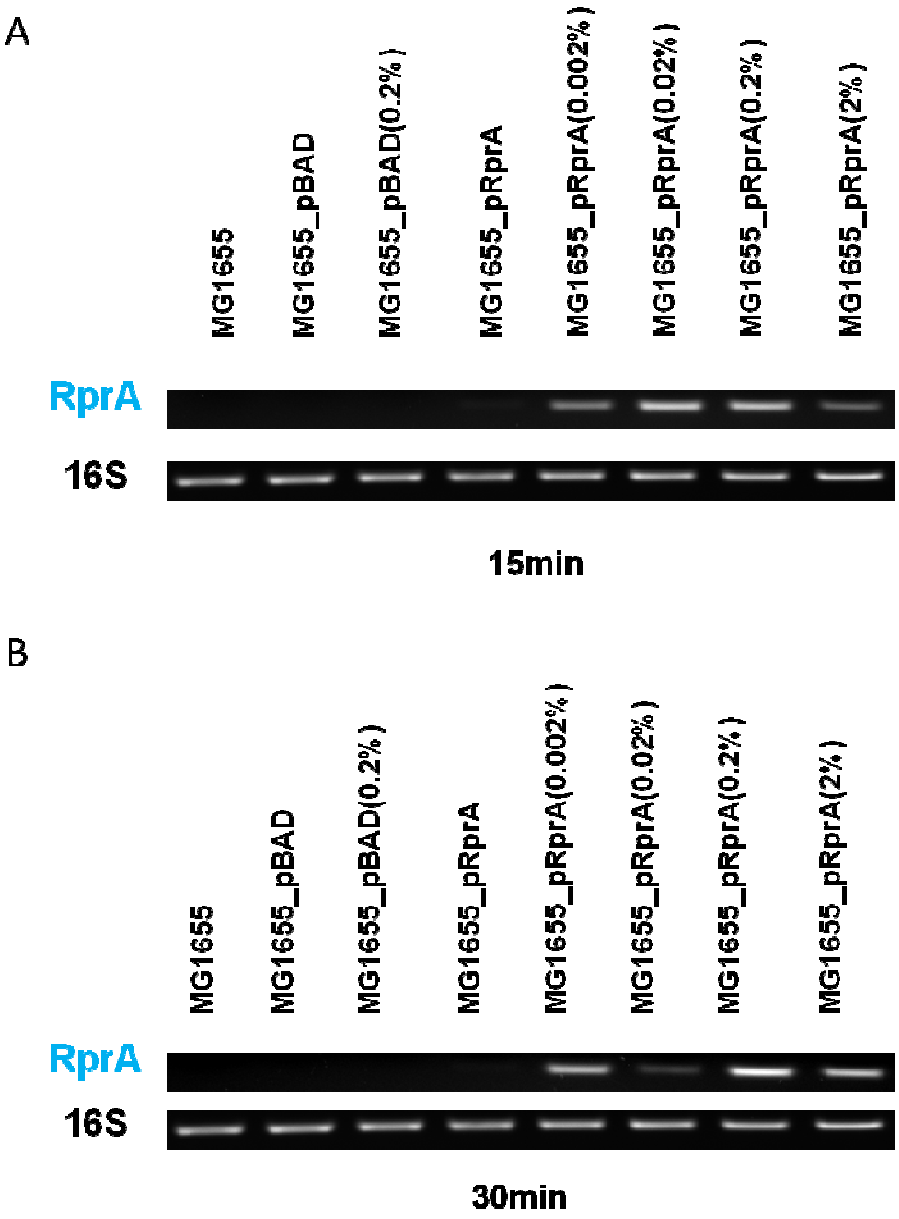

Figure 13 – RT-PCR results for RprA RNA overexpression in MG1655pRprA cells in response to arabinose. MG1655 was transformed with pRprA (pBAD-*rprA*) or a vector control (pBADHisA). Cells were grown at 37°C in LB with ampicillin (50 μg/μL) when required. A control with no vector was also grown. At OD_{600}0.3 cultures were induced with the indicated final arabinose concentrations. Samples were taken, total RNA was isolated and semi-quantitative RT-PCR was performed. RNA levels were analyzed at A) 15 minutes and B) 30 minutes. 16S transcript levels were used as a control. Bands were visualized on a 1% agarose gel using ethidium bromide and UV light.

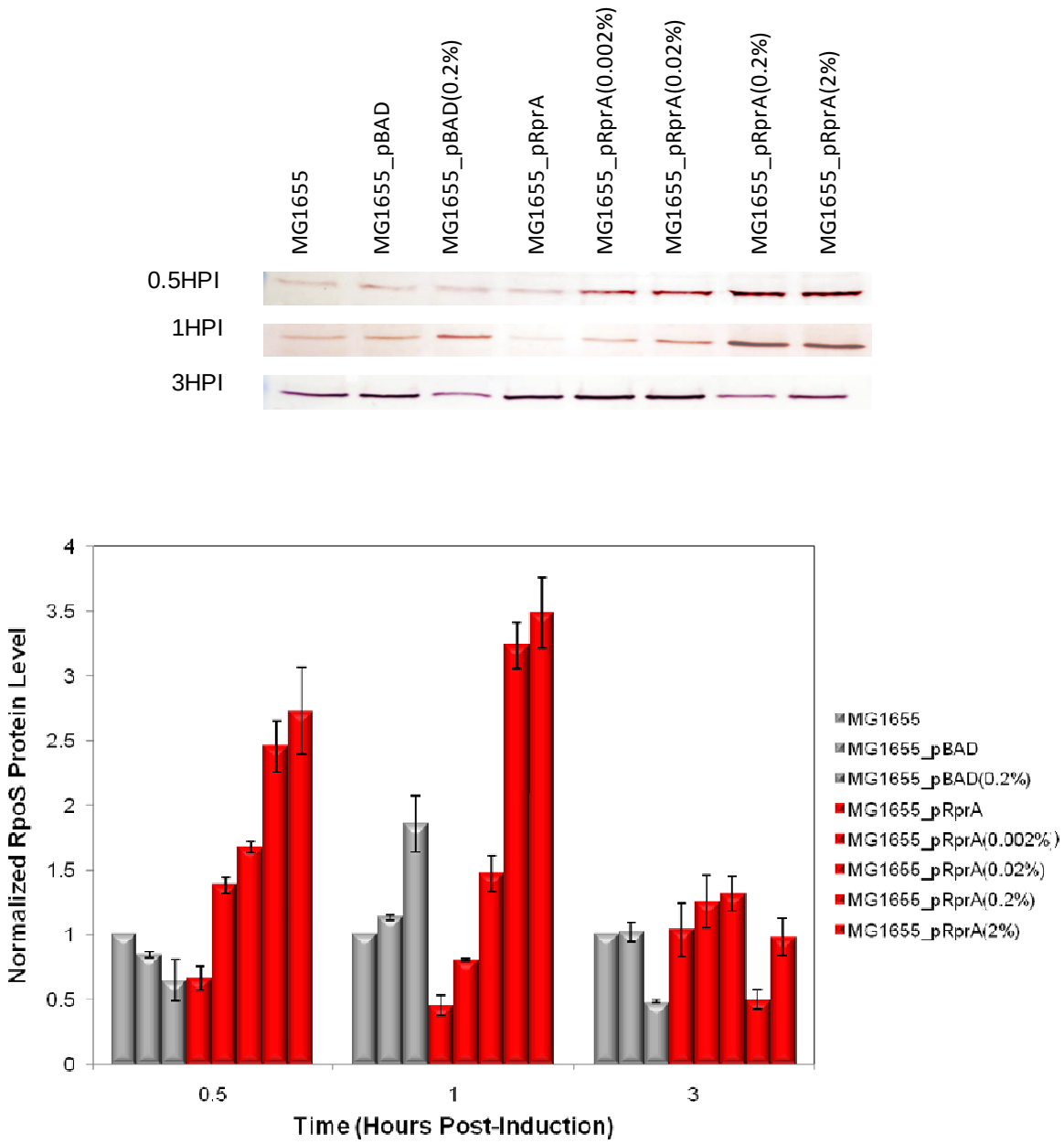

Figure 14 – RpoS Western blot analysis of MG1655pRprA. Cells were grown at 37°C in LB. At OD_{600}0.3 cultures were induced with the indicated final arabinose concentrations. Whole cell protein samples were taken at the indicated time points. Samples were resuspended in protein loading buffer. Ten microliter samples were used for SDS-PAGE and Western blotting. Representative Western blots for the samples are shown (top). Bands from the Western blots were quantified using Image J software and normalized against the Top10 control sample for each time point (bottom). Error bars represent the standard of deviation for two replicates each for at least two independent experiments.

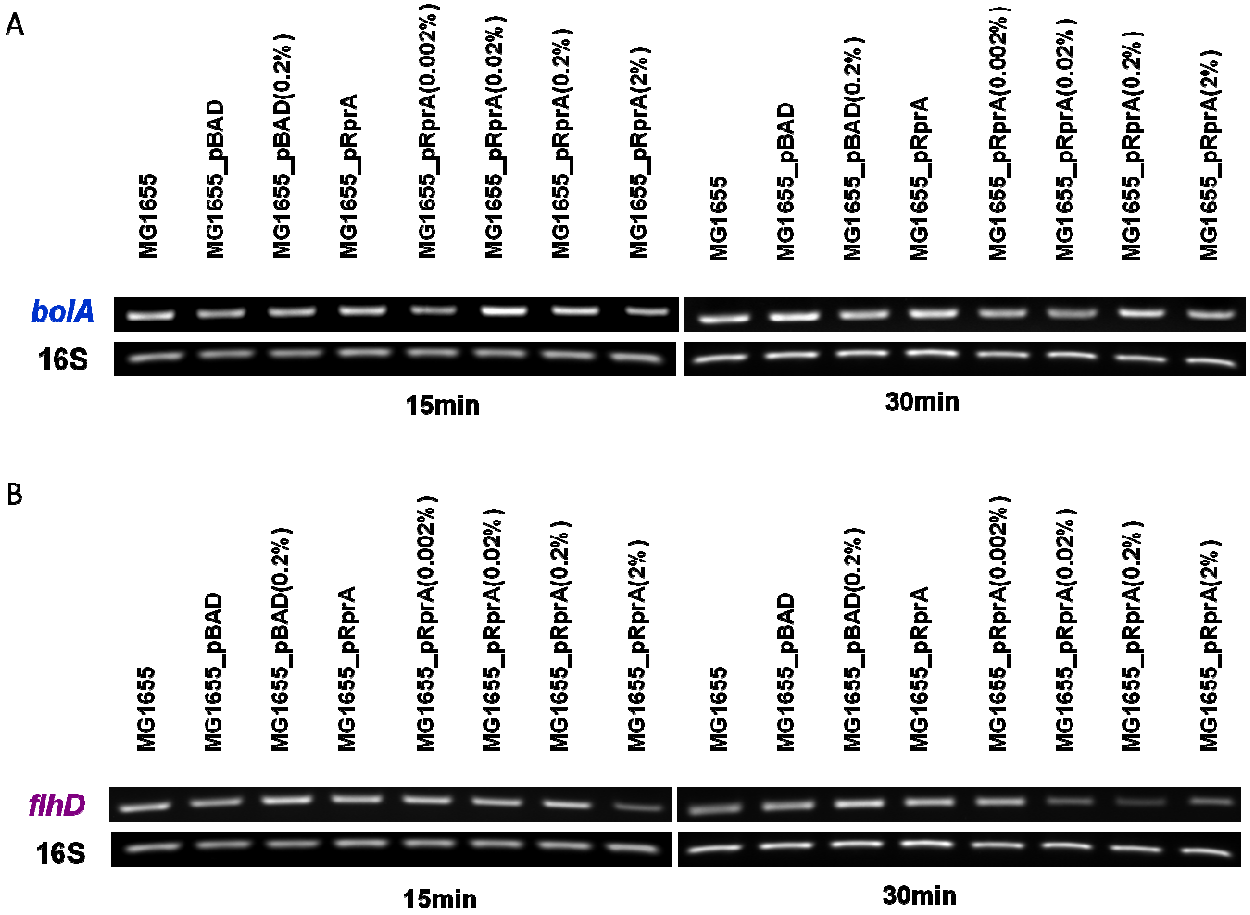

Figure 15 – *bolA and flhD* RT-PCR results for RprA RNA overexpression in MG1655pRprA. A) *bolA* and B) *flhD*. MG1655 was transformed with pRprA (pBAD-*rprA*) or a vector control (pBADHisA). Cells were grown at 37^{o}C in LB with ampicillin (50 μg/μL) when required. A control with no vector was also grown. At OD_{600}0.3 cultures were induced with the indicated final arabinose concentrations. Samples were taken, total RNA was isolated and semi-quantitative RT-PCR was performed. RNA transcript levels were analyzed at 15 minutes and 30 minutes. 16S transcript levels were used as a control. Bands were visualized on a 1% agarose gel using ethidium bromide and UV light.

DNA Microarray Results

The prior RT-PCR results indicated that there may be more genes that are affected unexpectedly in this system. To further investigate a microarray was done to get a complete genetic profile. We chose MG1655 as the host strain because of its wild- type status. MG1655 is capable of metabolizing arabinose, so in a four plex array, we compared the wild type, an induced wild-type containing the pBAD vector to negate the effects of arabinose, an uninduced wild-type housing the pRprA vector, and an induced pRprA vector. We chose an arabinose concentration of 0.2% because it has been shown to produce the high levels of RprA RNA from the *araBAD* promoter and because the previous data supported its efficacy for the pRprA system in particular. Samples equivalent to 1mL at an OD_{600} 1 were collected at 15 minutes and processed as described in the Materials and Methods section. Due to the extensive participation of RpoS in *E. coli* gene regulation many genes experienced significant fold changes, both negatively and positively. This work is still ongoing but did provide us with several genes to further test by semi-quantitative RT-PCR, *ibpA*, *pgaB*, *ycdT*, and *lon*. Each of these genes experienced at least a four-fold difference in the pRprA induced cultures. We also chose to compare each gene's semi-quantitative RT-PCR results in MG1655 with the results acquired for the Top10 strain.

The *ibpA* gene is a small heat shock protein that is associated with binding aggregated proteins and inclusion body formation upon recombinant protein production [143, 144].). It is associated in an operon along with *ibpB* and both are chaperone proteins under control of σ^{32}, as such it is transcribed under extreme

temperature upshift and two of the most induced during biofilm formation [145]. Mutants of *ibpA* and *ibpB* exhibit inhibited biofilm formation. Both genes were recently found to be substrates of the ATP protease Lon [146]. The microarray reported a decrease in these two genes in the pRprA system in MG1655. Analysis of pRprA in both Top10 and MG1655 using semi-quantitative RT-PCR displays interesting results. In Top10 (Figure 16A), there is a modest upshift in cultures with arabinose concentrations of 0, 0.002 and 0.02%, all other cultures were similar and by 30 minutes all the cultures were comparable. On the other hand, in MG1655 (Figure 16B) all the cultures with vectors expressed an increase at 15 minutes, with the 0.02%-2% being slightly higher, except the uninduced pBAD culture. This may make sense considering IbpA is a chaperone protein and while RprA is not a protein, it is still being overexpressed, perhaps creating a requirement for chaperone activity. The fact that this is in conflict with the microarray result will require further study. At 30 minutes, all of the induced pRprA cultures were like the wild-type with the pBAD and uninduced pRprA cultures modestly higher.

pgaB is part of a locus *pgaABCD* which is essential for the synthesis of a biofilm adhesin, PGA [147]. PgaB in particular is a predicted lipoprotein involved in the transfer of PGA across the outer membrane. Expression of the operon is highest in stationary phase but it has also been shown to be expressed in response to NaCl and ethanol [148]. In both strains, *pgaB* was shown to be upregulated in the pRprA cultures especially at the 0.02%, 0.2% and 2% concentrations at both 15 minutes and 30 minutes (Figures 17A and B). In MG1655 at 30 minutes, the only detectable transcript appears at the 0.2% arabinose induced pRprA culture. There does appear to

be some increase in the Top10 pBAD cultures indicating the possibility of plasmid effects on *pgaB* transcription however, this was dismissed when we compare this to the lack of transcription in the uninduced pRprA and the 0.002% induced pRprA cultures. Additionally, it was only a modest increase. Again, this plasmid produces a nonsense transcript which could lend to the emergence of secondary stress effects. Biofilm is usually produced in response to stressful conditions, in general. In this case the nonsense transcript could upregulate any number of stress related genes.

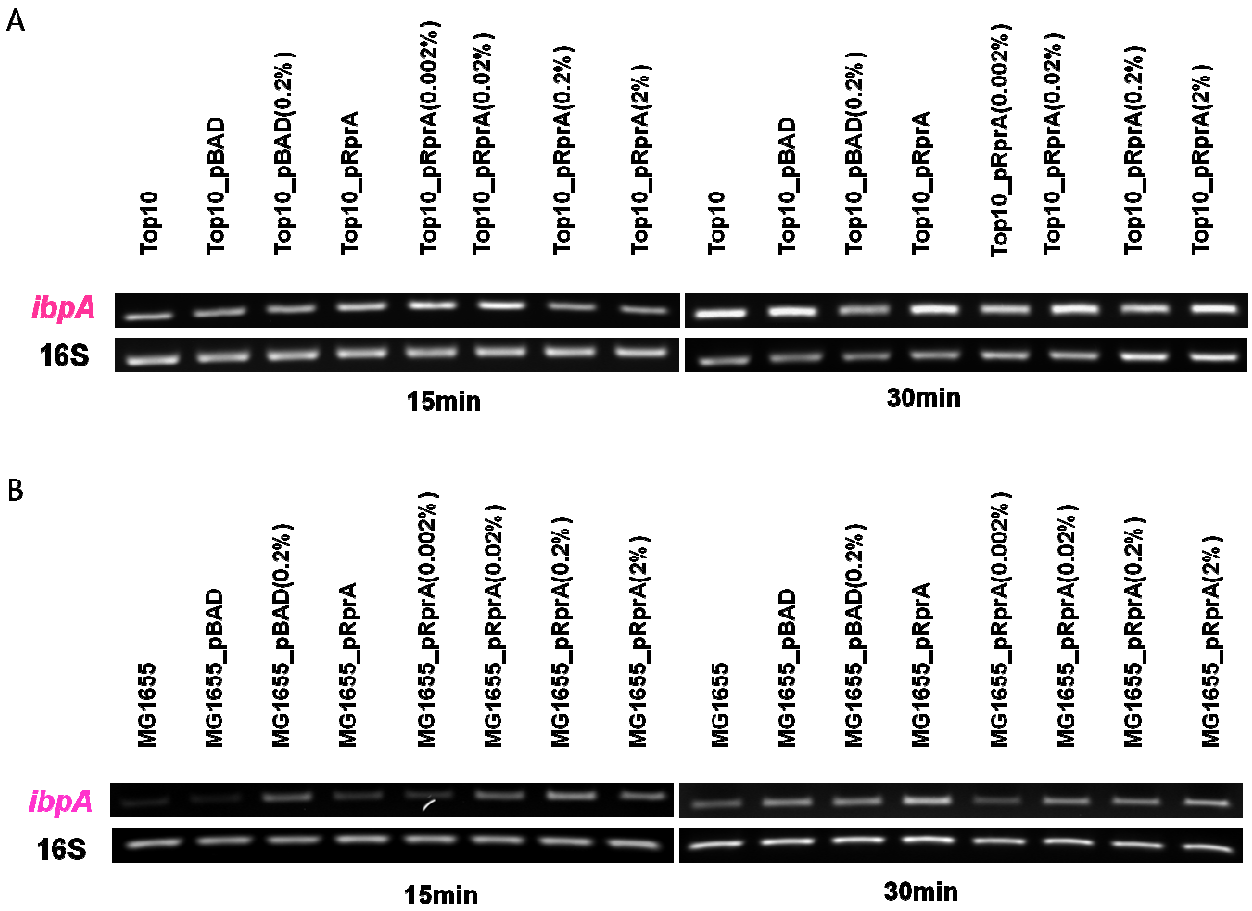

Figure 16 – *ibpA* RT-PCR results for RprA RNA overexpression in A) Top10pRprA and B) MG1655pRprA. Top10 was transformed with pRprA (pBAD-*rprA*) or a vector control (pBADHisA). Cells were grown at 37^{o}C in LB with ampicillin (50 μg/μL) when required. A control with no vector was also grown. At OD_{600}0.3 cultures were induced with the indicated final arabinose concentrations. Samples were taken, total RNA was isolated and semi-quantitative RT-PCR was performed. RNA transcript levels were analyzed at 15 minutes and 30 minutes. 16S transcript levels were used as a control. Bands were visualized on a 1% agarose gel using ethidium bromide and UV light.

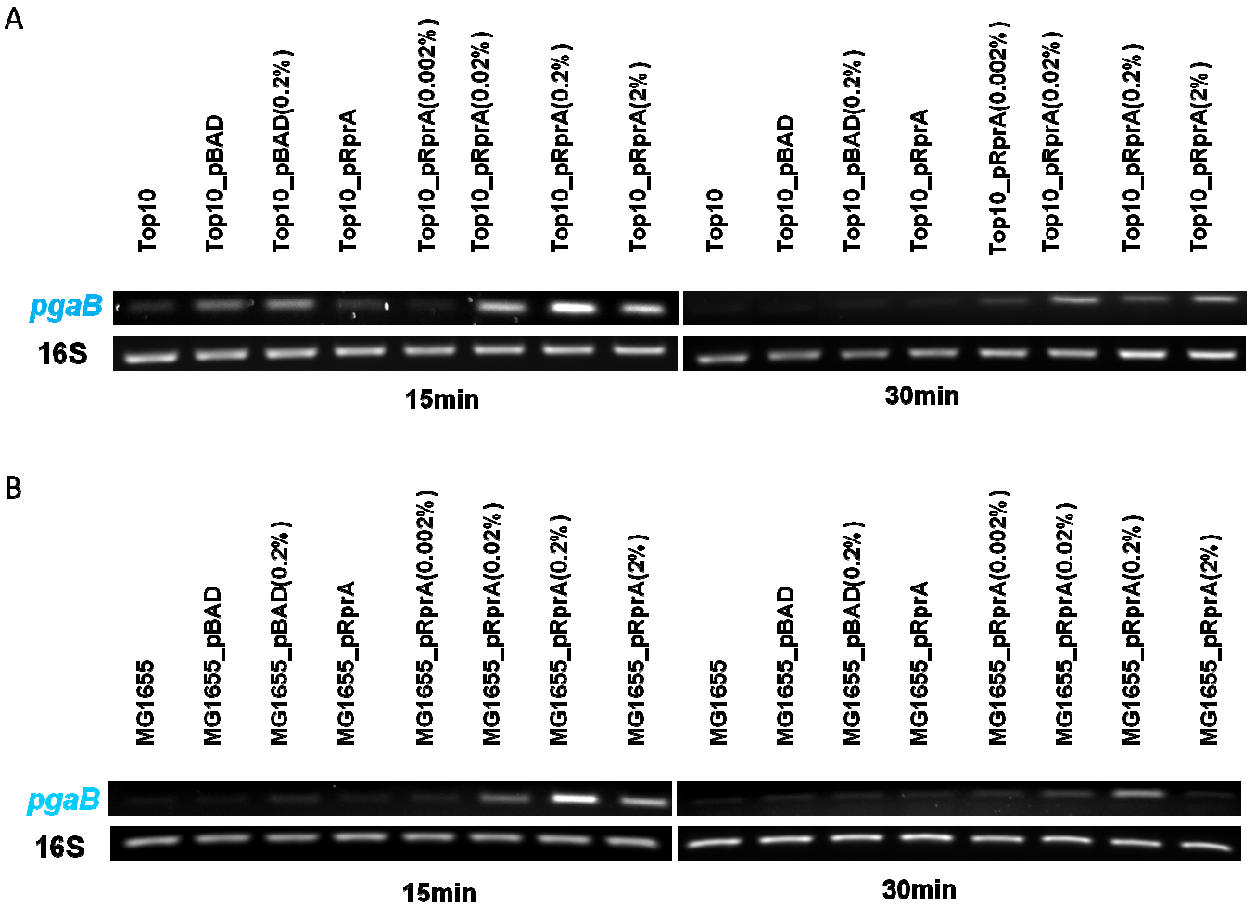

Figure 17 – *pgaB* RT-PCR results for RprA RNA overexpression in A) Top10pRprA and B) MG1655pRprA. Top10 was transformed with pRprA (pBAD-*rprA*) or a vector control (pBADHisA). Cells were grown at 37°C in LB with ampicillin (50 μg/μL) when required. A control with no vector was also grown. At OD_{600}0.3 cultures were induced with the indicated final arabinose concentrations. Samples were taken, total RNA was isolated and semi-quantitative RT-PCR was performed. RNA transcript levels were analyzed at 15 minutes and 30 minutes. 16S transcript levels were used as a control. Bands were visualized on a 1% agarose gel using ethidium bromide and UV light.

ycdT encodes a diguanylate cyclase that regulates motility in a cyclic-di-GMP (c-di-GMP) manner [149]. Overexpression of ycdT represses swimming behavior. In Figure 18A, *ycdT*, seemed to be upregulated in the Top10 cultures when plasmids are present, however, 0.2% and 2% cultures experience very minor decreases at 15 minutes. Top10 cultures at 30 minutes are pretty similar except for very modest increases in 0.02%, 0.2% and 2%. In MG1655, the addition of inducer to the cultures results at minor increases in ycdT transcripts only in the pRprA systems especially at 0.2% and 2% induction (Figure 18B). This behavior is the same for 30 minutes. There is also a minor increase in the induced pBAD culture suggesting there may be an effect from plasmid induction with higher concentrations of arabinose. It is important to note that total levels in both strains were not particularly high in general.

Finally, the *lon* gene, encodes for an ATP-dependent protease involved in the degradation of misfolded proteins. In the MG1655, lon levels are similar for 15 minutes but are appreciably lower by 30 minutes (Appendix). Lon has been found to be negatively regulated by RpoS [150]. Other genes involved in biofilm actually decreased, like *lldR* and *bssS [151, 152]*. This highlights the complexity involved in biofilm formation and maturation and the cell signaling pathways involved. It also supports why there are so many conflicting phenotypes observed. Other genes regulated were various inner and outer membrane proteins and nutrient transporters. Collectively, this data appears to suggest that the pRprA plays a part influencing the concerted regulation between motility, biofilm and stress response. Other microarrays are in the works and we hope these will finalize the genetic profile expressed by the inducible pRprA platform.

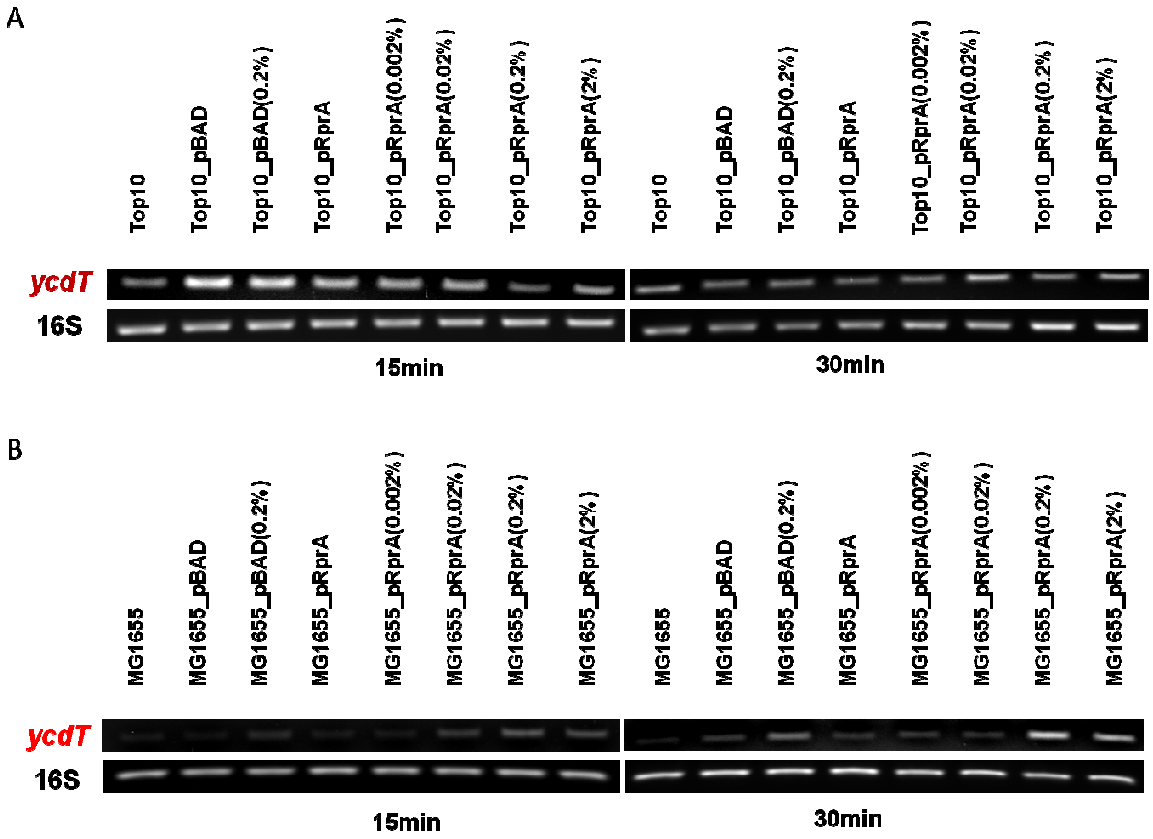

Figure 18 – ***ycdT* RT-PCR results for RprA RNA overexpression in A) Top10pRprA and B) MG1655pRprA**. Top10 was transformed with pRprA (pBAD-*rprA*) or a vector control (pBADHisA). Cells were grown at 37^{o}C in LB with ampicillin (50 μg/μL) when required. A control with no vector was also grown. At OD_{600}0.3 cultures were induced with the indicated final arabinose concentrations. Samples were taken, total RNA was isolated and semi-quantitative RT-PCR was performed. RNA transcript levels were analyzed at 15 minutes and 30 minutes. 16S transcript levels were used as a control. Bands were visualized on a 1% agarose gel using ethidium bromide and UV light.

pRprA system in other popular laboratory strains

We decided to transform the pRprA system into two other popular laboratory strains W3110 and MC4100. The strains of W3110 in our laboratory stocks did not express any detectable levels of RpoS when processed by Western blots up to three hours post-induction. This was not completely surprising considering the many studies revealing the numerous allelic versions of *rpoS* across this strain, as well as other strains. In original comparisons of the very similar genomes of MG1655 and W3110, *rpoS* was one of the genes with the greatest variation, even revealing nonfunctional alleles. It was also not surprising considering the various sources for attaining the strain and the polymorphic nature of *rpoS*, especially with respect to storage conditions.

MC4100 contains a chromosomal deletion in the *araD* gene. While this strain does not metabolize arabinose, the single deletion in the *ara* operon, without *araA* and *araB*, leaves this strain susceptible to arabinose toxicity [153]. We were intrigued by the idea of how this arabinose inducible pRprA overexpression system might perform in this strain. Curiously, even in this strain, at one hour the cultures containing pRprA were higher, whether arabinose is present or not (Figure 19). It is also interesting to note that the addition of arabinose to the pBAD culture does not result in the same kind of increase, indicating that the increase in RpoS levels the pRprA system is at least partially independent of any stress response induced from the arabinose addition.

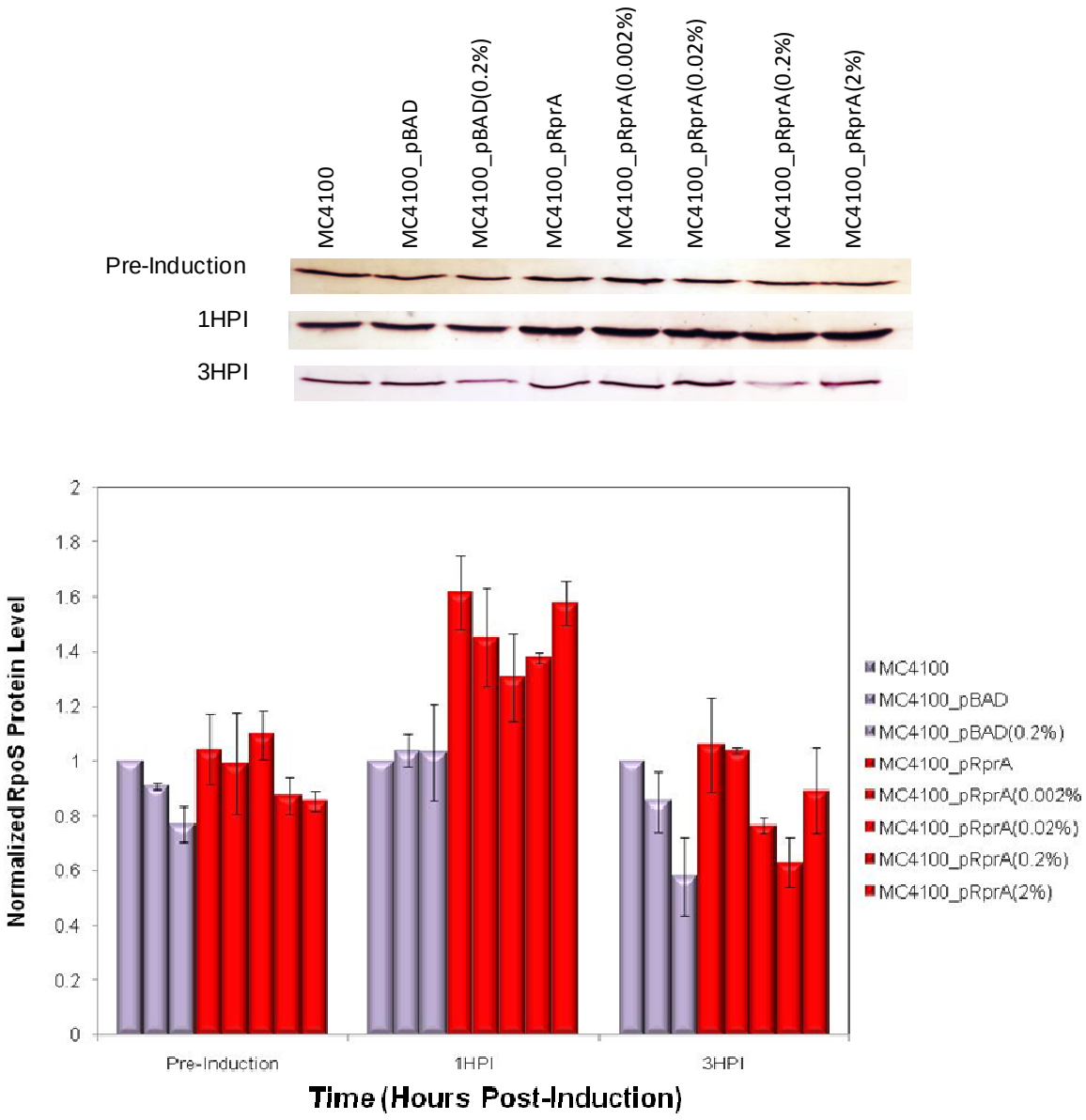

Figure 19 – RpoS Western blot analysis of MC4100pRprA. Cells were grown at 37°C in LB. At OD_{600}0.3 cultures were induced with the indicated final arabinose concentrations. Whole cell protein samples were taken at the indicated time points. Samples were resuspended in protein loading buffer. Ten microliter samples were used for SDS-PAGE and Western blotting. Representative Western blots for the samples are shown (top). Bands from the Western blots were quantified using Image J software and normalized against the Top10 control sample for each time point (bottom). Error bars represent the standard of deviation for two replicates each for at least two independent experiments.

pOxyS overexpression of oxyS transcript levels and corresponding RpoS protein levels

Overall the goal of the expression platform is that not only can various amounts of inducer be employed to express an sRNA, but that the small RNA, itself, can be easily substituted for a different effect on the RpoS genetic profile. Since the first scenario used an activator, RprA, reasonably the system should be evaluated with a repressor, OxyS. *oxyS* was cloned into the same site as *rprA* in the pBAD vector and tested under the same experimental conditions. As in the previous case, overexpression of pOxyS resulted in detectable levels of OxyS RNA (Figure 20). Initially at 15 minutes, there is no detectable level in the uninduced plasmid, however, a low level of transcript is observed by one hour post-induction. The level of transcript for all arabinose concentrations are similar except for the 0.002% culture which has moderately less. Thirty minutes reveals equivalent amounts across all of the cultures expressing detectable levels of transcript. Western blot analysis reveals that overexpression from pOxyS does reduce levels of RpoS protein levels especially in the 0.2% and 2% induced cultures by 30 minute post-induction (Figure 21). This difference is abolished by one hour post-induction and RpoS levels return to control level. This trend continues through three hour post-induction. There is also a more modest decrease in the pBAD cultures, as well; however, the bands for these were the same for the pRprA experiments. This result reveals that the overexpression of OxyS RNA from the pOxyS system can effectively downregulate RpoS protein levels, though this knockdown by the repressor seemed to be more transient than the upregulation by the activator.

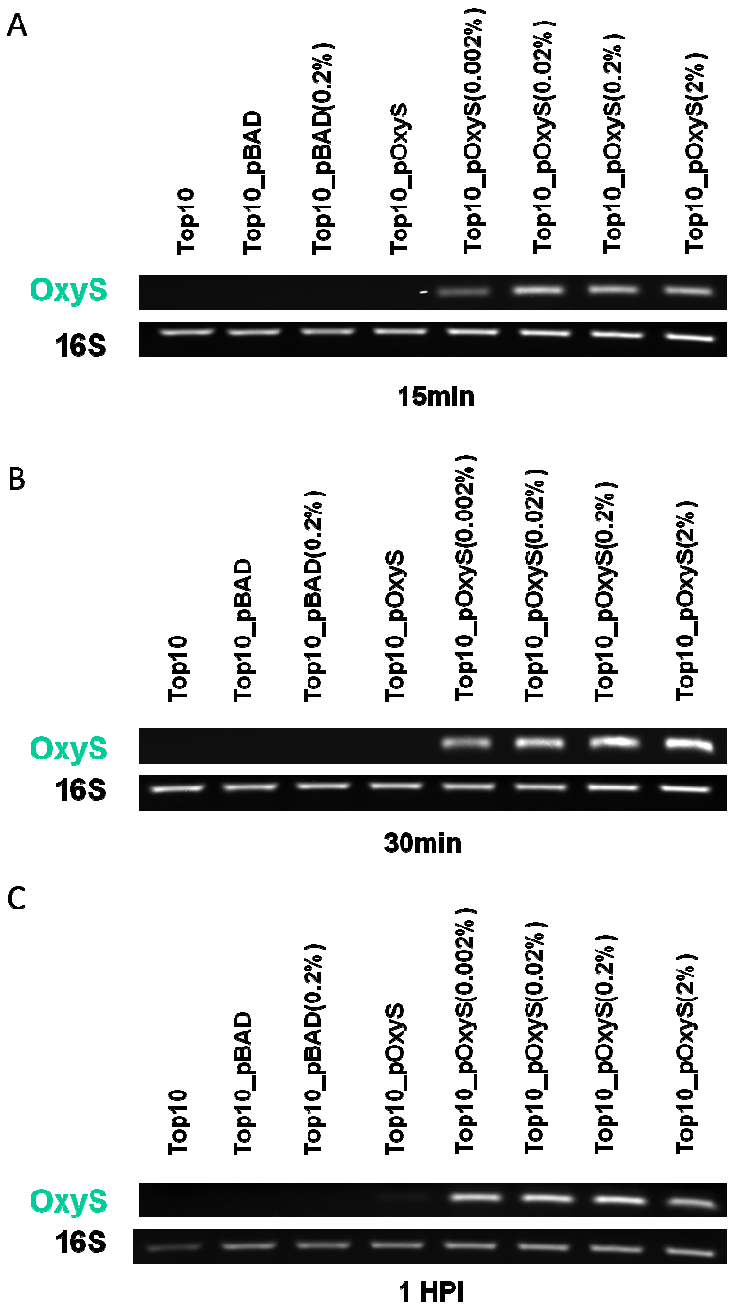

Figure 20 – RT-PCR results for OxyS RNA overexpression in Top10pOxyS cells in response to arabinose. Top10 cultures were transformed with pOxyS (pBAD-*oxyS*) or a vector control (pBADHisA). Cells were grown at 37°C in LB with ampicillin (50 μg/μL) when required. A control with no vector was also grown. At OD_{600}0.3 cultures were induced with the indicated final arabinose concentrations. Samples were taken, total RNA was isolated and semi-quantitative RT-PCR was performed. RNA levels were visualized at A) 15 minutes, B) 30 minutes and C) 1 hour post-induction (HPI). 16S transcript levels were used as a control. Bands were visualized on a 1% agarose gel using ethidium bromide and UV light.

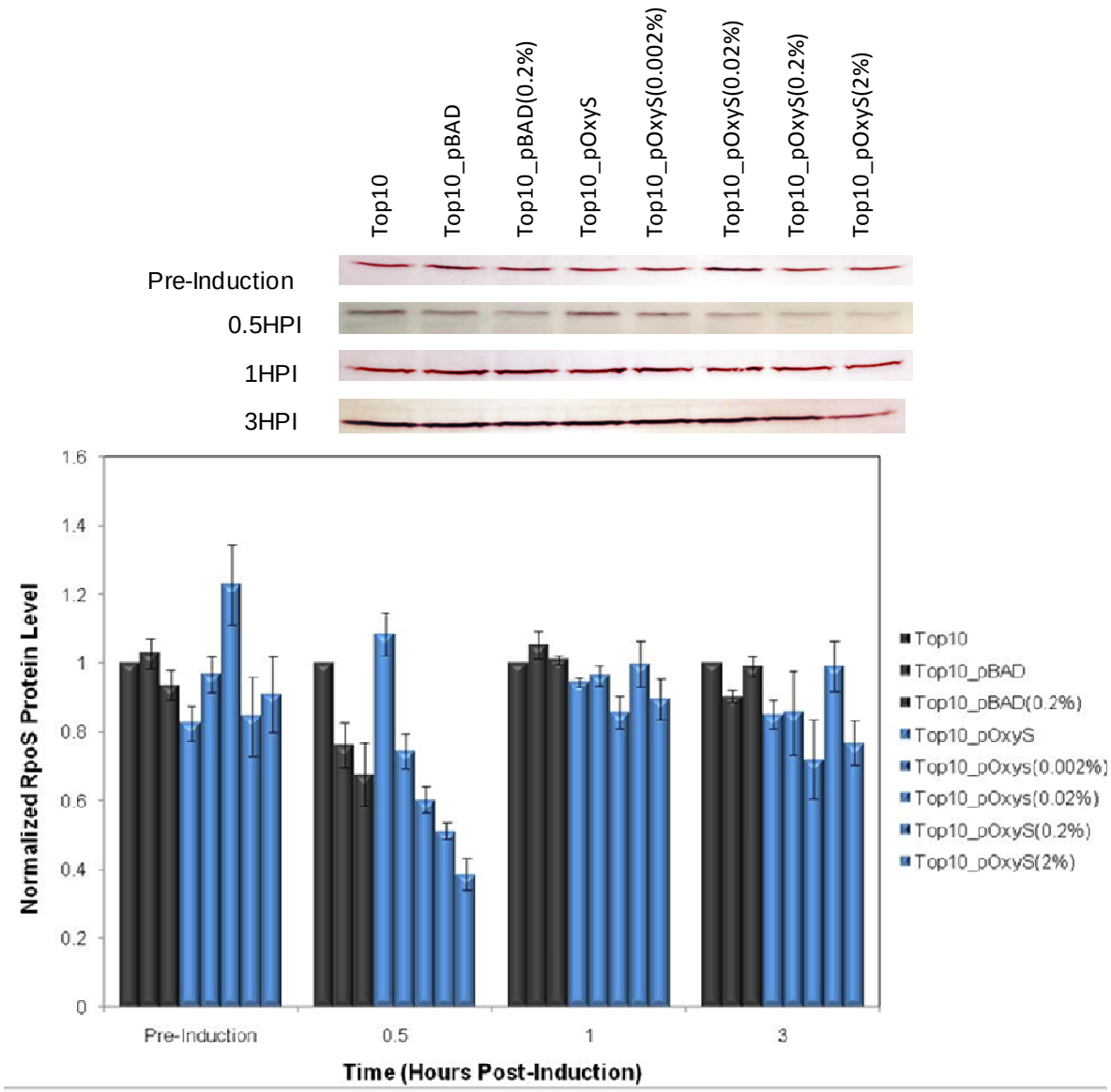

Figure 21 – RpoS Western blot analysis of Top10pOxyS. Cells were grown at 37°C in LB. At OD_{600}0.3 cultures were induced with the indicated final arabinose concentrations. Whole cell protein samples were taken at the indicated time points. Samples were resuspended in protein loading buffer. Ten microliter samples were used for SDS-PAGE and Western blotting. Representative Western blots for the samples are shown (top). Bands from the Western blots were quantified using Image J software and normalized against the Top10 control sample for each time point (bottom). Error bars represent the standard of deviation for two replicates each for at least two independent experiments.

Given that the level of RpoS protein was decreased in the first 30 minutes of inducing the pOxyS system, we wanted to see if this would result in the opposite trend in the two RpoS-dependent genes tested previously in the pRprA system, *bolA* and *flhD*. Based on semi-quantitative RT-PCR (Figure 22A), there were no appreciable differences in *bolA* transcription in any of the cultures containing a plasmid, though they all appear to be higher than Top10. All *bolA* transcript levels are the same at 30 minutes. For the *flhD* gene at 15 minutes, all induced cultures resulted in an obvious increase in *flhD* transcription levels indicating there may be an effect on flagellar synthesis just from the presence of a vector. In any case, the cultures containing the pOxyS plasmid were still the highest. When comparing the Top10 control here with the one in the pRprA system it appears that the levels detected in this culture is low. The difference seen here may just be an effect of experimental error and would most likely be rectified by repeating this experiment. What is most important here though is the fact that unlike the pRprA system, flhD does not decrease in the induced pOxyS cultures and is even markedly higher at 30 minutes in pOxyS cultures induced with 0.02%-2% arabinose (Figure 22B). This is the opposite trend experienced in the pRprA system. This confirms the fact that not only is the platform effective for RpoS regulation by exchanging the sRNA, but this regulation can be significantly altered depending on the sRNA used.

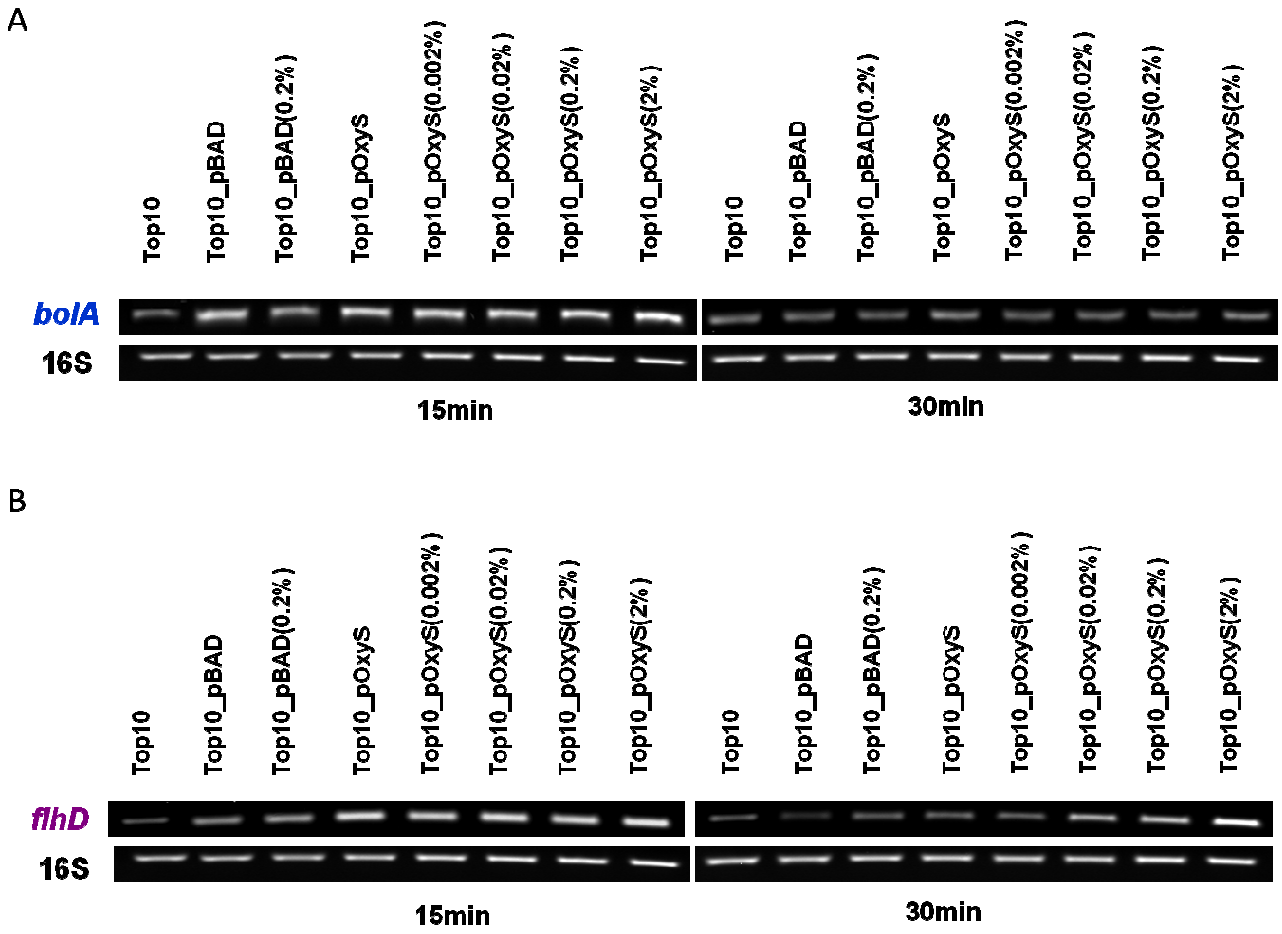

Figure 22 – *bolA and flhD* RT-PCR results for overexpression of Top10pOxyS. A) *bolA* and B) *flhD*. Top10 cultures were transformed with pOxyS (pBAD-*oxyS*) or a vector control (pBADHisA). Cells were grown at 37°C in LB with ampicillin (50 μg/μL) when required. A control with no vector was also grown. At OD_{600} 0.3 cultures were induced with the indicated final arabinose concentrations. RNA transcript levels were visualized at 15 minutes and 30 minutes. 16S transcript levels were used as a control. Overexpression of OxyS RNA had no effect on *bolA* transcript levels but effectively decreased *flhD* transcript levels in induced pRprA cultures at 30 minutes. Bands were visualized on a 1% agarose gel using ethidium bromide and UV light.

Conclusion

We have shown that the small RNA platform can effectively regulate RpoS protein levels both positively and negatively by the overexpression of both RprA and OxyS RNA from an arabinose inducible promoter and that this regulation can be further tuned by varying the concentration of the inducer. Moreover, the system also modulates the expression of both previously shown RpoS-dependent and independent genes. A comparison of the overexpression of RprA to the overexpression of RpoS also shows variations in gene and level of regulation. This is probably partially due to the fact that both systems will recruit different groups of genes for cellular interactions though they both increase RpoS levels. The extent of RpoS protein levels produced in the pRpoS plasmids no doubt elicits many stress response genes to address aggregation and inclusion bodies. RprA does not seem to increase the levels of traditionally identified heat shock proteins upon overproduction. RprA is a native RNA and the short transcripts may not be as taxing on overall cellular functions. Furthermore, RprA is induced upon sensing osmotic shock, meaning while the complete mechanism for how this signal transduction functions is unknown, it would be reasonable to assume that other genes are sequestered to address the particular stress as well. There may be a more specific assembly of genes that RprA enlists. This is another major goal of using such a platform because small RNAs may address stress from a more holistic perspective than trying to affect change at a single point. Assuming the small RNAs controlling RpoS regulation are not redundant than there may be a specific subset of different genes utilized by each sRNA to address its particular stress condition that can be purposefully exploited in this type of inducible

platform. RpoS has so many influences in the cell regulatory network, simply overexpressing RpoS would not necessarily be indicative of the stress that needs to be addressed. The pRpoS system alone may not upregulate the necessary genes to support effective stress relief in a particular condition.

Acknowledgement

I would like to acknowledge Christopher Byrd and Matthew Servinsky from the Army Research Laboratories who processed the DNA microarray.

Chapter 3: Downstream Phenotypic Effects Conferred by the Modulation of RpoS via Small RNA Overexpression Systems

Introduction

Escherichia coli have been used as a model organism for many years now and have garnered a great deal of both fundamental and specific information about cellular function. As more details are elucidated, however, the puzzle seems to grow more complex. Understanding of some of the elementary inner workings has led to the question of how such closely related strains have attained so much diversity, now referred to in some areas as genomic plasticity [154] . While there exist many universal concepts to bacterial regulation there are clearly just as many that have seemed to divergently evolve. This is very prominent when working with K-12 strains as the genomes for wild-type strains, where MG1655, W3110 and MC4100 have all been varied significantly in the laboratory setting even under the same conditions. This diversity has resulted in the realization that though similar, these strains may have differences in their regulatory networks, even within the same strain. Closer examination has revealed that in many cases, differential gene expression was at least partly due to RpoS and its dependent regulon. What has become even more fascinating is the extent to which this gene can influence these disparities in both gene expression and utility.

The evolution of the RpoS regulon has revealed that though its function is highly conserved across species it may be compiled of different genes depending on environmental conditions [155]. RpoS, as a global stress response, has the main goal of maintaining adaptability and survival in times of cellular duress and regulates its

surroundings as such, to the point that it even allows for its own mutation, or loss. Many of the genes in the RpoS regulon are considered nonessential in absence of stress which is why it is so amenable to selective pressure [156]. The ability to sense environmental stimuli and alter gene expression to adapt to dynamic surroundings rationally means RpoS, is dynamic itself, and as such it is difficult, if not impossible, to predict exactly how it will influence the cell to behave in any situation. Despite this many efforts have be launched to discern the expression profile of RpoS in a variety of scenarios under various growth phases (exponential, stationary and in biofilm), stress conditions, in different strains (e.g. MG1655 and MC4100) and media (e.g. LB and M9 minimal) and via different methods (e.g. DNA microarray and *lac*Z-fusions) [7, 50, 52, 55, 111]. While many conserved genes and cellular function have been elucidated, the fact remains that RpoS gene regulation is highly dependent on the given conditions. However, even in stating this fact, it is undeniable that RpoS regulation, and stress response obviously plays an immense role in the genotypic and phenotypic diversity witnessed today.

The duality of the RpoS stress regulation role, which is comprised of both preventive and acute strategies, is implicated in the variety witnessed in traditional, and engineered, cellular functions and behaviors. RpoS alternates these roles and works in concert with other global networks, such as quorum sensing, to affect cross-protection, pathogenicity, motility, biofilm and even in recombinant protein production. As RpoS plays a role in each of these conditions, modulation of RpoS could potentially modulate the given phenotype in turn.

Cross-protection

A major function of the general stress response is preventative. This fact becomes increasingly evident in incidences where microorganisms are exposed to short, sub-lethal doses of one particular stress. These same organisms then develop high resistance to subsequent sources of the same or different stress challenges [157]. Cross- protection is the term ascribed to this phenomenon and is at least partially mediated by σ^S, which renders cells broadly stress resistant [158, 159]. Several prior research efforts have shown that exposure to small amounts of one stress (e.g. carbon starvation, heat shock, metal toxicity, etc.) resulted in reduced hypersensitivity to a secondary stressor due to an amplification of *rpoS* mRNA translation [160].

Biofilm formation and antibiotic resistance

While bacteria grown in the lab is usually studied as planktonic cultures, bacteria by and large naturally exist as sessile, surface-adherent communities called biofilms [161]. Biofilm tolerance is of major importance because it directly relates to the decrease in antibiotic efficacy against acute infections and bacterial pathogenicity [162, 163]. This is of major concern because diverse kinds of polymicrobial infections are biofilm based including implant related infections, dental caries, and respiratory infections [162, 164]. The survival strategy of bacterial biofilms is similar to the stress tolerance experienced by planktonic cultures during stationary phase, including nutrient starvation and cessation of growth [53]. Current work indicates that refractory resistance to antibiotic killing is contributed to by induction of an RpoS-mediated stress response [165, 166]. RpoS potentially contributes to the

diversity that leads to antibiotic-tolerant variants or persistors. RpoS is an attractive target for new antimicrobial strategies because this regulator controls many of the genes important for bacterial adaptation to the host environment. It has been shown that RpoS has both direct and indirect roles in pathogenicity and virulence [167, 168]. This is true also because many stress-associated genes are expressed during biofilm development. In fact stress responses can increase biofilm formation. *RpoS* mutant *E. coli* have been shown to dramatically impaired in biofilm growth [53, 145, 151, 152]. On the other hand, the engineering of biofilms and their robustness has made them ideal candidates for biotechnology applications such as bioremediation, biofuels, the treatment of diseases and BioMEMS devices [169].

Recombinant DNA technology and recombinant protein-induced stress

Recombinant DNA technology has allowed the modification of microorganisms, such as *E. coli*, for the purpose of producing many therapeutic proteins, become the accepted mode of bioprocessing. The host organism then uses its own cellular machinery to manufacture the desired protein usually placed under the control of an inducible promoter system. The expression of this foreign protein however elicits a rapid stress response due to the metabolic burden incurred by the host to perform this additional task [170]. This stress response is partially mediated by σ^S, accompanying the heat shock sigma factor, σ^{32}, which is transiently induced and stabilized during recombinant protein production [171, 172]. A multitude of chaperone proteins and proteases respond to attempt to refold or degrade misfolded foreign proteins. The protein that remains often exists in an aggregated state ultimately decreasing the amount of viable protein available [173]. To this end, most

efforts at optimizing foreign protein production tend to focus on the exponential growth phase of the cells attempting to improve the growth conditions and maximizing protein yield before the stress response transpires (e.g. boosting aeration or nutrient supply). Other efforts focus on combating the stress response itself by transiently downregulating proteases, upregulating the production of chaperone proteins, or both [123]. At the same time, the resistance given to the cell through RpoS regulation creates a very robust organism that is capable of taking over younger cultures. Many successful approaches that help to negate this burden have been to use the intracellular molecules in quorum sensing communication [174]. It has also been shown there are situations when losing RpoS function becomes a selective advantage, or the growth advantage in stationary phase (GASP) phenotype [175]. This is due to the trade-off between growth and proliferation nurtured by σ^{70} and the survival promoted by σ^{S}. Additionally, other studies reveal that in early stationary phase *E. coli* cells are still fit for recombinant protein production probably due to RpoS mutations [176].

As RpoS plays a role in each of these conditions, modulation of RpoS could, in turn, potentially modulate the given associated phenotypes. It is becoming increasingly evident that impactful solutions to these types of issues must account for not only the cell itself but the cell in context of the entire surrounding area, because many cellular systems are working complementarily. The revelation that both the pRprA and pOxyS overexpression platforms can effectually modulate RpoS protein levels and downstream RpoS-dependent and independent genes inspired a study to identify some possible phenotypic effects that could be positively or negatively

modified by either system. For this purpose, we evaluated both platforms in tests of glycogen synthesis, aggregation, motility, biofilm formation and recombinant protein production.

Materials and Methods

Bacterial strains and growth conditions

Bacterial strains used are included in Table 2. Overnight cell cultures were inoculated in 5 mL of Luria-Bertani broth (LB) to an OD_{600} 0.01 in a 15 mL culture tube. Cultures were grown at 37^{o}C with 250 rpm shaking to an OD_{600} 0.3 at which point. Cultures were then induced for RprA or OxyS sRNA transcription with the appropriate concentration of 0.2% L-arabinose.

GFPuv plasmid construction

GFPuv was cloned from pTrcHisGFP [177] with new restriction sites KpnI and EcoRI to be reinserted into the pTrcHisB vector (Invitrogen) with the following primers: sRNAGFP_For (5'- TCG CCC ATG GAC GGT TAT AAA TCA AC- 3') and sRNAGFP_Rev (5'- ACT TAA GCT TAA AAA AAG CCC ATC GT- 3'). The entire segment from the *araC* gene until the transcription terminator site was cloned from both pRprA and pOxyS and inserted into sites of the pTrcHisB vector to give pTrcGFP_pRprA and pTrcGFP_pOxyS respectively with the following primer set: both used forward primer smallRNAKpnI_For (5'- CGT CGG TAC CTT ATG ACA ACT TGA- 3') and reverse primers RprAEcoRI_Rev (5'- CCG GAA TTC AAA AAA AGC CCA TCG') and OxySEcoRI_Rev (5'- CTT GAA TTC AGC GGA TCC TGG AGA-3') correspondingly. GFPuv is induced with 1 mM IPTG.

Motility Assay

Five milliliter cultures were inoculated to an OD_{600} 0.01 from overnight cultures at 37^{o}C and 250 rpm shaking. Cultures were grown to an OD_{600} 0.3 and a 10 μL drop of each culture was inoculated in the middle of individual motility plates (0.5% Bacto-tryptone, 0.5% NaCl, and 0.3% agar). An arabinose concentration of 0.2% was added to induce cultures and cultures were allowed to grow for one hour. 10 μL drops were inoculated on new motility plates. The process was repeated at two hours post-induction. All plates were place in the incubator after inoculation and allowed to grow for 48 hours at 37^{o}C. MG1655 experiments were the same except the induced culture was only allowed to grow for a half an hour because of the arabinose metabolism capability of the strain. These plates were also only incubated for 12 hours due to faster growth.

Biofilm Assay

Five milliliter cultures were inoculated to an OD_{600} 0.01 from overnight cultures at 30^{o}C and 250 rpm shaking. Cultures were grown to an OD_{600} 0.3 at which time 0.2% arabinose was added to induce cultures and all cultures were allowed to grow for one hour. Cultures were diluted to an OD_{600} 0.05 and 200 μL was added to a 96-well microtiter plate with five replicates each. Duplicate plates were made for each point of analysis, 24 hours and 48 hours. Plates were grown at 30^{o}C without shaking for 24 and 48 hours. After taking an OD_{600} reading, plates were washed with distilled water and 0.1% crystal violet was added to each well. After 20 minutes, the crystal violet was washed with DI water and briefly air dried. The biofilm-associated

crystal violet stain was solubilized by 200 μL 95% ethanol and the OD_{540} of the suspension was measured.

Aggregation Assay

Overnight cultures of the strains were adjusted to approximately the same optical density at OD_{600} 1, and 10 ml of each culture was placed in a sterile 15-ml Falcon tube. At the beginning of each experiment, all cultures were vigorously shaken for 10 seconds. Two 100-μl samples were taken from each tube, approximately 1 cm from the top, and transferred to two new tubes, each containing 1 ml of 0.9% NaCl. The OD_{600} was then measured for both samples at the indicted time points.

Glycogen synthesis and iodine vapor staining

Five milliliter cultures were inoculated to an OD_{600} 0.01 from overnight cultures at 37^{o}C and 250 rpm shaking. Cultures were grown to an OD_{600} 0.3 at which time 0.2% arabinose was added to induce cultures and all cultures were allowed to grow for one hour. Each experimental culture was streaked on a Kornberg medium (0.85% KH_2PO_4, 1.1% K_2HPO_4, 0.6% yeast extract, and 1.5% agar) plate in parallel with control cultures. Plates were grown overnight at 37^{o}C and then stored at 4^{o}C for 24 hours. The plates were then stained for 30 seconds with iodine vapor.

Results and Discussion

Glycogen accumulation

glgS is an RpoS-dependent gene responsible for glycogen synthesis in *E. coli*. There is still some speculation of the reason for glycogen accumulation in bacteria but it is thought to provide energy for organisms in unfavorable environments, especially if it can be accumulated. Overexpression of RpoS and consequently *glgS* results in higher glycogen biosynthesis levels that can be visualized by dark brown staining with iodine on glucose-rich Kornberg medium plates. In looking to attain an RpoS-dependent gene phenotype we tested for glycogen levels expressed in pRprA and pOxyS induced cultures in both Top10 and MG1655. Figure 23 reveals the darkest brown for Top10 and both pBAD cultures. The pRprA containing cultures exhibit slightly less glycogen accumulation, indicating a lower level of RpoS or an indirect effect on or production. On the other hand, Top10_pOxyS cultures, whether induced or not, contain much lower glycogen levels than any other producing cultures. This result suggests that RpoS may really be repressed upon expression of OxyS. There is also a level of leaky expression from the vectors without the addition of arabinose. This constitutive expression seems to be enough to repress glycogen synthesis. The same process was repeated with the MG1655 and in this case while there was no difference between induced and uninduced cultures the pRprA cultures were the same as the controls (Figure 24A). Interestingly, in MG1655 the pOxyS cultures have the opposite effect and actually stain darker indicating the activation, instead of repression, of more RpoS and an increase in *glgS* and glycogen

Figure 23 – Glycogen synthesis in Top10pRprA and Top10pOxyS. Top10 was transformed with pRprA (pBAD-*rprA*), pOxyS (pBAD-*oxyS*) or a vector control (pBADHisA). Cells were grown at 37^{o}C in LB with ampicillin broth. A control with no vector was also grown. At OD_{600}0.3 cultures the indicated cultures were induced with a 0.2% final concentration of arabinose. At 1 HPI cultures were plated on Kornberg medium plates. Plates were grown at 37^{o}C overnight, stored at 4^{o}C for 24 hours and vapor stained with iodine. The intensity of the brown stain reveals the extent of glycogen accumulation. There was no obvious difference between cultures that were induced or noninduced.

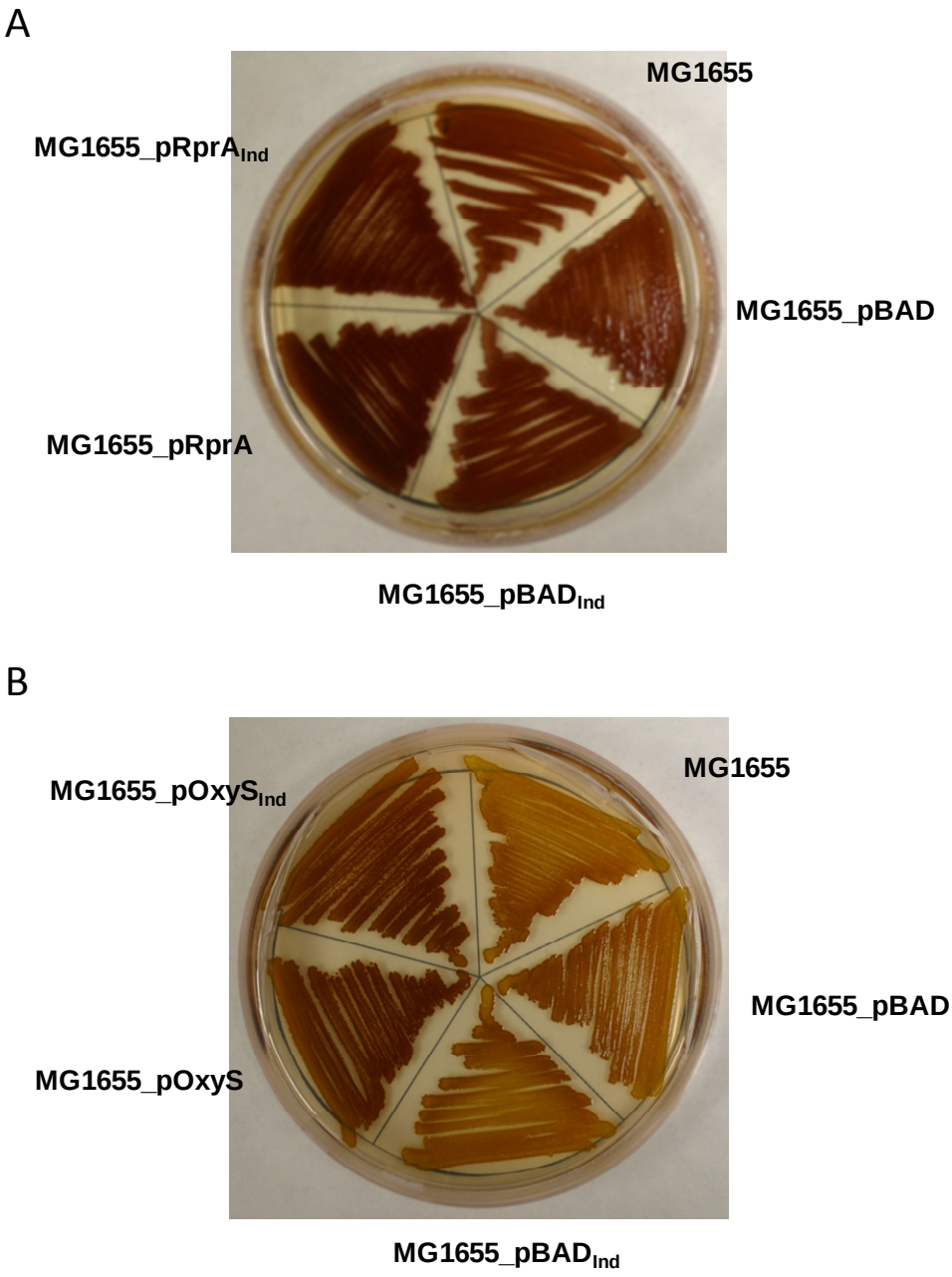

Figure 24 – Glycogen synthesis in MG1655pRprA and MG1655pOxyS. MG1655 was transformed with pRprA (pBAD-*rprA*), pOxyS (pBAD-*oxyS*) or a vector control (pBADHisA). Cells were grown at 37^{o}C in LB with ampicillin broth. A control with no vector was also grown. At OD_{600}0.3 cultures the indicated cultures were induced with a 0.2% final concentration of arabinose. At 0.5 HPI cultures were plated on Kornberg medium plates. Plates were grown at 37^{o}C overnight, stored at 4^{o}C for 24 hours and vapor stained with iodine. The intensity of the brown stain reveals the extent of glycogen accumulation in A) pRprA and B) pOxyS in MG1655. There was no obvious difference between cultures that were induced or noninduced.

accumulation. This indicates again that the same expression system has the capabilities of altering strains differently.

The small RNA overexpression system effects motility

Bacteria that move seem to try to in a purposeful way, especially in response to their surroundings. They are capable of moving away from toxins or towards nutrients in order to survive their ever changing settings. In the evaluation of the gene expression profiles in the sRNA platforms, *flhD*, was regulated in both strains and in opposite directions, a decrease for the activator and an increase for the repressor. *flhD* is part of the Class I master transcriptional regulator operon *flhDC*for flagellar synthesis. The Class I genes activate the Class II genes that encode the structural genes , which then activate the Class III genes that are responsible for chemotaxis and flagellar filament [142]. In light of these findings we chose to do motility tests to see is this translated into a logical motility phenotype. Each of the expression systems, pRprA and pOxyS were compared to control strains, Top10, Top10pBAD and a 0.2% induced Top10pBAD culture. The expression systems both had an induced and uninduced culture. Prior to, as well as, 1 and 2 hours after arabinose induction, a small drop was placed on motility plates and allowed to grow for 48 hours. There was no detectable difference in growth at 24 hours. Figure 25A displays the qualitative differences in the motility halos of each test culture at 48 hours post-induction. While initially all the cultures displayed large colony sizes, after induction, the cultures induced to overexpress RprA, had very small colonies. Comparison of the average colony diameters reveals that after induction, the pRprA

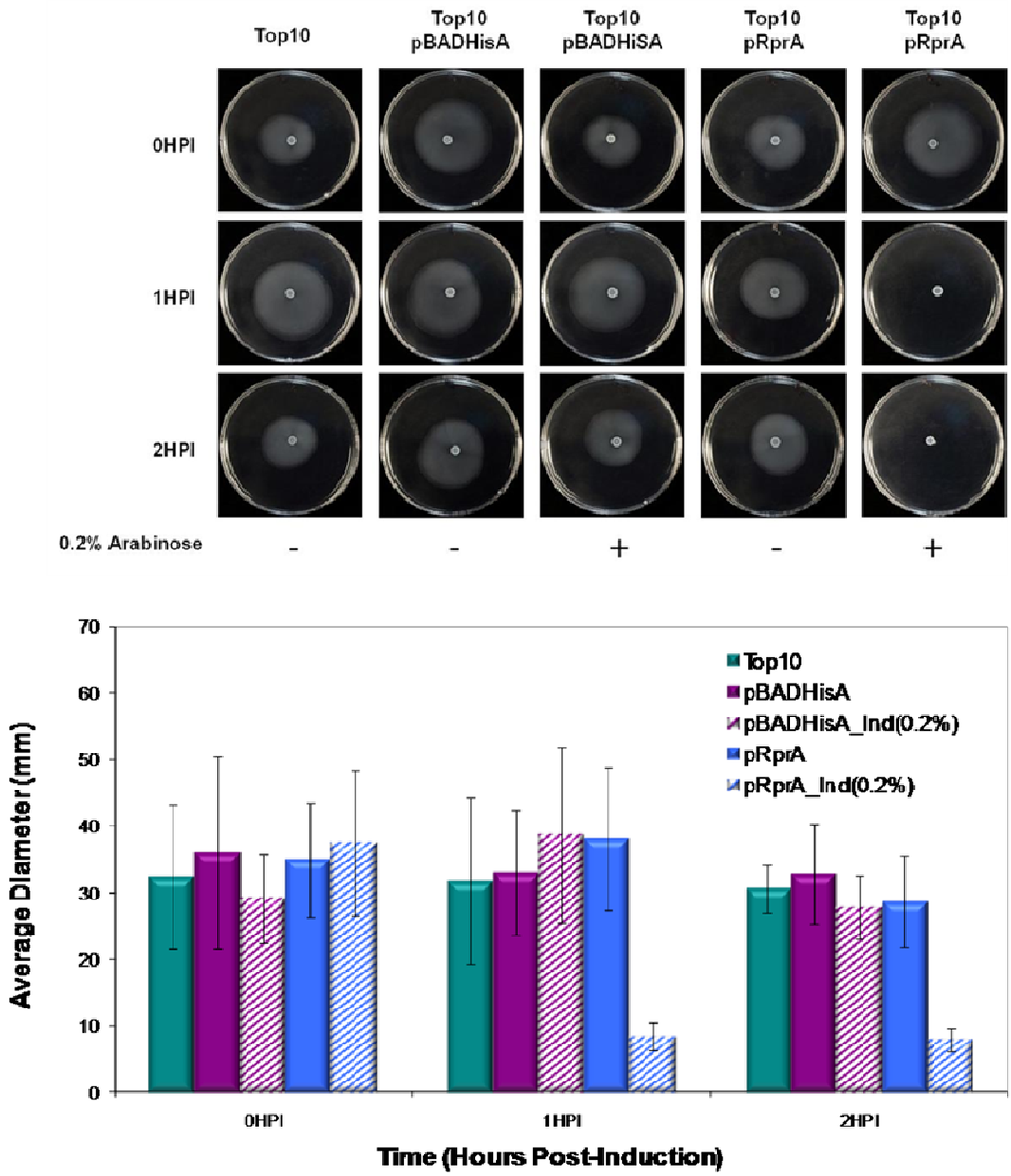

Figure 25 – Motility assay for Top10pRprA. A) Qualitative differences in motility halos grown at 30°C for 48 HPI on motility agar. B) Average diameters of motility halos prepared at each time post-induction. Values are the average of 2 to 4 plates from three independent experiments. Error bars represent the standard of deviation.

cultures are three fold lower than all the other cultures tested (Figure 25B). The same experiment with pOxyS showed no discernable differences in halo size.

The same experiment was repeated for MG1655 except plates were only prepared for one hour post-induction and the plates were grown for only 12 hours. Growth in MG1655 can be almost twice as fast as Top10. This is also reflected in the generally larger size of the halos. In Figure 26A, a quantitative representation of the differences in motility halos is shown. A very modest decrease may be detected in the induced pRprA culture. The same is true for both of the pOxyS cultures Figure 26B. The results from either expression system in MG1655 were not as dramatic as Top10pRprA. This strain also seemed to have greater instances of fluctuation that masked the actual behavioral differences (i.e. large error bars). The addition of either of the small RNA expression systems had no effect when induced in W3110, this too could be due to the lack of detectable RpoS, or perhaps at least a truncated form.

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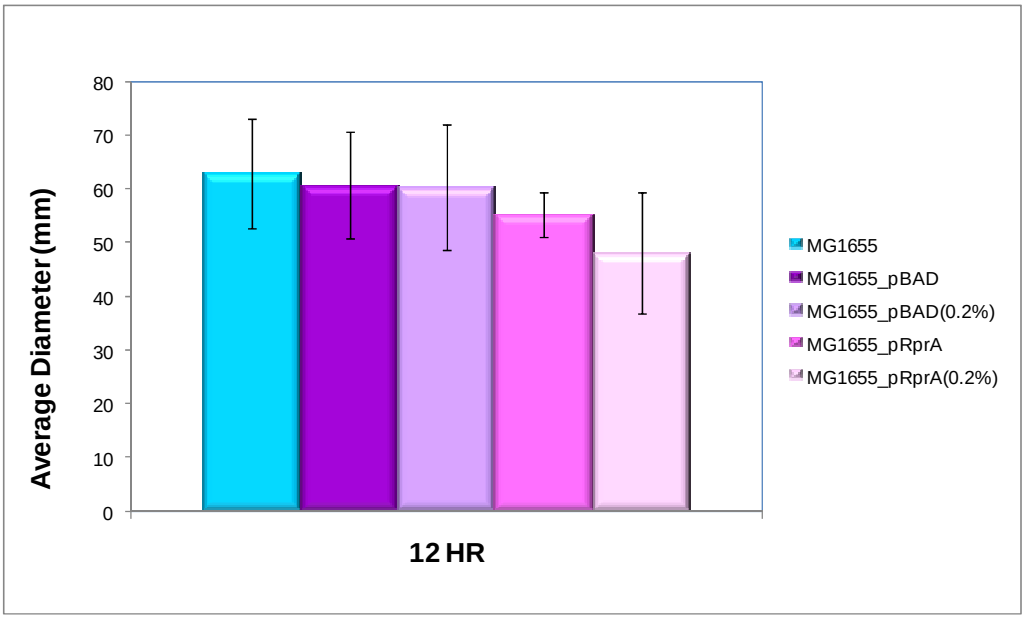

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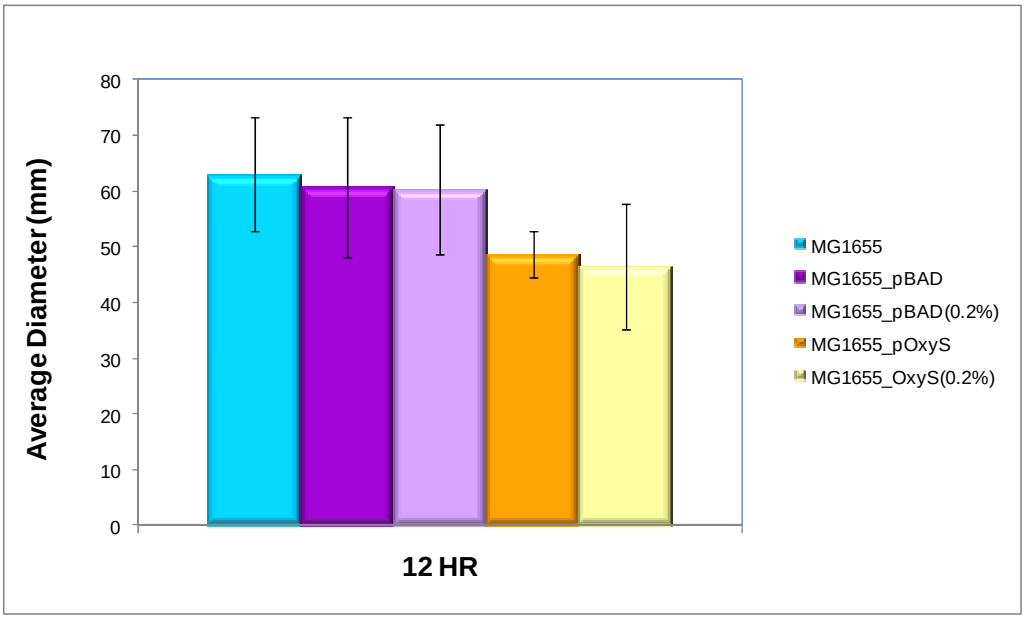

Figure 26 – Motility assay for MG1655 with A) pRprA and B) pOxyS. Average diameter measurements of motility halos of cultures inoculated on motility agar grown for 12 hours post-induction. Values are the average of 2 to 4 plates from three independent experiments. Error bars represent the standard of deviation.

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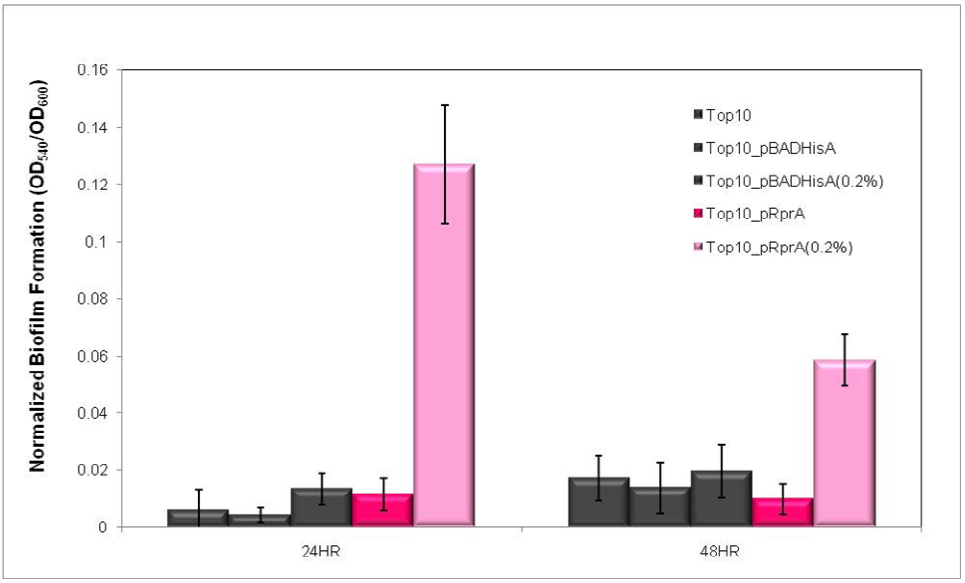

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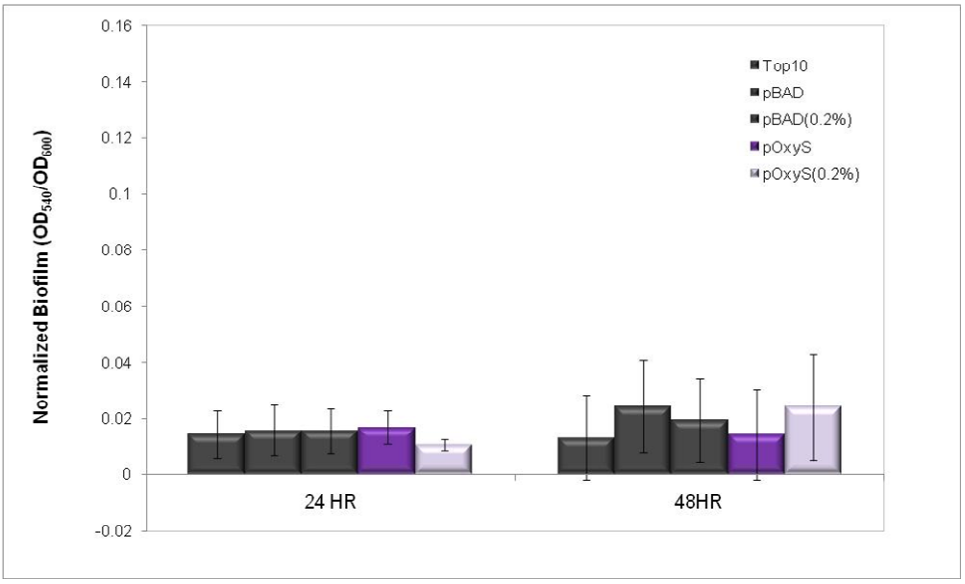

Figure 27 – Biofilm formation assay for Top10. A) pRprA and B) pOxyS. Biofilm was grown in LB in 96-well plates at 30°C with no shaking for the indicated time points. The biofilm was stained with 0.1% crystal violet and solubilized with 95% EtoH. The solubilized biomass was measured at OD_{540}. Reported biofilm is biomass measured at OD_{540} normalized to the OD_{600} value for that well at 24 or 48 hours. Values are the average of two plates with five replicates from at least two independent experiments. Error bars represent the standard of deviation

The pRprA expression system also has an effect on biofilm formation

If the pRprA had an effect on motility it would be reasonable to suspect a difference in biofilm formation, considering flagellar genes are required for biofilm formation. For the biofilm assay cultures were set up as described in the Materials and Methods section. The results are presented in Figure 28A and B, for pRprA and pOxyS respectively. While there is no difference in biofilm formation at all in the pOxyS cultures in Top10. An induced pRprA culture is almost 12-fold higher than the control cultures at 24 hours and almost 6-fold higher at 48 hours. This corresponds to what would be expected in cultures that both showed a decrease in *flhD* and motility.

As with the RT-PCR and Western blot data, we wanted to see if this huge increase in biofilm formation was solely due to an overexpression of RpoS protein levels. The biofilm assay was repeated comparing pRprA and pRpoS and the results are displayed in Figure 28. While the increase in biofilm formation previously observed in Top10pRprA cultures is present, a direct induction of RpoS alone does not increase biofilm formation. This also suggests what may be another advantage to increasing RpoS protein levels via sRNA overexpression systems is the potential to more effectively recruit other genes to facilitate the control. Overexpressing small RNAs mimics what may occur in the cell naturally when a stress is sensed.

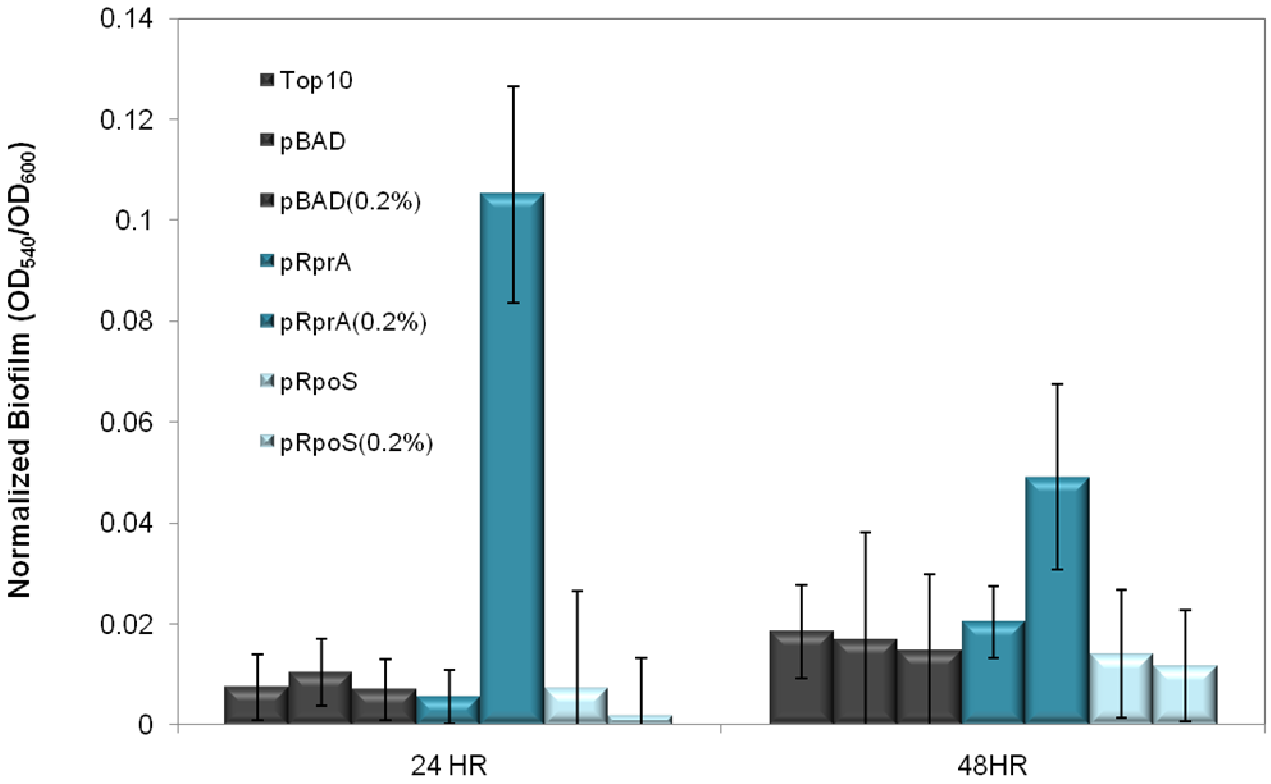

Figure 28 – Biofilm formation assay for Top10pRprA and Top10pRpoS. Biofilm was grown in LB in 96-well plates at 30°C with no shaking for the indicated time points. The biofilm was stained with 0.1% crystal violet and solubilized with 95% EtoH. The solubilized biomass was measured at OD_{540}. Reported biofilm is biomass measured at OD_{540} normalized to the OD_{600} value for that well at 24 or 48 hours. Values are the average of two plates with five replicates from at least two independent experiments. Error bars represent the standard of deviation.

The experiment was repeated with each system in the MG1655 strain. Figure 29A shows that the presence of any vector in this strain decreases biofilm formation regardless of induction, pBAD or pRprA. MG1655 is almost six times higher than all of the other cultures. MG1655, as the wild type, does produce good biofilm so it's interesting that a plasmid reduces it to barely detectable. Perhaps just the presence of the vector in this strain at 30^{o}C results in another stress and stress factor being recruited, confirming that several coordinated factors go into the formation of biofilm other than just the presence of RpoS. The biofilm formation is however recovered with the pOxyS expression system (Figure 29B). This is in contrast with what is expected from the repressor system but in line with the other phenotypes witnessed in this strain. It has also been shown recently that the regulation of motility is actually dependent on antagonistic control between RpoS and σ^{54}, RpoN which also controls flagellar genes. While a mutant of *rpoS* or *rpoN*, results in decreased and increased motility respectively, a double mutant abolishes motility [178]. Biofilm formation in W3110 remained unchanged from the wild type and in MC4100 biofilm formation only occurred in cultures induced with arabinose (not shown).

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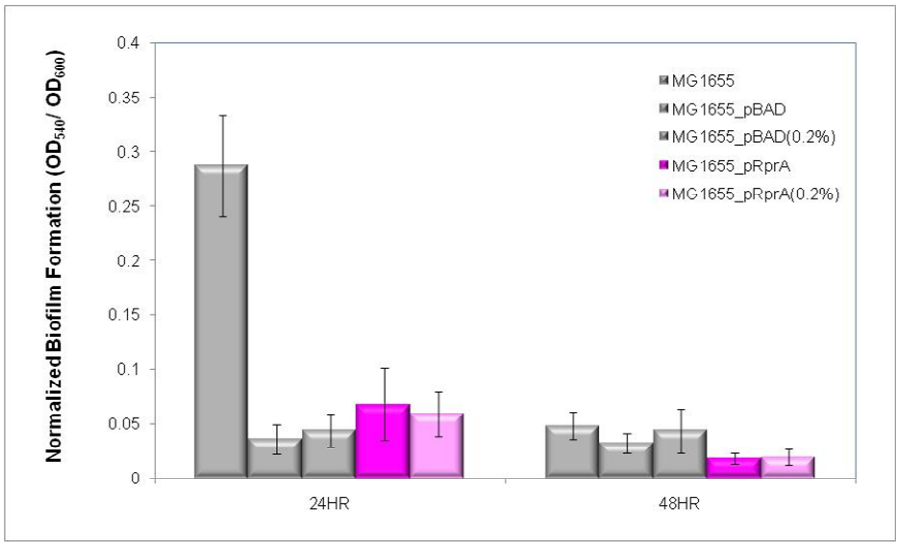

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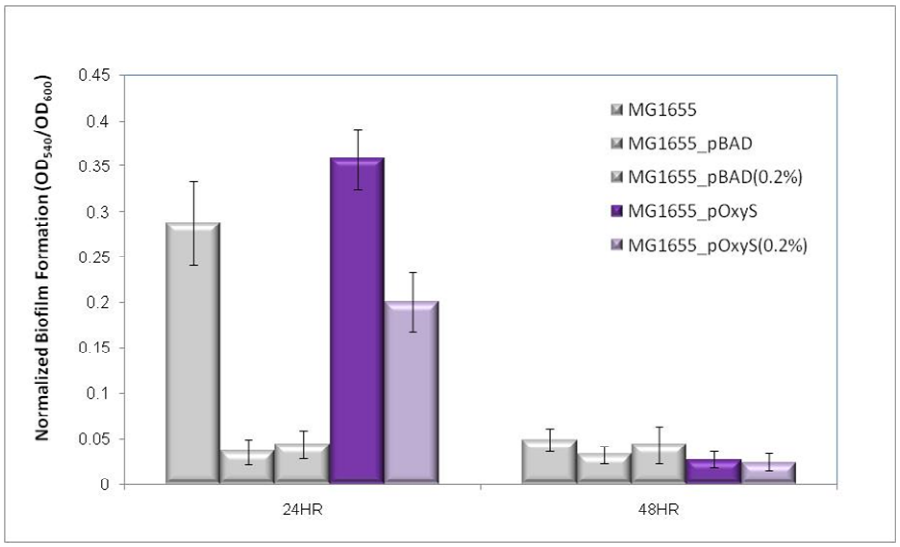

Figure 29 – Biofilm formation assay for MG1655. A) pRprA and B) pOxyS Biofilm was grown in LB in 96-well plates at 30°C with no shaking for the indicated time points. The biofilm was stained with 0.1% crystal violet and solubilized with 95% EtoH. The solubilized biomass was measured at OD_{540}. Reported biofilm is biomass measured at OD_{540} normalized to the OD_{600} value for that well at 24 or 48 hours. Values are the average of two plates with five replicates from at least two independent experiments. Error bars represent the standard of deviation

Autoaggregation and pRprA and pOxyS systems

Unexpectedly while performing other experiments, we noticed that there seemed to be a difference in settling from overnight culture in strain MC4100 with cultures containing pRprA remaining suspended long after the other cultures had settled. A study on biofilm and quorum sensing by our lab had revealed a role for the flu encoded, antigen 43 (Ag43) [179]. We decide we would perform an aggregation assay on this system. Experiments were conducted as described in the Materials and Methods but briefly overnight cultures were resuspended to OD_{600} 1. 100 μl samples were taken at the indicated time points and added to 1ml of 0.95 NaCl and the OD_{600} was read. As we suspected cultures containing the pRprA vector experienced a slower aggregation rate than the other cultures with the induced culture being almost 4 times slower than controls at its highest point (Figure 30A). The uninduced culture aggregated slightly faster. While the aggregation of the control cultures was resolved in about 24 hours, it took 2 days for the induced pRprA culture. The uninduced culture initially started off as high as the induced but by 36 hours it had settled as well, appearing to have a much steeper slope. Induced pOxyS culture experience about a 2-fold slower difference in aggregation rate as compared to the other controls but was also resolved by 24 hours (Figure 30B). If Ag43 positively mediates cell-cell aggregation and biofilm [180], this shows how RpoS is working in concert with many other genes to affect protective habits and survive. This gene could also have a part in the biofilm effect witnessed earlier with pRprA but also proves how much strains and conditions play a part in the level of regulation achieved.

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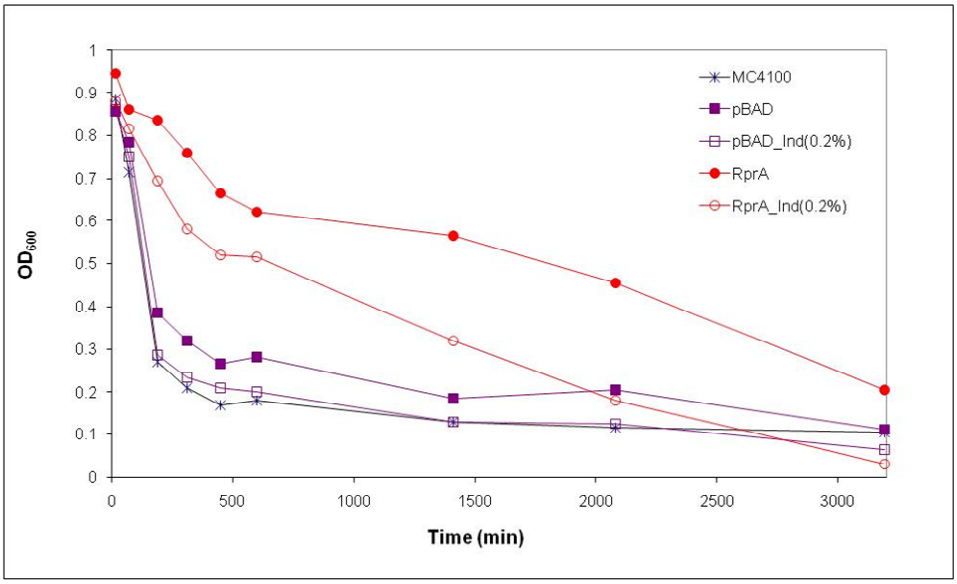

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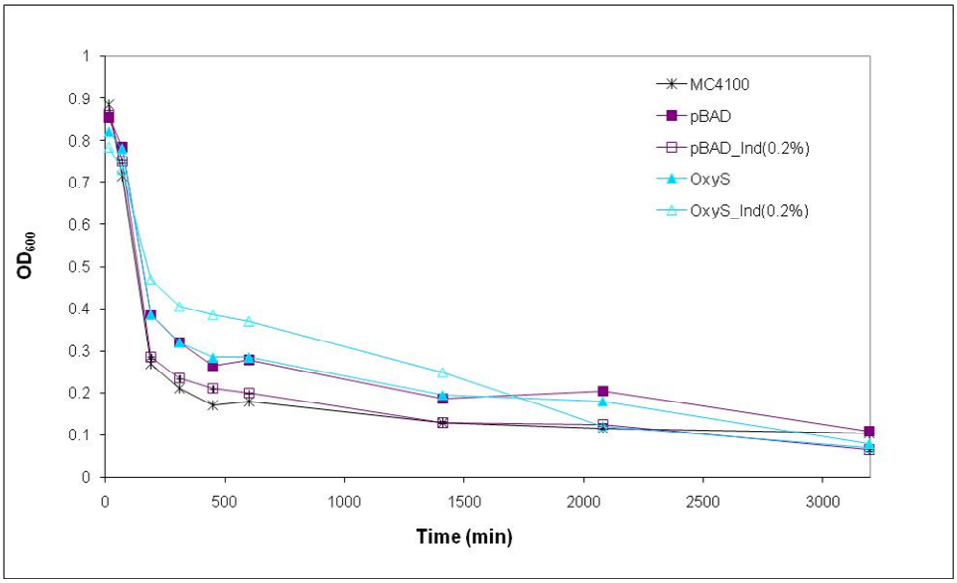

Figure 30 –Aggregation assay for MC4100. A) pRprA and B) OxyS. Time-resolved differences in rates of cell sedimation (see Materials and Methods). Values are the average of triplicate readings. Error bars represent the standard of deviation.

pRprA may attenuate GFPuv recombinant protein production

Finally, we wanted to test if maybe the overexpression of either expression platform could increase recombinant protein production, either by cross-protection or by repressing RpoS so that σ^{70} could continue to grow and proliferate. Figures 31A and B show that induction of either pRprA or pOxyS did not significantly increase protein production. In the pOxyS 0.2% culture, only 10HPI shows a modest increase in GFP production (Figure 31B). The lack of significant enhancement could signify that the pRprA and pOxyS systems are not useful for increasing recombinant protein production, even if the system may be increasing stress protective genes. However, it does seem that GFP production seems to be attenuated with increase of expression. By 10 hours all of the cultures share similar OD_{600} readings except for 2%. Perhaps the system can be considered a negative switch. Furthermore, GFPuv heterologous protein production has been shown to be very high and fairly soluble naturally. It could be that there was not much room left for optimization using the sRNA platform systems. To get a true evaluation of the system it must be tested further with other recombinant proteins.

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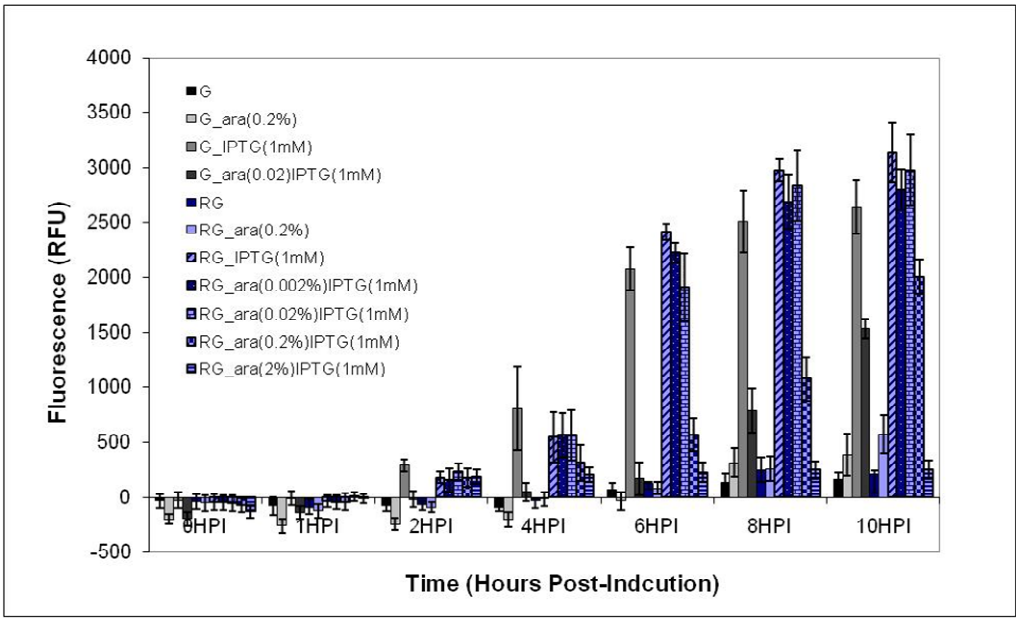

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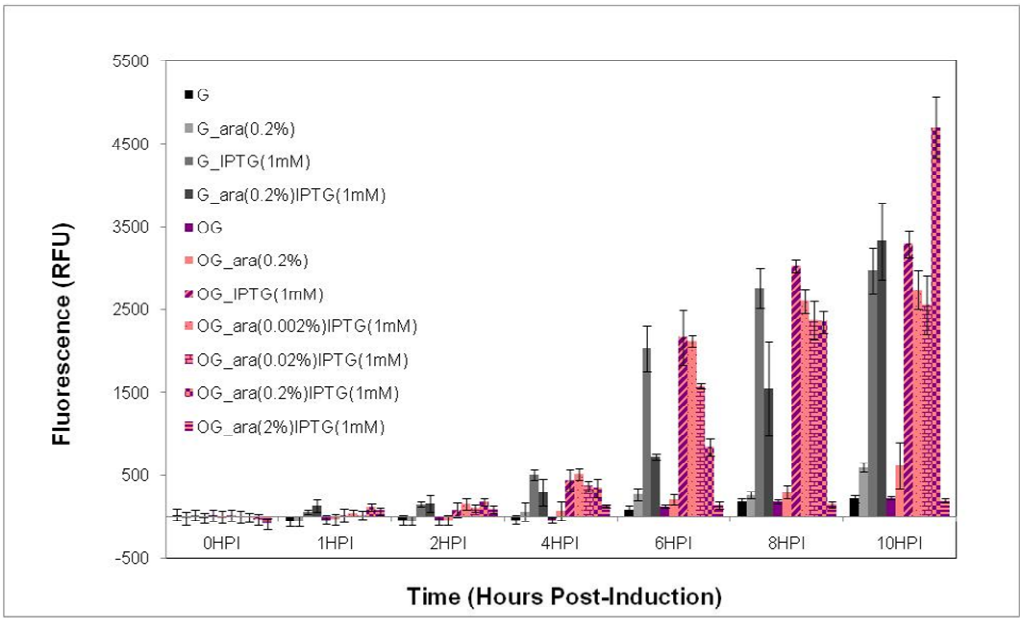


Figure 30 – Fluorescence for GFPuv recombinant protein production in MG1655 for A) pRprA and B) OxyS. Values represent experimental fluorescence value minus the background fluorescence of LB and are the average of triplicate readings from three independent studies. Error bars represent the standard of deviation.

Conclusion

In addition to controlling the genetic expression profile for RpoS, the pRprA and pOxyS riboregulatory platforms can effectively increase and decrease RpoS levels and effect downstream genes, even some which have not been identified as RpoS-dependent. This is due to both the polymorphic nature of RpoS and how it will adapt to its surroundings. Even with the same level of RpoS by different stresses, regulatory mechanisms will be different depending on the state of the environment. Depending on the sRNA used in the system, RprA or OxyS, the concentration of inducer and the strain we saw altered regulation of glycogen accumulation, motility, biofilm formation and potentially attenuation of recombinant protein production.

Chapter 4: Conclusions and Future Work

General Conclusions

We have successfully engineered an inducible sRNA riboregulatory platform that can be changed to direct desired phenotypic events. Explicit overexpression of different sRNAs allowed for the transient increase/attenuation of RpoS protein levels that altered the gene expression profile of the cell. We were able to show that in this time we could also upregulate a protective RpoS-dependent, *glgS*, which provides some evidence that conceivably we can purposefully upregulate other protective genes and enhance bacterial cell fitness. Concomitantly, this regulation was extended to specifically affect cell motility and aggregation, showing the capacity to direct the regulation of the cell's most fundamental behaviors for survival. From this study, one of the most exciting modes of modulation was in control of biofilm formation, which has also been shown to influence biofilm formation and architecture. If biofilms could be controlled by genetic manipulation then they could be formed at specific locations and engineered to treat disease or produce certain chemicals. Due to the protective state of biofilms and their robustness, they facilitate enhanced gene transfer and communication signaling. This has been successfully used to create biofilm reactors used for bioremediation of waste water and industrial streams using bacterial degradation of pollutants. Engineered biofilms have been used for delivery of drugs and nutrients. These biofilm have also been used for treatment of bacterial biofilm based diseases, like cystic fibrosis, to out compete the offending biofilm for survival

in patients suffering from the disease. Biofilm reactors have also been used for biofuel production.

The biggest limitation of the sRNA regulatory expression platform is that there will likely be altered expression of genes that result in unintended effects, especially considering the mechanisms of these small RNAs are still being investigated. Nonetheless, while the complete mechanism for how this signal transduction functions is unknown, it would be logical to assume that other genes are sequestered to address the particular stress as well. There may be a more specific assembly of genes that small RNAs enlist that are not solicited by overexpressing a single gene. Additionally, this gene set can potentially be changed to suit a desired application by changing the sRNA employed and changing inducer concentration. This is the major goal of using such a platform because small RNAs may address stress from a more holistic perspective than trying to affect change at a single point effecting global regulation. The inducible sRNA regulatory platform has shown that gene regulation can be globally tuned to have a range of physiological effects using variable amounts of inducer and diverse sRNA overexpression systems, providing tremendous potential for metabolic engineering and biotechnology applications.

Future Directions

Though this system needs more characterization it has shown promise in influencing RpoS regulation. It would be ideal to see a microarray done for the pOxyS system to get a complete genetic profile. Moreover, I think this work shows

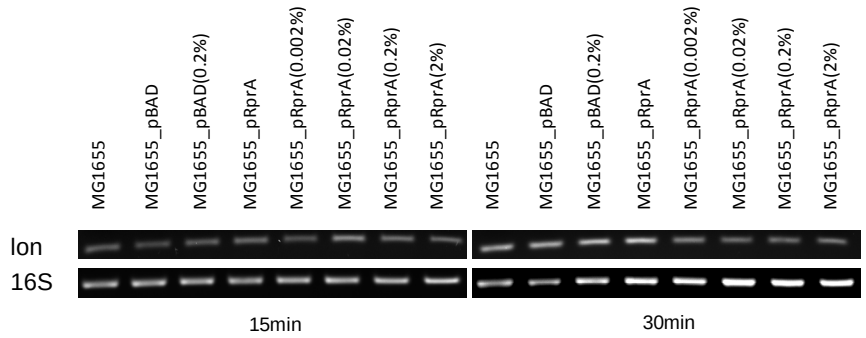
the promise in using microarrays for determining targets of small RNAs. Traditionally, gene regulatory functions are assessed by comparing the wild type expression to a mutant of that gene. This is not practical for small RNAs because they are not active unless a stress is encountered, so any changes in genetic profile would hardly be discernable. The small RNA overexpression system shows the promise in using microarray to collect a gene profile of a least potential targets for small RNAs provided proper controls are used. This was just employed by Susan Gottesman in the identification of a small RNA involved in *luxS* and quorum sensing regulation [181]. In addition, investigating the use of other inducers both chemical and natural would be desirable. This would allow even more fine tuning for particular systems. The idea that strain differences can be so dramatically alter gene expression profiles makes it interesting to evaluate the *rpoS* gene from one strain in another strain. One can then compare the potential differences of the same *rpoS* allele in different strains. This will be telling to see how many other systems are involved in many RpoS related behaviors. Finally, to create an optimized system by having different activator/repressor combinations would find many biotechnology applications.

Prospects for the future

The fact that RpoS has four sRNAs regulating its translation, under different conditions, raises the question of whether this phenomenon is distinctive, or indicative of a much greater potential for sRNA regulation. Knowledge garnered here may help to aid research that elucidates the function of the many sRNAs

identified with no known function, revealing higher levels of mechanistic regulation that affect both prokaryotic and eukaryotic global gene expression.

Appendix


MG1655
MG1655_pBAD
MG1655_pBAD(0.2%)
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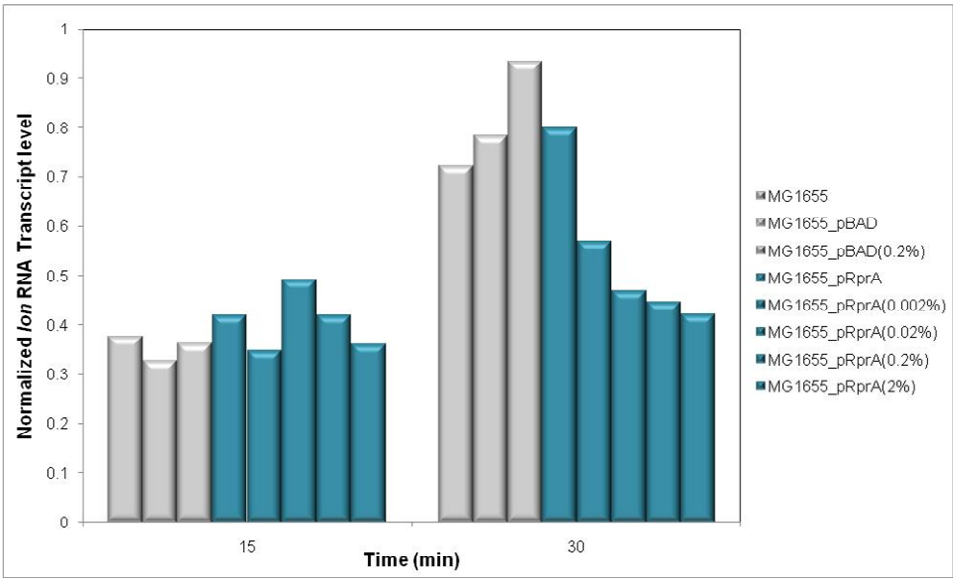
Normalized lon RNA Transcript level
1
0.9
0.8
0.7
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0.4
0.3
0.2
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MG1655
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Bibliography

1. Burgess RR: **Separation and characterization of the subunits of ribonucleic acid polymerase**. *The Journal of biological chemistry* 1969, **244**(22):6168-6176.
2. Zhang G, Darst SA: **Structure of the Escherichia coli RNA polymerase alpha subunit amino-terminal domain**. *Science* 1998, **281**(5374):262-266.
3. Wang Y, Severinov K, Loizos N, Fenyo D, Heyduk E, Heyduk T, Chait BT, Darst SA: **Determinants for Escherichia coli RNA polymerase assembly within the beta subunit**. *J Mol Biol* 1997, **270**(5):648-662.
4. Wigneshweraraj SR, Burrows PC, Severinov K, Buck M: **Stable DNA opening within open promoter complexes is mediated by the RNA polymerase beta'-jaw domain**. *J Biol Chem* 2005, **280**(43):36176-36184.
5. Ghosh P, Ishihama A, Chatterji D: **Escherichia coli RNA polymerase subunit omega and its N-terminal domain bind full-length beta' to facilitate incorporation into the alpha2beta subassembly**. *European journal of biochemistry / FEBS* 2001, **268**(17):4621-4627.
6. Mukherjee K, Chatterji D: **Studies on the omega subunit of Escherichia coli RNA polymerase--its role in the recovery of denatured enzyme activity**. *European journal of biochemistry / FEBS* 1997, **247**(3):884-889.
7. Weber H, Polen T, Heuveling J, Wendisch VF, Hengge R: **Genome-wide analysis of the general stress response network in Escherichia coli: sigmaS-dependent genes, promoters, and sigma factor selectivity**. *J Bacteriol* 2005, **187**(5):1591-1603.
8. Burgess RR, Travers AA, Dunn JJ, Bautz EK: **Factor stimulating transcription by RNA polymerase**. *Nature* 1969, **221**(5175):43-46.
9. Lange R, Hengge-Aronis R: **Identification of a central regulator of stationary-phase gene expression in Escherichia coli**. *Mol Microbiol* 1991, **5**(1):49-59.
10. McCann MP, Kidwell JP, Matin A: **The putative sigma factor KatF has a central role in development of starvation-mediated general resistance in Escherichia coli**. *J Bacteriol* 1991, **173**(13):4188-4194.
11. Reitzer L, Schneider BL: **Metabolic context and possible physiological themes of sigma(54)-dependent genes in Escherichia coli**. *Microbiol Mol Biol Rev* 2001, **65**(3):422-444, table of contents.
12. Grossman AD, Erickson JW, Gross CA: **The htpR gene product of E. coli is a sigma factor for heat-shock promoters**. *Cell* 1984, **38**(2):383-390.
13. Nonaka G, Blankschien M, Herman C, Gross CA, Rhodius VA: **Regulon and promoter analysis of the E. coli heat-shock factor, sigma32, reveals a multifaceted cellular response to heat stress**. *Genes Dev* 2006, **20**(13):1776-1789.
14. Arnosti DN, Chamberlin MJ: **Secondary sigma factor controls transcription of flagellar and chemotaxis genes in Escherichia coli**. *Proc Natl Acad Sci U S A* 1989, **86**(3):830-834.
15. Helmann JD: **Alternative sigma factors and the regulation of flagellar gene expression**. *Mol Microbiol* 1991, **5**(12):2875-2882.

16. Rezuchova B, Miticka H, Homerova D, Roberts M, Kormanec J: **New members of the Escherichia coli sigmaE regulon identified by a two-plasmid system**. *FEMS microbiology letters* 2003, **225**(1):1-7.
17. Dartigalongue C, Missiakas D, Raina S: **Characterization of the Escherichia coli sigma E regulon**. *J Biol Chem* 2001, **276**(24):20866-20875.
18. Mahren S, Braun V: **The FecI extracytoplasmic-function sigma factor of Escherichia coli interacts with the beta' subunit of RNA polymerase**. *Journal of bacteriology* 2003, **185**(6):1796-1802.
19. Paget MS, Helmann JD: **The sigma70 family of sigma factors**. *Genome Biol* 2003, **4**(1):203.
20. Lonetto M, Gribskov M, Gross CA: **The sigma 70 family: sequence conservation and evolutionary relationships**. *Journal of bacteriology* 1992, **174**(12):3843-3849.
21. Merrick MJ: **In a class of its own--the RNA polymerase sigma factor sigma 54 (sigma N)**. *Mol Microbiol* 1993, **10**(5):903-909.
22. Ishihama A: **Functional modulation of Escherichia coli RNA polymerase**. *Annu Rev Microbiol* 2000, **54**:499-518.
23. Maeda H, Fujita N, Ishihama A: **Competition among seven Escherichia coli sigma subunits: relative binding affinities to the core RNA polymerase**. *Nucleic Acids Res* 2000, **28**(18):3497-3503.
24. Grigorova IL, Phleger NJ, Mutalik VK, Gross CA: **Insights into transcriptional regulation and sigma competition from an equilibrium model of RNA polymerase binding to DNA**. *Proceedings of the National Academy of Sciences of the United States of America* 2006, **103**(14):5332-5337.
25. Kawakami K, Saitoh T, Ishihama A: **Biosynthesis of RNA polymerase in Escherichia coli. IX. Growth-dependent variations in the synthesis rate, content and distribution of RNA polymerase**. *Mol Gen Genet* 1979, **174**(2):107-116.
26. Jishage M, Ishihama A: **Regulation of RNA polymerase sigma subunit synthesis in Escherichia coli: intracellular levels of sigma 70 and sigma 38**. *J Bacteriol* 1995, **177**(23):6832-6835.
27. Raffaelle M, Kanin EI, Vogt J, Burgess RR, Ansari AZ: **Holoenzyme switching and stochastic release of sigma factors from RNA polymerase in vivo**. *Mol Cell* 2005, **20**(3):357-366.
28. Jishage M, Iwata A, Ueda S, Ishihama A: **Regulation of RNA polymerase sigma subunit synthesis in Escherichia coli: intracellular levels of four species of sigma subunit under various growth conditions**. *J Bacteriol* 1996, **178**(18):5447-5451.
29. Gentry DR, Hernandez VJ, Nguyen LH, Jensen DB, Cashel M: **Synthesis of the stationary-phase sigma factor sigma s is positively regulated by ppGpp**. *J Bacteriol* 1993, **175**(24):7982-7989.
30. Jishage M, Kvint K, Shingler V, Nystrom T: **Regulation of sigma factor competition by the alarmone ppGpp**. *Genes Dev* 2002, **16**(10):1260-1270.
31. Laurie AD, Bernardo LM, Sze CC, Skarfstad E, Szalewska-Palasz A, Nystrom T, Shingler V: **The role of the alarmone (p)ppGpp in sigma N**

competition for core RNA polymerase. *J Biol Chem* 2003, **278**(3):1494-1503.
32. Costanzo A, Ades SE: **Growth phase-dependent regulation of the extracytoplasmic stress factor, sigmaE, by guanosine 3',5'-bispyrophosphate (ppGpp)**. *J Bacteriol* 2006, **188**(13):4627-4634.
33. Gruber TM, Gross CA: **Assay of Escherichia coli RNA polymerase: sigma-core interactions**. *Methods in enzymology* 2003, **370**:206-212.
34. Gruber TM, Gross CA: **Multiple sigma subunits and the partitioning of bacterial transcription space**. *Annu Rev Microbiol* 2003, **57**:441-466.
35. Nystrom T: **Growth versus maintenance: a trade-off dictated by RNA polymerase availability and sigma factor competition?** *Mol Microbiol* 2004, **54**(4):855-862.
36. Kvint K, Farewell A, Nystrom T: **RpoS-dependent promoters require guanosine tetraphosphate for induction even in the presence of high levels of sigma(s)**. *J Biol Chem* 2000, **275**(20):14795-14798.
37. Typas A, Barembruch C, Possling A, Hengge R: **Stationary phase reorganisation of the Escherichia coli transcription machinery by Crl protein, a fine-tuner of sigmas activity and levels**. *Embo J* 2007, **26**(6):1569-1578.
38. Pratt LA, Silhavy TJ: **Crl stimulates RpoS activity during stationary phase**. *Mol Microbiol* 1998, **29**(5):1225-1236.
39. Gaal T, Ross W, Estrem ST, Nguyen LH, Burgess RR, Gourse RL: **Promoter recognition and discrimination by EsigmaS RNA polymerase**. *Mol Microbiol* 2001, **42**(4):939-954.
40. Hengge-Aronis R: **Stationary phase gene regulation: what makes an Escherichia coli promoter sigmaS-selective?** *Curr Opin Microbiol* 2002, **5**(6):591-595.
41. Becker G, Hengge-Aronis R: **What makes an Escherichia coli promoter sigma(S) dependent? Role of the -13/-14 nucleotide promoter positions and region 2.5 of sigma(S)**. *Mol Microbiol* 2001, **39**(5):1153-1165.
42. Bordes P, Conter A, Morales V, Bouvier J, Kolb A, Gutierrez C: **DNA supercoiling contributes to disconnect sigmaS accumulation from sigmaS-dependent transcription in Escherichia coli**. *Mol Microbiol* 2003, **48**(2):561-571.
43. Yuan AH, Gregory BD, Sharp JS, McCleary KD, Dove SL, Hochschild A: **Rsd family proteins make simultaneous interactions with regions 2 and 4 of the primary sigma factor**. *Mol Microbiol* 2008, **70**(5):1136-1151.
44. Ilag LL, Westblade LF, Deshayes C, Kolb A, Busby SJ, Robinson CV: **Mass spectrometry of Escherichia coli RNA polymerase: interactions of the core enzyme with sigma70 and Rsd protein**. *Structure* 2004, **12**(2):269-275.
45. Wassarman KM, Storz G: **6S RNA regulates E. coli RNA polymerase activity**. *Cell* 2000, **101**(6):613-623.
46. Trotochaud AE, Wassarman KM: **6S RNA function enhances long-term cell survival**. *J Bacteriol* 2004, **186**(15):4978-4985.
47. Nystrom T: **Stationary-phase physiology**. *Annu Rev Microbiol* 2004, **58**:161-181.

48. Hengge-Aronis R: **Signal transduction and regulatory mechanisms involved in control of the sigma(S) (RpoS) subunit of RNA polymerase**. *Microbiol Mol Biol Rev* 2002, **66**(3):373-395, table of contents.
49. Lacour S, Landini P: **SigmaS-dependent gene expression at the onset of stationary phase in Escherichia coli: function of sigmaS-dependent genes and identification of their promoter sequences**. *J Bacteriol* 2004, **186**(21):7186-7195.
50. Patten CL, Kirchhof MG, Schertzberg MR, Morton RA, Schellhorn HE: **Microarray analysis of RpoS-mediated gene expression in Escherichia coli K-12**. *Mol Genet Genomics* 2004, **272**(5):580-591.
51. Rahman M, Hasan MR, Oba T, Shimizu K: **Effect of rpoS gene knockout on the metabolism of Escherichia coli during exponential growth phase and early stationary phase based on gene expressions, enzyme activities and intracellular metabolite concentrations**. *Biotechnol Bioeng* 2006, **94**(3):585-595.
52. Dong T, Kirchhof MG, Schellhorn HE: **RpoS regulation of gene expression during exponential growth of Escherichia coli K12**. *Mol Genet Genomics* 2008, **279**(3):267-277.
53. Schembri MA, Kjaergaard K, Klemm P: **Global gene expression in Escherichia coli biofilms**. *Mol Microbiol* 2003, **48**(1):253-267.
54. Schellhorn HE, Audia JP, Wei LI, Chang L: **Identification of conserved, RpoS-dependent stationary-phase genes of Escherichia coli**. *J Bacteriol* 1998, **180**(23):6283-6291.
55. Vijayakumar SR, Kirchhof MG, Patten CL, Schellhorn HE: **RpoS-regulated genes of Escherichia coli identified by random lacZ fusion mutagenesis**. *J Bacteriol* 2004, **186**(24):8499-8507.
56. Lange R, Hengge-Aronis R: **The cellular concentration of the sigma S subunit of RNA polymerase in Escherichia coli is controlled at the levels of transcription, translation, and protein stability**. *Genes Dev* 1994, **8**(13):1600-1612.
57. Lange R, Barth M, Hengge-Aronis R: **Complex transcriptional control of the sigma s-dependent stationary-phase-induced and osmotically regulated osmY (csi-5) gene suggests novel roles for Lrp, cyclic AMP (cAMP) receptor protein-cAMP complex, and integration host factor in the stationary-phase response of Escherichia coli**. *J Bacteriol* 1993, **175**(24):7910-7917.
58. Hengge-Aronis R: **Interplay of global regulators and cell physiology in the general stress response of Escherichia coli**. *Curr Opin Microbiol* 1999, **2**(2):148-152.
59. Muffler A, Fischer D, Altuvia S, Storz G, Hengge-Aronis R: **The response regulator RssB controls stability of the sigma(S) subunit of RNA polymerase in Escherichia coli**. *Embo J* 1996, **15**(6):1333-1339.
60. Schweder T, Lee KH, Lomovskaya O, Matin A: **Regulation of Escherichia coli starvation sigma factor (sigma s) by ClpXP protease**. *J Bacteriol* 1996, **178**(2):470-476.

61. Hengge R: **Proteolysis of sigmaS (RpoS) and the general stress response in Escherichia coli**. *Research in microbiology* 2009, **160**(9):667-676.
62. Adams JL, McLean RJ: **Impact of rpoS deletion on Escherichia coli biofilms**. *Appl Environ Microbiol* 1999, **65**(9):4285-4287.
63. Chen G, Patten CL, Schellhorn HE: **Positive selection for loss of RpoS function in Escherichia coli**. *Mutat Res* 2004, **554**(1-2):193-203.
64. Dong T, Chiang SM, Joyce C, Yu R, Schellhorn HE: **Polymorphism and selection of rpoS in pathogenic Escherichia coli**. *Bmc Microbiology* 2009, **9**:-.
65. Gottesman S: **Stealth regulation: biological circuits with small RNA switches**. *Genes Dev* 2002, **16**(22):2829-2842.
66. Masse E, Majdalani N, Gottesman S: **Regulatory roles for small RNAs in bacteria**. *Curr Opin Microbiol* 2003, **6**(2):120-124.
67. Gottesman S: **The small RNA regulators of Escherichia coli: roles and mechanisms***. *Annu Rev Microbiol* 2004, **58**:303-328.
68. Wassarman KM, Repoila F, Rosenow C, Storz G, Gottesman S: **Identification of novel small RNAs using comparative genomics and microarrays**. *Genes Dev* 2001, **15**(13):1637-1651.
69. Vogel J, Sharma CM: **How to find small non-coding RNAs in bacteria**. *Biol Chem* 2005, **386**(12):1219-1238.
70. Brantl S: **Regulatory mechanisms employed by cis-encoded antisense RNAs**. *Current Opinion in Microbiology* 2007, **10**(2):102-109.
71. Aiba H: **Mechanism of RNA silencing by Hfq-binding small RNAs**. *Current Opinion in Microbiology* 2007, **10**(2):134-139.
72. Bouvier M, Sharma CM, Mika F, Nierhaus KH, Vogel J: **Small RNA Binding to 5 ' mRNA Coding Region Inhibits Translational Initiation**. *Molecular Cell* 2008, **32**(6):827-837.
73. Papenfort K, Bouvier M, Mika F, Sharma CM, Vogel J: **Evidence for an autonomous 5 ' target recognition domain in an Hfq-associated small RNA**. *Proceedings of the National Academy of Sciences of the United States of America* 2010, **107**(47):20435-20440.
74. Opdyke JA, Kang JG, Storz G: **GadY, a small-RNA regulator of acid response genes in Escherichia coli**. *Journal of Bacteriology* 2004, **186**(20):6698-6705.
75. Kawamoto H, Koide Y, Morita T, Aiba H: **Base-pairing requirement for RNA silencing by a bacterial small RNA and acceleration of duplex formation by Hfq**. *Molecular Microbiology* 2006, **61**(4):1013-1022.
76. Majdalani N, Cunning C, Sledjeski D, Elliott T, Gottesman S: **DsrA RNA regulates translation of RpoS message by an anti-antisense mechanism, independent of its action as an antisilencer of transcription**. *Proceedings of the National Academy of Sciences of the United States of America* 1998, **95**(21):12462-12467.
77. Zhang A, Wassarman KM, Rosenow C, Tjaden BC, Storz G, Gottesman S: **Global analysis of small RNA and mRNA targets of Hfq**. *Mol Microbiol* 2003, **50**(4):1111-1124.

78. Sledjeski DD, Whitman C, Zhang A: **Hfq is necessary for regulation by the untranslated RNA DsrA**. *J Bacteriol* 2001, **183**(6):1997-2005.
79. Link TM, Valentin-Hansen P, Brennan RG: **Structure of Escherichia coli Hfq bound to polyriboadenylate RNA**. *Proceedings of the National Academy of Sciences of the United States of America* 2009, **106**(46):19286-19291.
80. Valentin-Hansen P, Eriksen M, Udesen C: **The bacterial Sm-like protein Hfq: a key player in RNA transactions**. *Molecular Microbiology* 2004, **51**(6):1525-1533.
81. Moller T, Franch T, Hojrup P, Keene DR, Bachinger HP, Brennan RG, Valentin-Hansen P: **Hfq: a bacterial Sm-like protein that mediates RNA-RNA interaction**. *Molecular Cell* 2002, **9**(1):23-30.
82. Brescia CC, Mikulecky PJ, Feig AL, Sledjeski DD: **Identification of the Hfq-binding site on DsrA RNA: Hfq binds without altering DsrA secondary structure**. *Rna* 2003, **9**(1):33-43.
83. Zhang A, Wassarman KM, Ortega J, Steven AC, Storz G: **The Sm-like Hfq protein increases OxyS RNA interaction with target mRNAs**. *Mol Cell* 2002, **9**(1):11-22.
84. Mikulecky PJ, Kaw MK, Brescia CC, Takach JC, Sledjeski DD, Feig AL: **Escherichia coli Hfq has distinct interaction surfaces for DsrA, rpoS and poly(A) RNAs**. *Nat Struct Mol Biol* 2004, **11**(12):1206-1214.
85. Soper T, Mandin P, Majdalani N, Gottesman S, Woodson SA: **Positive regulation by small RNAs and the role of Hfq**. *Proceedings of the National Academy of Sciences of the United States of America* 2010, **107**(21):9602-9607.
86. Kajitani M, Kato A, Wada A, Inokuchi Y, Ishihama A: **Regulation of the Escherichia-Coli Hfq Gene Encoding the Host Factor for Phage Q-Beta**. *Journal of Bacteriology* 1994, **176**(2):531-534.
87. Rajkowitsch L, Schroeder R: **Dissecting RNA chaperone activity**. *Rna-a Publication of the Rna Society* 2007, **13**(12):2053-2060.
88. Geissmann TA, Touati D: **Hfq, a new chaperoning role: binding to messenger RNA determines access for small RNA regulator**. *Embo Journal* 2004, **23**(2):396-405.
89. Wartell RM, Updegrove TB, Correia JJ, Chen YF, Terry C: **The stoichiometry of the Escherichia coli Hfq protein bound to RNA**. *Rna-a Publication of the Rna Society* 2011, **17**(3):489-500.
90. Fender A, Elf J, Hampel K, Zimmermann B, Wagner EGH: **RNAs actively cycle on the Sm-like protein Hfq**. *Genes & Development* 2010, **24**(23):2621-2626.
91. Hopkins JF, Panja S, McNeil SAN, Woodson SA: **Effect of salt and RNA structure on annealing and strand displacement by Hfq**. *Nucleic Acids Research* 2009, **37**(18):6205-6213.
92. Hussein R, Lim HN: **Disruption of small RNA signaling caused by competition for Hfq**. *Proceedings of the National Academy of Sciences of the United States of America* 2011, **108**(3):1110-1115.

93. Hwang W, Arluison V, Hohng S: **Dynamic competition of DsrA and rpoS fragments for the proximal binding site of Hfq as a means for efficient annealing**. *Nucleic Acids Res*.
94. Sauter C, Basquin J, Suck D: **Sm-like proteins in Eubacteria: the crystal structure of the Hfq protein from Escherichia coli**. *Nucleic Acids Research* 2003, **31**(14):4091-4098.
95. Worhunsky DJ, Godek K, Litsch S, Schlax PJ: **Interactions of the non-coding RNA DsrA and RpoS mRNA with the 30 S ribosomal subunit**. *J Biol Chem* 2003, **278**(18):15815-15824.
96. Repoila F, Majdalani N, Gottesman S: **Small non-coding RNAs, co-ordinators of adaptation processes in Escherichia coli: the RpoS paradigm**. *Mol Microbiol* 2003, **48**(4):855-861.
97. Sledjeski D, Gottesman S: **A small RNA acts as an antisilencer of the H-NS-silenced rcsA gene of Escherichia coli**. *Proc Natl Acad Sci U S A* 1995, **92**(6):2003-2007.
98. Sledjeski DD, Gupta A, Gottesman S: **The small RNA, DsrA, is essential for the low temperature expression of RpoS during exponential growth in Escherichia coli**. *Embo J* 1996, **15**(15):3993-4000.
99. Repoila F, Gottesman S: **Signal transduction cascade for regulation of RpoS: temperature regulation of DsrA**. *J Bacteriol* 2001, **183**(13):4012-4023.
100. Lease RA, Smith D, McDonough K, Belfort M: **The small noncoding DsrA RNA is an acid resistance regulator in Escherichia coli**. *J Bacteriol* 2004, **186**(18):6179-6185.
101. Majdalani N, Cunning C, Sledjeski D, Elliott T, Gottesman S: **DsrA RNA regulates translation of RpoS message by an anti-antisense mechanism, independent of its action as an antisilencer of transcription**. *Proc Natl Acad Sci U S A* 1998, **95**(21):12462-12467.
102. Lease RA, Cusick ME, Belfort M: **Riboregulation in Escherichia coli: DsrA RNA acts by RNA:RNA interactions at multiple loci**. *Proc Natl Acad Sci U S A* 1998, **95**(21):12456-12461.
103. Lease RA, Belfort M: **Riboregulation by DsrA RNA: trans-actions for global economy**. *Mol Microbiol* 2000, **38**(4):667-672.
104. Mandin P, Gottesman S: **Integrating anaerobic/aerobic sensing and the general stress response through the ArcZ small RNA**. *Embo Journal* 2010, **29**(18):3094-3107.
105. Vecerek B, Rajkowitsch L, Sonnleitner E, Schroeder R, Blasi U: **The C-terminal domain of Escherichia coli Hfq is required for regulation**. *Nucleic Acids Research* 2008, **36**(1):133-143.
106. Resch A, Afonyushkin T, Lombo TB, McDowall KJ, Blasi U, Kaberdin VR: **Translational activation by the noncoding RNA DsrA involves alternative RNase III processing in the rpoS 5 '-leader**. *Rna-a Publication of the Rna Society* 2008, **14**(3):454-459.
107. Vecerek B, Beich-Frandsen M, Resch A, Blasi U: **Translational activation of rpoS mRNA by the non-coding RNA DsrA and Hfq does not require ribosome binding**. *Nucleic Acids Research* 2010, **38**(4):1284-1293.

108. Madhugiri R, Basineni SR, Klug G: **Turn-over of the small non-coding RNA RprA in E. coli is influenced by osmolarity**. *Mol Genet Genomics* 2010, **284**(4):307-318.
109. Majdalani N, Hernandez D, Gottesman S: **Regulation and mode of action of the second small RNA activator of RpoS translation, RprA**. *Mol Microbiol* 2002, **46**(3):813-826.
110. Davalos-Garcia M, Conter A, Toesca I, Gutierrez C, Cam K: **Regulation of osmC gene expression by the two-component system rcsB-rcsC in Escherichia coli**. *Journal of bacteriology* 2001, **183**(20):5870-5876.
111. Ferrieres L, Thompson A, Clarke DJ: **Elevated levels of sigma S inhibit biofilm formation in Escherichia coli: a role for the Rcs phosphorelay**. *Microbiology* 2009, **155**(Pt 11):3544-3553.
112. Papenfort K, Said N, Welsink T, Lucchini S, Hinton JCD, Vogel J: **Specific and pleiotropic patterns of mRNA regulation by ArcZ, a conserved, Hfq-dependent small RNA**. *Molecular Microbiology* 2009, **74**(1):139-158.
113. Papenfort K, Said N, Welsink T, Lucchini S, Hinton JC, Vogel J: **Specific and pleiotropic patterns of mRNA regulation by ArcZ, a conserved, Hfq-dependent small RNA**. *Mol Microbiol* 2009, **74**(1):139-158.
114. Argaman L, Altuvia S: **fhlA repression by OxyS RNA: kissing complex formation at two sites results in a stable antisense-target RNA complex**. *J Mol Biol* 2000, **300**(5):1101-1112.
115. Basineni SR, Madhugiri R, Kolmsee T, Hengge R, Klug G: **The influence of Hfq and ribonucleases on the stability of the small non-coding RNA OxyS and its target rpoS in E. coli is growth phase dependent**. *RNA Biol* 2009, **6**(5):584-594.
116. Altuvia S, Weinstein-Fischer D, Zhang A, Postow L, Storz G: **A small, stable RNA induced by oxidative stress: role as a pleiotropic regulator and antimutator**. *Cell* 1997, **90**(1):43-53.
117. Breaker RR: **Natural and engineered nucleic acids as tools to explore biology**. *Nature* 2004, **432**(7019):838-845.
118. Blake WJ, Isaacs FJ: **Synthetic biology evolves**. *Trends Biotechnol* 2004, **22**(7):321-324.
119. Davidson EA, Ellington AD: **Engineering regulatory RNAs**. *Trends Biotechnol* 2005, **23**(3):109-112.
120. Barrick JE, Corbino KA, Winkler WC, Nahvi A, Mandal M, Collins J, Lee M, Roth A, Sudarsan N, Jona I *et al*: **New RNA motifs suggest an expanded scope for riboswitches in bacterial genetic control**. *Proc Natl Acad Sci U S A* 2004, **101**(17):6421-6426.
121. Isaacs FJ, Collins JJ: **Plug-and-play with RNA**. *Nat Biotechnol* 2005, **23**(3):306-307.
122. Franch T, Petersen M, Wagner EG, Jacobsen JP, Gerdes K: **Antisense RNA regulation in prokaryotes: rapid RNA/RNA interaction facilitated by a general U-turn loop structure**. *J Mol Biol* 1999, **294**(5):1115-1125.
123. Srivastava R, Cha HJ, Peterson MS, Bentley WE: **Antisense downregulation of sigma(32) as a transient metabolic controller in Escherichia coli: effects**

on yield of active organophosphorus hydrolase. *Appl Environ Microbiol* 2000, **66**(10):4366-4371.
124. Atlung T, Nielsen HV, Hansen FG: **Characterisation of the allelic variation in the rpoS gene in thirteen K12 and six other non-pathogenic Escherichia coli strains**. *Mol Genet Genomics* 2002, **266**(5):873-881.
125. Tsui HC, Feng G, Winkler ME: **Negative regulation of mutS and mutH repair gene expression by the Hfq and RpoS global regulators of Escherichia coli K-12**. *J Bacteriol* 1997, **179**(23):7476-7487.
126. Horst JP, Wu TH, Marinus MG: **Escherichia coli mutator genes**. *Trends Microbiol* 1999, **7**(1):29-36.
127. Layton JC, Foster PL: **Error-prone DNA polymerase IV is controlled by the stress-response sigma factor, RpoS, in Escherichia coli**. *Mol Microbiol* 2003, **50**(2):549-561.
128. McKenzie GJ, Lee PL, Lombardo MJ, Hastings PJ, Rosenberg SM: **SOS mutator DNA polymerase IV functions in adaptive mutation and not adaptive amplification**. *Mol Cell* 2001, **7**(3):571-579.
129. McKenzie GJ, Rosenberg SM: **Adaptive mutations, mutator DNA polymerases and genetic change strategies of pathogens**. *Curr Opin Microbiol* 2001, **4**(5):586-594.
130. Storvik KA, Foster PL: **RpoS, the stress response sigma factor, plays a dual role in the regulation of Escherichia coli's error-prone DNA polymerase IV**. *J Bacteriol*, **192**(14):3639-3644.
131. Subbarayan PR, Sarkar M: **A comparative study of variation in codon 33 of the rpoS gene in Escherichia coli K12 stocks: implications for the synthesis of sigma(s)**. *Mol Genet Genomics* 2004, **270**(6):533-538.
132. Jishage M, Ishihama A: **Variation in RNA polymerase sigma subunit composition within different stocks of Escherichia coli W3110**. *J Bacteriol* 1997, **179**(3):959-963.
133. Subbarayan PR, Sarkar M: **A stop codon-dependent internal secondary translation initiation region in Escherichia coli rpoS**. *RNA* 2004, **10**(9):1359-1365.
134. King T, Ishihama A, Kori A, Ferenci T: **A regulatory trade-off as a source of strain variation in the species Escherichia coli**. *J Bacteriol* 2004, **186**(17):5614-5620.
135. Bailey JE: **Toward a science of metabolic engineering**. *Science* 1991, **252**(5013):1668-1675.
136. Isaacs FJ, Dwyer DJ, Ding C, Pervouchine DD, Cantor CR, Collins JJ: **Engineered riboregulators enable post-transcriptional control of gene expression**. *Nat Biotechnol* 2004, **22**(7):841-847.
137. Chen G, Patten CL, Schellhorn HE: **Controlled expression of an rpoS antisense RNA can inhibit RpoS function in Escherichia coli**. *Antimicrob Agents Chemother* 2003, **47**(11):3485-3493.
138. Kemmer C, Neubauer P: **Antisense RNA based down-regulation of RNaseE in E. coli**. *Microb Cell Fact* 2006, **5**:38.
139. Breaker RR: **RNA switches out in the cold**. *Mol Cell*, **37**(1):1-2.

140. Guzman LM, Belin D, Carson MJ, Beckwith J: **Tight regulation, modulation, and high-level expression by vectors containing the arabinose PBAD promoter**. *J Bacteriol* 1995, **177**(14):4121-4130.
141. Santos JM, Freire P, Vicente M, Arraiano CM: **The stationary-phase morphogene bolA from Escherichia coli is induced by stress during early stages of growth**. *Mol Microbiol* 1999, **32**(4):789-798.
142. Kalir S, McClure J, Pabbaraju K, Southward C, Ronen M, Leibler S, Surette MG, Alon U: **Ordering genes in a flagella pathway by analysis of expression kinetics from living bacteria**. *Science* 2001, **292**(5524):2080-2083.
143. Allen SP, Polazzi JO, Gierse JK, Easton AM: **Two novel heat shock genes encoding proteins produced in response to heterologous protein expression in Escherichia coli**. *J Bacteriol* 1992, **174**(21):6938-6947.
144. Gill RT, Valdes JJ, Bentley WE: **A comparative study of global stress gene regulation in response to overexpression of recombinant proteins in Escherichia coli**. *Metab Eng* 2000, **2**(3):178-189.
145. Ren D, Bedzyk LA, Thomas SM, Ye RW, Wood TK: **Gene expression in Escherichia coli biofilms**. *Appl Microbiol Biotechnol* 2004, **64**(4):515-524.
146. Bissonnette SA, Rivera-Rivera I, Sauer RT, Baker TA: **The IbpA and IbpB small heat-shock proteins are substrates of the AAA+ Lon protease**. *Mol Microbiol* 2010, **75**(6):1539-1549.
147. Itoh Y, Rice JD, Goller C, Pannuri A, Taylor J, Meisner J, Beveridge TJ, Preston JF, 3rd, Romeo T: **Roles of pgaABCD genes in synthesis, modification, and export of the Escherichia coli biofilm adhesin poly-beta-1,6-N-acetyl-D-glucosamine**. *J Bacteriol* 2008, **190**(10):3670-3680.
148. Cerca N, Jefferson KK: **Effect of growth conditions on poly-N-acetylglucosamine expression and biofilm formation in Escherichia coli**. *FEMS Microbiol Lett* 2008, **283**(1):36-41.
149. Jonas K, Edwards AN, Simm R, Romeo T, Romling U, Melefors O: **The RNA binding protein CsrA controls cyclic di-GMP metabolism by directly regulating the expression of GGDEF proteins**. *Mol Microbiol* 2008, **70**(1):236-257.
150. Ranquet C, Gottesman S: **Translational regulation of the Escherichia coli stress factor RpoS: a role for SsrA and Lon**. *J Bacteriol* 2007, **189**(13):4872-4879.
151. Beloin C, Valle J, Latour-Lambert P, Faure P, Kzreminski M, Balestrino D, Haagensen JA, Molin S, Prensier G, Arbeille B *et al*: **Global impact of mature biofilm lifestyle on Escherichia coli K-12 gene expression**. *Mol Microbiol* 2004, **51**(3):659-674.
152. Domka J, Lee J, Wood TK: **YliH (BssR) and YceP (BssS) regulate Escherichia coli K-12 biofilm formation by influencing cell signaling**. *Appl Environ Microbiol* 2006, **72**(4):2449-2459.
153. Englesberg E, Anderson RL, Weinberg R, Lee N, Hoffee P, Huttenhauer G, Boyer H: **L-Arabinose-sensitive, L-ribulose 5-phosphate 4-epimerase-deficient mutants of Escherichia coli**. *J Bacteriol* 1962, **84**:137-146.

154. Vijayendran C, Polen T, Wendisch VF, Friehs K, Niehaus K, Flaschel E: **The plasticity of global proteome and genome expression analyzed in closely related W3110 and MG1655 strains of a well-studied model organism, Escherichia coli-K12**. *J Biotechnol* 2007, **128**(4):747-761.
155. Chiang SM, Schellhorn HE: **Evolution of the RpoS regulon: origin of RpoS and the conservation of RpoS-dependent regulation in bacteria**. *J Mol Evol* 2010, **70**(6):557-571.
156. King T, Seeto S, Ferenci T: **Genotype-by-environment interactions influencing the emergence of rpoS mutations in Escherichia coli populations**. *Genetics* 2006, **172**(4):2071-2079.
157. Venturi V: **Control of rpoS transcription in Escherichia coli and Pseudomonas: why so different?** *Mol Microbiol* 2003, **49**(1):1-9.
158. Muffler A, Barth M, Marschall C, Hengge-Aronis R: **Heat shock regulation of sigmaS turnover: a role for DnaK and relationship between stress responses mediated by sigmaS and sigma32 in Escherichia coli**. *J Bacteriol* 1997, **179**(2):445-452.
159. Tosun H, Gonul SA: **The effect of acid adaptation conditions on heat resistance of Escherichia coli O157: H7**. *Pol J Microbiol* 2005, **54**(4):295-299.
160. Jenkins DE, Chaisson SA, Matin A: **Starvation-induced cross protection against osmotic challenge in Escherichia coli**. *J Bacteriol* 1990, **172**(5):2779-2781.
161. Stanley NR, Lazazzera BA: **Environmental signals and regulatory pathways that influence biofilm formation**. *Mol Microbiol* 2004, **52**(4):917-924.
162. O'Connell HA, Kottkamp GS, Eppelbaum JL, Stubblefield BA, Gilbert SE, Gilbert ES: **Influences of biofilm structure and antibiotic resistance mechanisms on indirect pathogenicity in a model polymicrobial biofilm**. *Appl Environ Microbiol* 2006, **72**(7):5013-5019.
163. Fux CA, Costerton JW, Stewart PS, Stoodley P: **Survival strategies of infectious biofilms**. *Trends Microbiol* 2005, **13**(1):34-40.
164. Costerton JW, Montanaro L, Arciola CR: **Biofilm in implant infections: its production and regulation**. *Int J Artif Organs* 2005, **28**(11):1062-1068.
165. Dhar N, McKinney JD: **Microbial phenotypic heterogeneity and antibiotic tolerance**. *Curr Opin Microbiol* 2007, **10**(1):30-38.
166. Beloin C, Ghigo JM: **Finding gene-expression patterns in bacterial biofilms**. *Trends Microbiol* 2005, **13**(1):16-19.
167. Dong T, Schellhorn HE: **Role of RpoS in virulence of pathogens**. *Infect Immun* 2010, **78**(3):887-897.
168. Fang FC, Libby SJ, Buchmeier NA, Loewen PC, Switala J, Harwood J, Guiney DG: **The alternative sigma factor katF (rpoS) regulates Salmonella virulence**. *Proc Natl Acad Sci U S A* 1992, **89**(24):11978-11982.
169. Wood TK, Hong SH, Ma Q: **Engineering biofilm formation and dispersal**. *Trends Biotechnol*, **29**(2):87-94.
170. Bentley WE, Mirjalili N, Andersen DC, Davis RH, Kompala DS: **Plasmid-Encoded Protein - the Principal Factor in the Metabolic Burden**

Associated with Recombinant Bacteria. *Biotechnol Bioeng* 1990, **35**(7):668-681.
171. Rockabrand D, Livers K, Austin T, Kaiser R, Jensen D, Burgess R, Blum P: **Roles of DnaK and RpoS in starvation-induced thermotolerance of Escherichia coli**. *J Bacteriol* 1998, **180**(4):846-854.
172. Schweder T, Lin HY, Jurgen B, Breitenstein A, Riemschneider S, Khalameyzer V, Gupta A, Buttner K, Neubauer P: **Role of the general stress response during strong overexpression of a heterologous gene in Escherichia coli**. *Appl Microbiol Biotechnol* 2002, **58**(3):330-337.
173. Thomas JG, Baneyx F: **Protein misfolding and inclusion body formation in recombinant Escherichia coli cells overexpressing Heat-shock proteins**. *J Biol Chem* 1996, **271**(19):11141-11147.
174. Tsao CY, Hooshangi S, Wu HC, Valdes JJ, Bentley WE: **Autonomous induction of recombinant proteins by minimally rewiring native quorum sensing regulon of E. coli**. *Metab Eng*, **12**(3):291-297.
175. Notley-McRobb L, King T, Ferenci T: **rpoS mutations and loss of general stress resistance in Escherichia coli populations as a consequence of conflict between competing stress responses**. *Journal of Bacteriology* 2002, **184**(3):806-811.
176. Ou J, Wang L, Ding X, Du J, Zhang Y, Chen H, Xu A: **Stationary phase protein overproduction is a fundamental capability of Escherichia coli**. *Biochem Biophys Res Commun* 2004, **314**(1):174-180.
177. Cha HJ, Wu CF, Valdes JJ, Rao G, Bentley WE: **Observations of green fluorescent protein as a fusion partner in genetically engineered Escherichia coli: monitoring protein expression and solubility**. *Biotechnol Bioeng* 2000, **67**(5):565-574.
178. Dong T, Yu R, Schellhorn H: **Antagonistic regulation of motility and transcriptome expression by RpoN and RpoS in Escherichia coli**. *Mol Microbiol* 2011, **79**(2):375-386.
179. Li J, Attila C, Wang L, Wood TK, Valdes JJ, Bentley WE: **Quorum sensing in Escherichia coli is signaled by AI-2/LsrR: effects on small RNA and biofilm architecture**. *J Bacteriol* 2007, **189**(16):6011-6020.
180. Danese PN, Pratt LA, Dove SL, Kolter R: **The outer membrane protein, antigen 43, mediates cell-to-cell interactions within Escherichia coli biofilms**. *Mol Microbiol* 2000, **37**(2):424-432.
181. De Lay N, Gottesman S: **The Crp-activated small noncoding regulatory RNA CyaR (RyeE) links nutritional status to group behavior**. *J Bacteriol* 2009, **191**(2):461-476.